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Probiotic therapy for heart failure: Investigating the potential anti-hypertrophic properties of probiotics

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

PROBIOTIC THERAPY FOR HEART FAILURE: INVESTIGATING THE POTENTIAL ANTI-HYPERTROPHIC PROPERTIES OF PROBIOTICS

(Thesis format: Monograph)

by

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Abstract

Heart failure patients face a five-year 50% mortality rate, due to impaired cardiac function and hypertrophy of the heart. Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host. Considering the established cardiovascular benefits of some probiotics, including reduction of cholesterol and hypertension, it was hypothesized that probiotics can improve outcomes of heart failure. Probiotics or controls were administered orally to an animal model for heart failure. Those receiving probiotics showed a significant improvement in cardiac hypertrophy and an attenuation of heart failure compared to control. No changes in gut microbial composition occurred. An *in vitro* model for cardiomyocyte hypertrophy examined the mechanistic action of probiotics in preventing cardiac hypertrophy. Studies showed that probiotics confer a direct anti-hypertrophic benefit to cardiomyocytes. These results support the hypothesis that probiotics can improve outcomes of heart failure and present a novel treatment strategy for heart failure.

Keywords

Probiotics, lactobacilli, *Lactobacillus rhamnosus*, heart failure, cardiac hypertrophy, cardiomyocytes, microbiota, microbiome.

Co-Authorship Statement

The work herein contains material from one previously published manuscript:

Gan XT, **Ettinger G**, Huang CX, Burton JP, Haist JV, Rajapurohitam, V, Sidaway JE, Martin G, Gloor GB, Swann JR, Reid G, Karmazyn M. Probiotic administration attenuates myocardial hypertrophy and heart failure following myocardial infarction in the rat. *Circ Heart Fail*. 2014; 7: 491.

My contributions included: design of the study, probiotic treatment preparation, microbial cultivation and next-generation sequencing of digesta, preparation of the heart tissue for metabonomic analysis (not included in this thesis), blood cytokine analysis, and manuscript preparation.

The surgeries, echocardiography, hemodynamic measurements, leptin analysis, and heart tissue processing, as well as housing and maintenance of the rats was performed by laboratory staff of Dr. Morris Karmazyn: Tracey Gan, Cathy Huang, James Haist, and Venkatesh Rajapurohitam.

Dr. Greg Gloor provided bioinformatic expertise and designed the pipeline for 16S rRNA next-generation sequencing analysis.

Dr. Jonathon Swann and James Sidaway, and Glynn Martin performed the ^{1}H NMR spectroscopic analysis of heart tissue (not included in this thesis) and manuscript preparation.

Drs. Burton and Reid and Karmazyn were responsible for the conceptualization and design of the study, as well as manuscript preparation.

This thesis refers to the above publication in the following sections:

Chapter 2, section 2.1

Chapter 3, section 3.1-3.5

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List of Abbreviations

ACE	angiotensin converting enzyme
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
ASD	Autism Spectrum Disorder
aSKA	α-skeletal actin
AV	atrial ventricular
BD	Becton and Dickinson
BP	base pairs
BSH	bile salt hydrolase
CAL	coronary artery ligation
CFU	colony forming units
CHD	coronary heart disease
CLA	conjugated linoleic acid
CVD	cardiovascular disease
DMP	Disease Management Program
E/A	Early/atrial
ECM	extracellular matrix
EF	ejection fraction
FA	fusidic acid

FS	fractional shortening
HD	heat denatured/denaturation
HF	heart failure
HK	heat killed
HMP	Human Microbiome Project
I/R	ischemia/reperfusion
IFN	interferon
IL	interleukin
LDL-C	low-density lipoprotein cholesterol
LPSM	*Lactobacillus plantarum* selective media
LV	left ventricle
LVEDP	left ventricular end diastolic pressure
LVID	left ventricular internal diameter
LVIDd	diastolic left ventricular internal diameter
LVIDs	systolic left ventricular internal diameter
LVSP	left ventricular end systolic pressure
LVW/BW	left ventricle weight/bodyweight
MAPK	mitogen activated protein kinase
MCP	monocyte chemoattractant protein
MI	myocardial infarction

MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
MRS	Man, Rogosa, Sharpe
MSP	Major Secreted Protein
NF	nuclear factor
NVCM	neonatal rat ventricular cardiomyocytes
NYHA	New York Heart Association
OTU	operational taxonomic unit
PBS	phosphate buffered saline
PCM	probiotic-conditioned media
PCoA	Principal coordinate analysis
PCR	polymerase chain reaction
PE	phenylephrine
qPCR	quantitative PCR
ROS	reactive oxygen species
RQ	relative quantification
RT	reverse transcription
SA	sinoatrial
SD	standard deviation
SDS	sequence detection system

SEM	standard error of the mean
TGF	transforming growth factor
TMA	trimethylamine
TMAO	trimethylamine-N-oxide
TNF	tumor necrosis factor
WT	wild-type

Chapter 1 : Introduction

1 Focus of the thesis

The purpose of this research was to investigate the potential for probiotic lactobacilli as a therapy for heart failure. While research has shown that lactobacilli can influence cardiovascular health through modulating cholesterol and inflammation, a study showing that pretreatment of animals with probiotic *Lactobacillus* could ameliorate damage caused by coronary artery ligation injury and subsequent perfusion, formed the impetus for the present thesis. Until our work, no studies had tested whether probiotic administration post-ligation injury could improve outcomes of heart failure. Therefore, the focus of the thesis was a series of *in vivo* and *in vitro* experiments that explored the ability of probiotic *Lactobacillus* to reduce the hallmark features of heart failure, namely ventricular remodeling and the associated cardiac hypertrophy, and to investigate what mechanism may be involved.

1.1 Heart anatomy and physiology

The human heart is a muscular pump comprised of 4 chambers: The left and right atria, and the left and right ventricles. Deoxygenated and nutrient-poor blood is delivered to the heart from the superior and inferior vena cava into the right atrium. Atrial systole pushes blood from the right atrium through the tricuspid valve and into the right ventricle. Ventricular systole pumps blood through the pulmonary valve into the pulmonary artery, and blood eventually reaches the lungs for gas exchange. Oxygenated blood then returns to the heart through the pulmonary vein into the left atrium. Atrial systolic contractions push blood through the bicuspid valve into the left ventricle (LV). The LV is the largest and strongest chamber of the heart, and is responsible for pumping oxygenated blood into the aorta during ventricular systole contractions. The aorta then bifurcates and delivers oxygenated blood to tissues throughout the body.

Rhythmic muscle contractions are an involuntary event controlled by electrical impulses originating from the sinoatrial (SA) node in the right atrium. The atrial ventricular (AV) node relays the impulses to the Purkinje fibers of the right and LV, which coordinate ventricular systole. In ventricular diastole, muscle relaxation and reduced volume of blood in the ventricles allows blood to passively flow from the atria through the tricuspid

and bicuspid valves into the right and LV respectively. This change in pressure triggers atrial systole, and the contraction forces the remaining blood out of the atria. Ventricular filling completes the cardiac cycle, and after the diastolic relaxation period, ventricular systole occurs again through electrical impulses from the SA node.

The muscle tissue of the heart is called the myocardium. It is a thick, middle layer of heart tissue enclosed by the external-most and protective pericardial layer, and the inner-most endocardium. The basic cellular unit of the myocardium is the single-nucleated cardiomyocyte. These form striated chains of sarcomeres – the basic contractile unit of cardiac muscle. Repeating sections of sarcomeres, make up myofibrils – the basic unit of muscle. Similar to skeletal muscle, the striation of sarcomeres is formed by alternating thick and thin protein filaments, actin and myosin. Involuntary electrical impulses originating at the SA node cause a depolarization of the cardiomyocyte membrane, opening calcium ion channels.

Muscle contraction is triggered by an influx of calcium ions, allowing myosin to bind to actin. This causes a shortening of the sarcomere, similar to the sliding filament model in skeletal muscle. The repolarization of cellular calcium ion concentration across the membrane causes myosin to unbind actin, resulting in muscle relaxation. Coordinated contraction and relaxation of the myocardium allows for steady movement of blood in and out of the chambers of the heart. This rhythmic flow of blood is required to sustain life. As tissues throughout the body continuously consume oxygen and nutrients, it is essential for blood to properly replenish this supply. Any mechanical dysfunction in blood flow presents serious implications to the patient. A cardiovascular even causing a limited oxygen supply to the heart can result in impaired cardiac function and blood pumping. Decreased cardiac output overall puts a patient at a major health risk.

1.2 Methods for cardiovascular research

Cardiovascular research involves methods and avenues of research that range from cardiomyocyte cell culture to human clinical trials. Each has its own unique advantages, disadvantages and appropriate applications.

1.2.1 Cell culture

Since the first isolation of the perfused mammalian heart in 1895, advances in cell culture techniques have allowed direct manipulation of cardiomyocytes and provide a wealth of knowledge in cardiac physiology[1]. The obvious advantage of using cardiomyocyte cell culture is the increased versatility, reduced cost, and convenient methodology as compared to animal or clinical studies[1-3]. While immortalized cardiac cell lines, carcinoma cells, and embryonic cardiac cell lines are available, the use of primary cells has more physiological relevance to animal studies[2]. Cardiomyocyte isolation, culture, and maintenance has brought insight on cellular structure and physiology using techniques such as microscopy, electrophysiology, calcium imaging, RNA and protein biochemistry, and immunohistochemistry[1]. Primary cardiomyocytes isolated from murine animals are most commonly used because the murine model is a widely accepted small animal cardiac model[4-6], the animals are easy to handle, relatively inexpensive, and produce a sufficient amount of cells that can be used for studies within 24 hours of isolation[1,2]. Primary cardiomyocytes are isolated from either the neonatal or adult heart, both of which cell types are highly differentiated non-regenerating[2,3,7]. This allows for pharmacological and molecular manipulation in a controlled environment without the interference of cell division or the influence of other cell types. A caveat is the need to harvest fresh cells for each experiment without the convenience of passing and storing cells. Two of the major differences between the neonatal and adult cardiomyocytes lie in isolation techniques and their distinct adaptations to culture conditions. Litters of 10-20 pups are often sufficient for neonatal cardiomyocyte studies and are less expensive than adults[8]. Neonatal cardiomyocytes are generally described as easier to harvest and culture than adult cardiomyocytes because they are less sensitive to Ca^{2+} in the isolation medium[2,7,8]. Neonatal cardiomyocytes attach readily to culture dishes and adapt quickly to culture conditions, as evidenced by the beating of cells as early as 2-3 days after plating[7,9]. This is advantageous and important because experimentation can begin soon after isolation, minimizing the length of time required in culture. Consistent isolation methods and culture conditions are essential for neonatal cardiomyocyte culture because, depending on the age of the cells, they can still possess some embryonic gene expression and might divide in culture[9,10]. The gene expression and protein production of these

immature and mitotic neonatal cardiomyocytes is different from their post-mitotic adult counterparts, therefore comparing cells from different cultures types should be taken with care[1,9,10]. Ideally the experimental design for neonatal cardiomyocyte studies requires isolation of cardiomyocytes from litters of the exact same age and identical culture conditions. Once this is controlled for, neonatal cardiomyocytes are very useful tools for cardiovascular research. They respond to pharmacological and physiological treatments similar to adult cardiomyocytes, and are widely used for gene and protein expression, histological, and contractility experiments[1].

Adult cardiomyocytes represent mature, differentiated heart cells that must undergo elaborate cytoskeletal adjustments in culture[7]. This makes them less flexible in cell culture, but without the complication of potential mitotic activity. Isolated adult cardiomyocytes are commonly used to model cardiac ischemia and hypoxia, as well as contractile mechanics, gene and protein expression, and electrophysiology[3]. These cells also have the advantage of representing the *in vivo* cardiac model closer than neonatal cardiomyocytes, as *in vivo* rat experiments are usually performed on adults.

1.2.2 Animal models

The ideal animal model for cardiovascular research is one that closely mimics the anatomy and physiology of the human heart and responds to stimuli similar to humans[5]. Factors such as phylogenetics, size, life span, cost, and development of end-stage diseases are all important when choosing an animal model for cardiovascular research.

The use of large animals presents several advantages and disadvantages. Large animal models are physically, and in some cases phylogenetically, closer to humans than small animal models, making studies more physiologically relevant[4,6]. Larger animals are often easier to perform surgical procedures and imaging assessments, [11] however, their longer life span and high cost of maintenance makes them very expensive and time consuming to use[4].

Small animal models are popular for cardiovascular research primarily because they are inexpensive, convenient to house and maintain, have a relatively short gestation period,

and therefore can produce large sample sizes over a short period of time[11,12]. The rat, in particular, is one of the most popular models as its size compared to mice makes it easier to perform open-chest surgical procedures such as coronary ligation, as well as MRI and echocardiography imaging and the use of conductance catheters[12]. However, with the development of transgenic and knock-out mouse strains, the murine model is often used to investigate novel targets for pharmacological and molecular therapy[12]. Blood pressure monitoring is feasible in rats and mice using the tail-cuff method, whether other rodent models without a long tail, such as the guinea pig, are more difficult to use for these types of measurements.

1.3 Overview of cardiovascular disease

Cardiovascular disease (CVD) is defined as a group of disorders of the heart and the blood vessels that supply the heart, brain, and extremities[13]. CVD is accompanied by a myriad of symptoms and dysfunctions, from hypertension to cardiomyopathy. Its prevalence is increasing across the globe[13], representing an immense burden to health care systems. In Canada, CVD is the leading cause of hospitalization and accounts for 29 per cent of all deaths per year[14]. Treating CVD costs the Canadian economy $21 billion per annum[15]. The steady increase in CVD cases internationally is largely attributed to an imbalanced or poor diet and sedentary lifestyle[13]. Overnutrition and lack of regular physical activity leading to obesity have negative consequences for the heart[13]. While widespread campaigns for awareness and the promotion of improved diet and lifestyle habits are among the principal strategies for reducing this trend, from a clinical perspective, innovative approaches are needed to help prevent, intervene, and manage CVD. Heart failure (HF) is defined as “the inability of the heart to pump a sufficient amount of blood to meet the demands of the body at normal filling pressures”[16]. HF is a complex syndrome and in many cases is considered the final outcome of several manifestations of CVD. Myocardial infarction (MI) or heart attack, coronary heart disease, hypertension and chronic inflammation, are all examples of confounding factors that instigate and perpetuate HF.

1.3.1 Risk factors and prevalence of heart failure

The rate of mortality from CVD has decreased over recent years, due to improved acute medical care, primarily rapid reperfusion[17]. However, the irreversible damage to the heart associated with coronary heart disease and MI resulting in death of tissue, places patients at high risk for HF. As a result, there are a growing number of patients surviving MI but at high risk of HF. The prevalence of HF increases with age: 1 percent of Canadians over 65 and 4 percent of Canadians over 70 have HF[16]. With the ageing trend of the Canadian population, HF will impact the lives of many people in the years to come. Currently in Canada, 55,000 individuals are diagnosed with HF yearly, joining the half million already living with the condition[16]. Once HF has manifested, the prognosis is bleak: 50 percent of patients will die within the first five years of diagnosis[18]. One explanation is that a portion of patients do not visit the doctor until the pathologies become symptomatic and affect their everyday life. By then, the disease has progressed into later stages of HF with irreversible damage. Research into mechanisms for reversing the many features of HF is of great interest, as the standard treatments today are generally unable to rescue the damage caused by MI and HF, and instead are targeted at reducing co-morbidities of HF, including hypertension and arrhythmia. For these reasons, novel approaches to preventing HF are desired.

1.3.2 Symptoms and the diagnosis of heart failure

The pathologies of heart failure can be acute, but by-and-large they manifest as a chronic deterioration of heart function[19]. Several criteria and classification systems are recognized for diagnosing HF[19]. The New York Heart Association (NYHA) classification is the most commonly used system based on self-reports of patient's daily activities and quality of life[20]. Physicians classify patients with heart failure into 4 categories (I-IV), from stage I representing mild, asymptomatic HF without any limitations on physical activity, to stage IV representing severe HF with the inability to perform any physical exercise without significant discomfort[20]. While this diagnostic system is popular, it is limited to ability of the patient to recognize a symptom and consult a physician. Patients in stage I and II HF do not experience symptoms severe enough to motivate a visit to the doctor. Meanwhile, cardiac dysfunction progresses in the absence of intervention. Other

classification and diagnosis systems require physical exams detecting evidence of cardiac dysfunction based on chest radiographs[19]. While these methods have proven to be sensitive and predictive, they require the patient to experience symptoms and discomfort and then consult with a physician. Efforts to improve the early detection and treatment of HF in Canada rely on disease management programs (DMPs), comprising intensive therapy that includes drugs, education, monitoring, and caregiver assignment[16]. Patients in the emergent and urgent categories of HF are primary candidates for DMPs and are targeted to undergo evaluation within 24 hours to 2 weeks of the presentation of risks or signs and symptoms. These categories include post-heart transplant, post-MI, and myocarditis patients. Timely intervention for HF in the emerging and urgent categories has resulted in a 30% reduction in death and re-hospitalization rates after 18 months of follow-up and disease management[16]. This emphasizes the imperative to provide timely access to intervention and treatment in high-risk HF patients, ultimately reducing mortality and the costs associated with HF.

1.3.3 Hallmark features, treatments, and outcomes of heart failure

HF as a disease has several features, all in which result in the impaired ability of the ventricles to fill and/or eject blood. Heart failure occurs most commonly in patients with previous MI[21]. In the event of MI, the blocked blood flow from the coronary arteries to the myocardium leads to necrosis and ischemia of cardiomyocytes[22]. Treatment strategies for MI involve the prompt restoration of blood flow, using thrombolytic drugs, stents and catheters, or bypass surgery, all in an effort to reduce the infarct size. Any duration spent with blocked blood flow to the myocardium, will inevitably cause oxidative stress, ischemia, necrosis and infarction. As a result, the heart undergoes a healing process in efforts to recover from the damage sustained. While the process of post-MI repair and remodeling is not entirely understood, there are highly regulated events involved that become pathological in HF[23].

1.3.4 The immune response

The degradation by matrix metalloproteinases (MMPs) of the existing extracellular matrix (ECM) and coronary vasculature in the infarction region occurs within the one

week post-MI[21]. Remodeling of the ECM is shown to play a significant role in several vascular disorders, and MMP expression increases in HF[23]. With the production of reactive oxygen species (ROS), necrosis, and ECM degradation, there follows an immune reaction[22]. Pro-inflammatory cytokines and chemokines are released and macrophages are recruited to clear debris, and promote cardiac healing and scar formation around the damaged tissue[24]. However, chronic inflammation and macrophage recruitment can lead to additional cell death and perpetuate the myocardial remodeling process. Some important pro-inflammatory cytokines implicated in the progression of HF include tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6, macrophage inflammatory protein (MIP)-2, monocyte chemoattractant protein (MCP)-1[24,25]. These cytokines exacerbate myocardial and hemodynamic dysfunction and are typically up-regulated in chronic HF. While the repair and healing process involving these agents are necessary post-MI, there is a fine balance between promoting and preventing inflammation. IL-10 is an important anti-inflammatory cytokine known to down-regulate the production of TNF-α, IL-1, and IL-6[25]. The antagonistic role of IL-10 is thought to be beneficial in HF patients. Increased expression and plasma concentration of IL-10 has been shown to improve cardiac mechanical function in rats and patients with HF[25,26]. However, the protective nature of IL-10 is disputed as HF patients show varying levels of increased or decreased plasma IL-10[25]. Overall, evidence suggests that the short-term and initial pro-inflammatory response becomes maladaptive in HF over time. Thus, anti-inflammatory mediators such as IL-10 play an important role in the immune response to MI and HF, and use of such mediators is being considered as a therapy[27]. It remains to be seen if these will be successful. In inflammatory bowel disease, administration of anti-TNF-α antibodies (Infliximab) has shown some benefits[28], while administration of IL-10 by a genetically engineered food-grade bacterium, *Lactococcus lactis*, has some pre-clinical evidence but awaits confirmation in patients with colonic inflammation[29].

1.3.5 Ventricular remodeling and hypertrophy

Following the immediate inflammatory reaction post-MI, a regulated process of cardiac remodeling takes place. Ventricular remodeling is defined as genome expression resulting in molecular, cellular, and interstitial changes[30]. Clinically, this process is

manifested as changes in size, shape, and function of the heart, and is considered a determinant of heart failure[30]. MI stands as the most common instigator of cardiac remodeling, and the changes also occur in patients with other cardiac pathologies such as stenosis, hypertension, and myocarditis[30]. In post-MI patients, within the first week of ECM degradation by MMPs, phagocytic macrophages are recruited to the infarct site for digestion and removal of infarcted tissue[21]. Fibrogenic mediators, such as transforming growth factor (TGF)-β, then increase the synthesis of collagen by myofibroblasts to replace the degraded ECM and form scar tissue[21,31]. Scar tissue formation is evident around week 2 post-MI and continues for over 8 weeks[21]. This process of wound healing provides some potential for the infarct site to function again as the contractile properties of myofibroblasts generate small and strong scars with the potential to bear load[31]. However, the inevitable build-up of collagen in the infarct area and beyond also causes ventricular stiffness and dysfunction[31].

The loss of functional myocardium causes an increase in blood volume in the ventricle that is not properly cleared with contraction. The increased load and ventricular wall stress is sensed by cardiomyocytes which undergo compensatory changes to the cellular architecture of the ventricle. Because cardiomyocytes are terminally differentiated and cannot divide, they instead respond to wall stress by cell stretching/lengthening to increase wall thickness[32-34]. This abnormal growth of cardiomyocytes, termed cardiac hypertrophy, is a hallmark feature of HF[35-37]. Hypertrophy is an adaptive response that initially increases the mass of the functional myocardium, distributes wall stress, and normalizes resting cardiac output[36,38]. Chronically, this response becomes maladaptive, due to poor contractility of hypertrophic cardiomyocytes, which leads to a decrease in cardiac output, increased rate of apoptosis and fibrosis, and increased risk for cardiac arrhythmias[35,36]. For these reasons, cardiac hypertrophy is a very important therapeutic target for heart failure patients. Modern treatments of heart failure, including β-blockers and angiotensin converting enzyme (ACE)-inhibitors have been shown to improve hypertrophy by reducing the heart rate and hemodynamic load, which reduces the stress stimulus for cardiomyocyte growth[37]. More targeted approaches to attenuating maladaptive hypertrophy are desired.

1.3.6 The hormone response

Post-MI, a hormone-mediated recovery process takes place in an effort to maintain mechanical function of the heart. These hormones are often considered biomarkers of HF and represent the compensatory response. Atrial natriuretic peptide (ANP) is a hormone secreted primarily by the atria that has diuretic, natriuretic, and vasodilator properties[39,40]. Ventricular expression of ANP can be induced in conditions of hypertension, wall stress and ventricular load, and increased ventricular mass[39,40]. ANP production is strongly correlated to cardiomyocyte size, where a higher incidence of ANP expressing cardiomyocytes is found in cells with larger diameters[41,42]. Increased levels of ventricular ANP expression in animal and cell models of cardiac hypertrophy and HF have been thoroughly documented[41,43,44]. In these conditions, it is thought that ANP acts to reduce cardiac load and wall stress through its anti-hypertensive properties[42]. Accordingly, ventricular ANP expression is strongly correlated with cardiac hypertrophy and is used extensively *in vitro* and *in vivo* as an indicator of hypertrophy and HF[39,41].

In HF, contractile dysfunction leads to a decrease in cardiac output[45] which then determines arterial circulation. This is sensed by β-adrenergic receptors in the ventricle walls which drive sympathetic nervous system output to increase contractility, cardiac load, and heart rate[30]. Decreased cardiac output also triggers the renin-angiotensin-aldosterone system. These renal hormones promote water retention, vasoconstriction and increase blood pressure to maintain blood flow to vital organs. To the heart, activation of this system has deleterious effects and exacerbate pre-existing co-morbidities of HF such as hypertension. Angiotensin II, a potent vasoconstrictor, autocrine and endocrine hormone produced by heart cells, induces cardiac hypertrophy through its hypertensive properties[36,46]. Cardiomyocytes enlarge due to the increased vascular resistance of blood flow and, as a consequence, ventricular ANP production is activated[32,46]. It has also been suggested that angiotensin II activity via the β-adrenergic pathway causes cardiac hypertrophy independent of hypertension[45-47]. For these reasons, drugs blocking the action of the hormones in the renin-angiotensin-aldosterone system have become some of the most effective therapies for HF[45,48-50]. ACE-inhibitors reduce the activity of the renin-angiotensin-aldosterone system by preventing the conversion of angiotensin I to

angiotensin II. As a result, vasoconstrictive and hypertrophic action of angiotensin II is reduced. Short-term β-adrenergic blockage also improves ventricular function and decreases morbidity and mortality in patients with HF[51]. β-blockers overall have improved survival in post-MI and chronic HF patients, and in combination with ACE-inhibitors are considered the first-line drug therapy for HF patients[33,49,50].

Hormones that are unrelated to hemodynamic regulation are also involved in HF. Leptin is a hormone chiefly secreted by adipocytes that plays an important role in energy balance, appetite, and metabolism. Leptin has been associated with several mechanisms of pathogenesis in obesity and type II diabetes, both of which are risk factors for HF[52,53]. Furthermore, elevated plasma leptin levels are associated with increased risk coronary heart disease (CHD) and hypertension, independent of obesity and diabetes[53,54]. As such, leptin is a proposed biomarker and independent risk factor for myocardial infarction, ischemic heart diseases, CHD and HF[52,55-57]. While adipocytes are the major source of leptin, evidenced by high plasma leptin levels in individuals with increased adiposity[58], the heart has shown to both secrete and express receptors for leptin[56]. The cardiovascular actions of leptin are in part based on the stimulation of the sympathetic nervous system that is thought to cause a hypertensive effect[54,55]. The stimulation of the β-adrenergic pathway by leptin receptors in the heart upregulates angiotensin II expression, which in turn stimulates increased leptin secretion by adipocytes[52]. Leptin can also potentiate secretion of pro-inflammatory cytokines such as TNF-α, IL-2, and IL-6[52], all of which are upregulated in HF, as previously discussed. Leptin is has been shown to promote atherogenesis, cardiac hypertrophy, and is elevated in post-MI and HF patients[52,57,59,60]. Overall, leptin participates in a viscous cycle of inflammation and β-adrenergic activation, causing cardiac pathologies that are deleterious to HF patients.

1.4 The human microbiome: a dynamic ecosystem

The human body is host to dynamic microbial communities that have an increasingly recognized impact on human health. The gut, skin, vagina, urinary tract and oral cavity are among sites where microbial communities exist in an equilibrium that is required for proper function and health[61]. As a whole, the genomes, activity, size, compositions and surrounding ecosystems of these communities represent the human microbiome[61].

Formerly, the organisms in body sites were investigated using traditional laboratory cultivation techniques. This limited the identification and enumeration of the communities due to the inability to cultivate many species. With the advancement of culture-independent techniques, the composition of the human microbiome and the role it plays in our health, have become better understood. Recent research, notably championed by the Human Microbiome Project (HMP) consortium[62], has attempted to characterize "normal" microbiota composition of various sites throughout the body[63-65]. Using high-throughput next-generation DNA sequencing of the 16S rRNA genome, the analysis of over 4000 specimens collected from 242 adults revealed that each site has a characteristic microbiome, with constituents adapted to specific niches[61,63,64]. The gut, for example, is generally dominated by two phyla: Firmicutes and Bacteriodetes, while other phyla, including Proteobacteria and Actinobacteria, exist in smaller proportions[66,67]. The gut bacteria perform a multitude of functions from the transformation of bile acids, breakdown of insoluble fibers, to the production of specific vitamins and cofactors[68]. Despite individuals having somewhat unique microbiota patterns in the gut, many functions appear to be shared. Even with large numbers of organisms (greater than 10^{14}) present, the addition of organisms in comparatively low numbers (10^{9} or less) such as in the form of probiotics or alternatively pathogens, can still significantly affect the host through their production of metabolic by-products- toxins as in the case of the latter[69,70], bacteriocins, modulation of other host factors such as the immune system[71-73]

1.4.1 Microbial dysbiosis: Etiology for disease?

The critical role of the microbiome in host health is illustrated by the fact that a shift to dysbiosis is now associated to several gastrointestinal diseases including inflammatory bowel disease, *C. difficile* associated diarrhea, colon cancer and other forms of enteritis[74-76]. In the oral cavity, a disruption of the homeostatic function of commensal bacteria caused by the colonization of a single pathogenic species can lead to periodontitis and other gingival diseases[77,78]. In the vagina, a displacement of commensal *Lactobacillus* species by various anaerobes is characteristic of bacterial vaginosis, a condition that causes an increased risk of endometriosis, pregnancy complications, and pre-term labour[79-81].

While the incidence of microbial-associated disease at the primary site of colonization is intuitive, the impact of dysbiosis is much more far-reaching. There is mounting evidence that bacteria in the gut can influence distal sites, and for example contribute to the development of metabolic syndrome[82,83]. In addition, individuals with type II diabetes and obesity have a gut microbial composition of less diversity compared to that of healthy individuals[84] and the balance of Firmicutes and Bacteriodetes shifts to one with a dominance of the former[84-86]**.** Studies also demonstrate a link between the brain and gut microbiota associated with a variety of neurological conditions, including appetite disorders, multiple sclerosis, hepatic encephalopathy, and Autism Spectrum Disorder (ASD)[87-91]. Several mouse models have demonstrated that the gut microbiota can convey neurological signals via vagus nerve stimulation, immune-modulatory signaling, and neuroendocrine pathway signalling[92,93]. Furthermore, is has been anecdotally reported that antibiotic and probiotic use, presumably altering the gut microbiome, causes behavioral changes and clinical improvements in children with ASD[94,95]. Although the mechanisms for these relationships are not entirely elucidated, it is clear that microbes in one site can influence overall health status.

1.4.2 The oral microbiome and CVD

The oral microbiome comprises a population that can withstand exposure to the external environment and a diverse range of conditions. With over 700 species of bacteria identified in the oral cavity, and diverse surfaces in which the organisms can colonize, from the hard teeth to the deep gingival tissue pockets, the options for propagating are many, and the result is distinct patterns in the different niches[96]. The oral microbiome plays an important role in the health status of the host, and a shift in microbial composition can cause numerous local infections including periodontitis, caries, gingivitis, and tonsillitis[96-98]. In addition, it has been implicated in several systemic diseases, including CVD. There is a strong clinical association with chronic periodontal disease and increased risk for atherosclerosis, hyperlipidaemia, CHD, stroke, and MI[98-101].

Periodontitis is a severe infection that attacks both the soft tissue and bone supporting the teeth[101]. Opportunistic oral pathogens overgrow in periodontal pockets and release

proteolytic enzymes that break down host tissue[101]. These pathogens are extremely difficult to eradicate and the disease often manifests over chronic periods of time. Pathogens implicated in periodontitis are often detected in blood cultures, as they are able to enter systemic circulation via the compromised oral mucous membrane[102,103]. The access to organs throughout the body represents a major risk for inflammation and infection. It is the chronic nature, however, of periodontitis that is thought to increase the risk for CVD[104]. Cross-sectional studies suggest that the inflammatory factors associated with periodontitis, such as C-reactive protein, plasma fibrinogen, IL-6, and hyperlipidaemia, are responsible for the increased risk of CVD[98,101,104,105]. Cohort studies have found positive dose-dependent associations between chronic periodontitis and the incidence of CHD in men less than 60 years old, independent of pre-existing CVD risk factors (including old age)[103,105,106]. While these associations have been documented for well over 25 years, there is significant variability among studies and subjects[104]. The advent of deep genome sequencing has led to an increased effort to track shifts in the microbial community between health and disease. Changes in the oral microbiota occur in response to treatment for periodontal disease, with several genera of periodontal pathogens depleted in patients who had undergone treatment[107]. This line of research may shed light on the mechanistic role of periodontal pathogens in CVD, by comparing the plaque microbiota of periodontal patients at risk for CVD with healthy, matched controls.

1.4.3 Gut microbiota, diet, CVD

In the so-called ‘Western world’, the prevalence of CVD coincides with other chronic conditions such as obesity, type II diabetes, and gut inflammation. Metabolic disease, which includes hypercholesterolemia, diabetes, and obesity, is largely diet-dependent and is a key preventable risk factor for CVD. It has been well documented that individuals with obesity have a gut microbiome profile distinct from those of lean subjects[66,86,108,109]. In diet-induced obesity, overnutrition can alter composition of the gut microbiome, with dietary nutrients influencing the growth of certain species. Diets rich in cholesterol, saturated fats, and simple carbohydrates are associated with gut microbiomes rich in the Firmicutes phylum[67,110]. In obese individuals, the decreased proportion of Bacteriodetes in comparison to Firmicutes is normalized with a low-calorie diet-associated weight

loss[67]. Conceivably, these obesity-associated microbiome profiles feature organisms that are more adept at processing the energy-rich diets. This theory is supported by metagenomic and biochemical analyses showing that the core gut microbiome of obese individuals has an increased capacity for energy harvesting, compared to lean individuals[111]. This capacity was characterized by the genomic expression of KEGG pathways associated with starch/sucrose metabolism, galactose metabolism, and butanoate metabolism[111]. When the gut microbiota from normal mice is transplanted into germ-free recipients, there is an increase in weight and adiposity without any increase in food consumption[67]. This indicates that the increased energy harvesting capacity is transmissible. The modern Western diet, high in refined sugars and lipids but lacking complex fermentable fibers, is seemingly mismatched with the capacity of our ancestral microbiota. This consequently results in less diversity and a shifted microbiome profile[110]. It has been suggested that a return to ancestral diets, consisting mainly of plant-based complex fibers and low in red meat, can help promote the proliferation of the beneficial microbes considered indigenous to our gut[112].

Epidemiological studies indicate that vegetarians and vegans have lower blood cholesterol and lower risk for CVD compared to omnivores[112,113]. The elimination of red meat from the diet can be beneficial for cardiovascular health[114,115]. It has been reported recently that gut microbes play a significant role in the metabolic processing of red meat. Dietary carnitine and phosphotidyl choline, predominantly from red meat, is converted to trimethylamine (TMA) by gut microbes[116]. TMA is then converted to the proatherogenic metabolite trimethylamine-*N*-oxide (TMAO), which accelerates atherosclerosis in mice[116]. The conversion of dietary carnitine to TMAO is gut microbe dependent, as individuals receiving oral antibiotics for a week prior to consuming red meat experienced a complete suppression of endogenous TMAO production[116]. The same study also reported that vegetarians and vegans had significantly lower fasting baseline TMAO levels, compared to omnivores[116]. Correspondingly, vegetarian and vegans had significantly higher abundance of *Bacteriodes* and lower abundance of *Prevotella* species in the gut microbiome than omnivores, and a decreased risk for coronary heart disease and the traditional risk factors for CVD such as hypertension, atherosclerosis, peripheral artery disease, and stenosis[116]. As vegetarian and vegan diets typically consist of very

high portions of plant fibres, fermentable substrates and low dietary carnitine, all of which are metabolized by gut microbes, this provides strong evidence for a role of the microbiota in CVD.

In terms of prevention and treatment strategies, these findings data are extraordinarily valuable. The increased adiposity, angiogenesis, blood flow, and cardiac output associated with overnutrition and obesity is a major risk factor for hypertension and hyperlipidemia; major risk factors for atherosclerosis, MI and coronary heart disease, all of which predispose to congestive HF[117]. If modulation of the gut microbiome can interrupt this progression at any point, there is a potential to improve an individual's cardiovascular health. Developing the most efficacious method of manipulating a dysbiotic microbiota with no detriment to the host, represents a novel area of investigation for diseases like CVD.

1.5 Modulation of the human microbiome

As our understanding of the elaborate relationship between the human microbiome and the host expands, strategies for modulating the microbiome have evolved. Modern medicine has relied heavily on the prescription of antibiotics in efforts to eradicate infectious pathogens. Some episodes induced by *C. difficile, E. coli, Salmonella* spp., and *H. pylori* can be controlled with the use of antibiotics[118], however not without significant detriment to the host. Antibiotics are relatively non-discriminating agents, unable to distinguish between pathogenic and non-pathogenic bacteria. As a result, antibiotic use disrupts the microbiome and eliminates endogenous bacteria that perform vital functions for our health. Antibiotic-associated diarrhea occurs in at least 20% of antibiotic users, due to the decreased fermentation capacity and malabsorption in the gut[119,120]. Antibiotic use can lead to secondary infections, when opportunistic pathogens normally suppressed by endogenous microbes are able to proliferate. For these reasons, the widespread prescription of antibiotics has been discouraged, and alternative methods are being explored.

Probiotic therapy is one such method that has gained increasing attention among consumers, health practitioners, and researchers. The accumulating reports of the health

benefits of probiotic products in the laboratory and the clinical setting have catapulted novel strategies for probiotic use for diseases to the gastrointestinal system and beyond.

1.5.1 Probiotics to restore homeostasis

The consumption of bacteria is not a modern trend; ancient diets featured fermented food products from various grains, vegetables, beans, fish, and dairy products. In fact, it was the observation of the overall good health and longevity of certain ethnic Europeans consuming fermented foods that brought modern-day prominence to probiotics[121]. Today, the United Nations and World Health Organization and many other organizations define probiotics as live microorganisms which when administered in adequate amounts confer a health benefit on the host[79].

Despite most regulatory authorities not allowing disease claims for foods without pharmaceutical level supporting studies, there is strong clinical evidence supporting probiotic foods and supplements treating bacterial vaginosis[122], diarrhea[123,124], and irritable bowel syndrome[124-128], as well as improving the management of *Helicobacter pylori*[129] and *Clostridium dificile*-associated diarrhea[130]. In most cases, the effects are strain specific and the precise mechanisms for the action not well elucidated. There are several ways in which probiotics interact with the host. First, the introduction of a probiotic species into a bacteria-harboring environment can alter the existing community dynamic. Probiotics have demonstrated competition against pathogens and deleterious organisms, interfering with their ability to infect the host[131,132]. Various mechanisms have been studied including production of bacteriocins, biosurfactants and simple competitive exclusion[72,133,134]. The net effect of suppressing pathogenic activity can be restoration to a 'healthy' equilibrium of the microbial community. Second, probiotics participate in epithelial cross talk with the host immune system[135]. The gut epithelium is a major barrier against foreign pathogens and antigens. Probiotics not only improve the integrity of the epithelial barrier[136], but also interact with toll-like receptors and transcription factors that regulate inflammatory responses[137,138]. This immune-modulating effect has proven beneficial not only for local gastrointestinal diseases such as inflammatory bowel disease and necrotizing enterocolitis[135,137], but also distal conditions such as rheumatoid arthritis[139]. Third, probiotics are producers of many metabolites,

enzymes, co-factors and vitamins that are active in modulating our health. For example, the fermentation of carbohydrates by probiotics results in the production of short chain fatty acids, such as acetate or propionate, which are used as energy sources by the host[68]. Certain probiotic strains produce vitamin K, B2, B12, and folate[140-143], which are utilized by the host.

1.6 Probiotics: Evidence for reducing risk factors for CVD

1.6.1 Via interference with periodontal disease

The use of probiotics in the oral cavity has been investigated for reducing pathogenic agents in several types of oral disease. For example, *L. rhamnosus* and *L. reuteri* strains have shown to reduce the number of *S. mutans*, a caries pathogen, when consumed in yogurt or milk[144,145]. Similarly, the consumption of probiotic cheese and *S. salivarius* reduced the prevalence of oral *Candida* in an elderly population[146]. *Streptococcus salivarius* K12 is a probiotic strain shown to release large amounts of bacteriocins into the saliva that compete with pathogenic microbes[147]. The strain is commercially available in lozenge and chewing gum forms and has been shown to reduce oral candidiasis in a mouse model[148], malodor in patient with halitosis[149], and inhibit baseline secretion of IL-8 while activating the anti-inflammatory pathway of nuclear factor (NF)-κB in human bronchial cells challenged with *Pseudomonas aeruginosa*[150].

Although periodontitis is a very difficult disease to cure, there is encouraging evidence that probiotics can contribute through reduction of periodontal pathogens and inflammation. In two independent clinical studies, *L. reuteri* significantly reduced several clinical indices of periodontal disease, including gingival bleeding, plaque, and microbial levels of periodontal pathogens[151,152]. Similar results have been reported using other lactobacilli and *Bifidobacterium* products, where normalization of the oral microbiota occurred with treatment of gingivitis and periodontitis patients[153,154]. *L. reuteri* also reduces the pro-inflammatory cytokines TNF-α and IL-8 in patients with gingival inflammation, when taken in a chewing gum for two weeks[155]. These studies provide

strong support for the use of probiotics as a therapy for oral diseases, yet mechanistically speaking, the data are incomplete.

The ability of probiotic organisms to attach and colonize the oral cavity are believed to be important, but long-term colonization is rare[156]. There are conflicting reports on the persistence of probiotic strains in the oral cavity once treatment has been removed and it does not seem that colonization is a prerequisite for effective treatment in some cases[157]. Since periodontal disease is a contributing factor in CVD, the ability of probiotics to alleviate the former could influence the latter, although no studies with this specific aim have been performed.

1.6.2 Obesity and adiposity

The increased risk and prevalence of CVD can largely be attributed to diet and lifestyle-derived disorders. Obesity is one of the primary risk factors for CVD, as the progression from overweight to obese introduces a slew of comorbidities that are detrimental to cardiovascular health[158]. The excessive energy intake and fat accumulation in obesity presents major risk for type II diabetes, chronic inflammation, and hyperlipidemia, all of which predispose to coronary heart disease, cardiac arrest, and HF[158]. The accumulating *in vitro*, animal, and human research suggests an opportunity for probiotics to improve these risks. It has been established that obese individuals have a different gut microbiome profile from normal weight individuals[67,85,86,108], indicating a partial role of the microbiome in obesity. The use of probiotics to re-set the dysbiotic obese gut microbiome is one proposed mechanism of action. A study performed on healthy weight mice found that there were significant changes in the gut microbiome as well as significant weight reduction in mice receiving either *L. rhamnosus* GG or *L. sakei* NR28 daily by oral gavage for 3 weeks[159]. Overall, both probiotic treatments resulted in a reduction of the total number of microorganisms in the gut, and the Firmicutes/Bacteriodetes ratio was significantly reduced[159]. This change in ratio was seemingly due to a reduction in *Clostridium* species belonging to the Firmicutes phylum, rather than an increase in Bacteriodetes organisms. There was no significant difference in food consumption between control and probiotic treatment groups, indicating that the significant reduction in epididymal fat mass was not due to a reduction of energy consumption[159]. Results from

this study indicate that the modulation of the gut microbiome with probiotic administration produces an anti-obesity effect that directly reduces epididymal fat mass. However, efforts to significantly alter the human gut microbiota using probiotics have shown less subtle effects[160].

In humans, the twice daily administration of a multi-strain probiotic capsule (*Streptococcus thermophilus*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium lactis*, *Bifidobacterium longum,* and *Bifidobacterium breve*) to overweight individuals with a body mass index greater than 25 resulted in a significant reduction in weight, waist circumference, and serum cholesterol after 8 weeks[161]. These metabolic changes positively correlated with a significant increase in *Lactobacillus plantarum* populations and the overall ratio of gram negative bacteria, presumably representing the Bacteriodetes phylum[161]. Probiotic-induced changes to the gut microbiome composition is not an exclusive prerequisite for weight and adiposity reduction. There are numerous reports of improved body fat mass and weight loss without any differences in gut microbial composition, suggesting and explicit relationship between probiotic and adipose tissue. Certain metabolites have been implicated to directly reduce adipose tissue mass. Conjugated linoleic acid (CLA) produced by certain *Lactobacillus* species have shown to reduce obesity and arteriosclerosis in mice[108,162]. Studies investigating CLA-producing probiotic strains have demonstrated that *Lactobacillus rhamnosus* PL60 and *Lactobacillus plantarum* PL62 reduce body weight and adipose tissue mass in mice on a high fat diet in a CLA-dependent manner, without any changes in food intake[163,164]. Probiotics have directly reduced adipocyte cell size in high fat diet mice[165,166], which can improve oxidative stress and the subsequent chronic inflammation associated with inadequate blood supply to enlarged adipocytes in obesity.

1.6.3 Cholesterol and hypertension

One of the most thoroughly investigated applications of probiotic therapy for CVD is for reduction of serum cholesterol. Elevated low-density lipoprotein cholesterol (LDL-C) is a major risk factor for CVD[167]. The accumulation of LDL-C in the blood is a precursor to hypertension, hyperlipidaemia, and causes the formation and build-up of atherosclerotic

plaque in the arteries[167]. Meta-analyses of randomized controlled clinical trials have been performed to evaluate the effect of probiotic consumption on serum LDL-C and total cholesterol levels. Pooled data from a total 485 total participants with 'high' 'borderline high' and 'normal' serum cholesterol levels found that probiotic consumption significantly lowered LDL-C and total cholesterol levels among all categories, compared to the control[168]. The cholesterol-lowering properties of probiotics are strain and species specific; and among these select probiotics, there are several postulated mechanisms of action. Probiotics can remove cholesterol from the gut by incorporating cholesterol in to the cellular membrane during growth and proliferation. *In vitro* studies using fluorescently tagged cholesterol molecules have tracked the incorporation into the cellular membrane by several strains of lactococci and lactobacilli[169,170]. Cholesterol can also be converted to coprostanol by probiotics expressing cholesterol reductase[170]. Coprostanol is subsequently excreted in feces, potentially reducing cholesterol absorption in the gut.

Perhaps the most accepted mechanism is the processing of bile acids in the gut by Gram-positive organisms including *Lactobacillus, Bifidobacterium,* and *Bacteriodetes*[171]. Metabolism of cholesterol, a precursor of bile acids, is mediated through gut microbes expressing the enzyme bile salt hydrolase (BSH). Probiotic strains with high BSH activity promote the deconjugation of bile acids to secondary amino acid conjugates[172]. When these are then excreted, and cholesterol is broken down to replace the processed bile salts. Overall, this process promotes the catabolism of cholesterol and reduces serum cholesterol levels[171]. There is considerable variability in BSH activity among probiotic species, indicating that genes, which encode it, are likely to be horizontally acquired[173]. It is also known that many probiotic strains express more than one BSH homologue[171]. This might maximize survival because of the variable exposure to different forms of bile salt conjugation in the gut. Further research is required to precisely identify the mechanism of the BSH gene and activity, especially with regards to the kinetics of bile salt transport.

Most of the above mechanisms have been exclusively explored *in vitro,* and more animal and human trials are required. However, regardless of the mechanism implied, the

extensive meta-analyses on human clinical trials indicate that the use of probiotics to lower serum cholesterol is a promising prevention strategy for CVD.

Hypertension, closely tied to hypercholesterolemia, is a major risk factor for CVD[174]. About 30% of Americans are hypertensive, doubling their risk for developing the disease[174]. Reducing hypertension alone, using diuretics, ACE-inhibitors and β-blockers is about 30% less effective than reducing hypertension by treating hypercholesterolemia in hypertensive patients[174]. This suggests that not only does probiotic therapy improve lipid blood chemistry, but it also can improve hypertension and outcomes for CVD patients. Meta-analysis of fourteen randomized placebo-controlled clinical trials with 702 participants showed that probiotic fermented milk significantly reduced both systolic and diastolic blood pressure in pre-hypertensive and hypertensive patients[175]. Certain probiotic strains produce peptides with ACE-inhibitory activity through the proteolysis and fermentation of milk proteins[176,177]. When growth of certain *Lactobacillus* and *Bifidobacterium* strains was enhanced using fermentation substrates, or prebiotics (inulin, pectin, fructo-oligosaccharides, and mannitol), proteolytic activity and ACE inhibition was proportionally increased[177]. Further research on maximizing the production and evaluating the activity of these peptides *in vivo* is required.

By reducing cholesterol and hypertension, two of the most prevalent risk factors for CVD, the risk for developing coronary heart disease, atherosclerosis, heart attack, and stroke is reduced by nearly half[174]. The strong clinical evidence for the attenuation of hypercholesterolemia and hypertension with probiotic consumption brings encouraging support for the treatment of CVD with probiotics.

1.6.4 Direct cardioprotection against apoptosis and ischemic injury

Until recently, probiotic applications for cardiovascular health were limited to metabolic and diet-associated processes. The aforementioned evidence for probiotic therapy for CVD pertains mostly to symptoms of CVD that are precursory to direct heart damage incurred by coronary artery disease, myocarditis, MI, and HF. We wanted to explore whether probiotics can provide a direct, cardioprotective effect to the heart that results in reduced ischemic injury and improved cardiac function, following an ischemic event. If

coronary arteries become occluded, the blocked blood supply to the heart muscle can cause tissue ischemia and infarction. If a patient survives MI, there is a risk for progression to HF and chronic inflammation, as the damaged heart struggles to maintain its regular work capacity. Reduction of ischemic injury and infarction is critical to improving the outcomes of MI patients.

The protective role of probiotics against apoptotic injury was first investigated in intestinal cells. While exploring the mechanisms of action against inflammatory bowel disease, it was found the *L. rhamnosus* GG prevented TNF-α, IL-1α, and interferon (IFN)-γ-induced apoptosis in mouse colon cells[178]. This was achieved through both activation of the anti-apoptotic Akt pathway, and inhibition of the pro-apoptotic p38 mitogen-activated protein kinase pathway[178]. The purification of *L. rhamnosus* GG supernatant identified a novel protein, p75, to be responsible[136]. The effect of this protein on ischemia/reperfusion (I/R) induced heart cell injury was evaluated using a rat model. The pre-treatment of rats with the purified p75 protein isolated from *L. rhamnosus* GG 30 minutes prior to I/R surgery significantly attenuated heart tissue infarction in a dose-dependent manner. This phenotype was reportedly generated by enhanced expression of heat shock proteins with p75 pretreatment[179], suggesting that proteins produced by *Lactobacillus* have a direct cardioprotective effect against ischemic injury. Further mechanistic research is required, as the isolated p75 protein delivered in a bolus, bypassed the gastrointestinal system. Studies examining the production and kinetics of p75 from *L. rhamnosus* GG within the gut could contribute to an understanding of the role of the microbiome in this phenotype.

Recently, a rat study demonstrated that oral consumption of a probiotic could be cardioprotective. Animals administered the probiotic drink marketed as “GoodBelly”, containing *Lactobacillus plantarum* 299v, in their drinking water for up to 14 days before I/R heart surgery, had a 29% reduction in ischemia and a 23% improvement in post-ischemic mechanical function, as measured by left ventricular diastolic pressure[180]. This cardioprotection seemed to be gut microbiome-dependent, as administration of vancomycin generated the same phenotype. It was found that the attenuated ischemia was independent of cytokine mediation, but dependent on serum leptin reduction. There was a

significant increase in serum leptin post-I/R that was significantly attenuated with GoodBelly and vancomycin pretreatment[180]. Pretreatment with leptin abolished the cardioprotection confirming that this molecule played a key role in the process. Leptin is a hormone mainly produced by adipocytes but also by cardiomyocytes, and is typically upregulated in CVDs such as HF and hypertension. This novel finding linking communication between the gut and heart through hormone signaling, warranted further research into the cardioprotective properties of probiotics.

We decided to determine whether probiotic treatment post-ligation injury without reperfusion could confer benefits reported with probiotic pre-treatment. In addition, we wanted to further investigate the mechanisms by which probiotic activity worked.

1.7 Hypothesis and objectives

My hypothesis was that the administration of *Lactobacillus* probiotic strains will have a beneficial effect on the outcome of myocardial infarction-induced heart failure and provide a direct benefit to cardiac tissue through gut-bloodstream mediators.

Objective 1: Assess the effect of oral probiotic administration on the outcomes of gut microbial composition and MI-induced heart failure in a rat coronary artery ligation model.

My aim was to determine the effect of probiotic administration after the MI event. This study assessed changes in the gut microbiota associated with probiotic administration.

Objective 2: Assess the effect of direct probiotic administration on cardiomyocytes expressing the hypertrophic response associated with heart failure.

Using chemical agents to induce hypertrophy, my aim was to evaluate the effect of co-culturing cardiomyocytes with probiotic lactobacilli. I examined both morphological and molecular changes of cardiomyocytes in co-culture. Using a variety of tools for generating different probiotic treatments, this *in vitro* model tested if probiotics directly interact with and benefit cardiomyocytes and help elucidate potential mechanisms.

Chapter 2 : Materials and Methods

2 Overview of experiments

The experiments performed for this thesis can generally be divided into two groups: *in vivo* animal studies, and mechanistic *in vitro* studies. The specific designs for all experiments performed are described in detail in the next sections.

2.1 The effect of probiotic administration on the outcome of heart failure in the rat

In collaboration with Dr. Morris Karmazyn (Department of Pharmacology and Physiology, Western University), a study was designed to investigate the outcome of probiotic therapy for heart failure in the rat. There were two stages: First, a 4 week pilot study was performed on 15 animals using 2 different probiotic strains. This served as a proof-of-concept assessment. Next, a 6 week comprehensive study using 60 animals and only one probiotic treatment was performed. The methods described in this section have been published and are described in detail below[181].

2.1.1 Probiotic culture and treatment preparation

In the 4 week pilot study, two different probiotic strains were prepared as treatments: *Lactobacillus rhamnosus* GR-1 or *Lactobacillus plantarum* 299v. *L. plantarum* 299v was chosen because it was the probiotic strain in the commercially available drink (GoodBelly, NextFoods, Boulder, CO, USA) that was demonstrated to have cardioprotective effects against I/R injury to the heart[180]. *L. rhamnosus* GR-1 was chosen because it has been extensively characterized by several laboratories and has been shown benefit the gastrointestinal and urogenital tracts[182,183]. In addition, the genome sequence for *L. rhamnosus* GR-1 was available within our group, which may have provided further tools for analysis.

All media for bacterial culture were acquired from Becton and Dickinson (BD, Mississauga, ON). All strains were grown under anaerobic conditions at 37°C, using the GasPak™ system (BD). Details on each strain are provided in table 1.

L. plantarum 299v was isolated from a sample of GoodBelly by inoculating de Man, Rogosa, Sharpe (MRS) broth with the drink using an inoculation loop. The broth was

grown for 15 hours then streaked onto MRS agar and grown again for another 15 hours. After 3 subsequent sub-cultures were performed, 2-3 single isolated colonies were added to MRS broth (20% glycerol) in a sterile cyrotube for long term storage at -80°C. Freezer stocks of *L. rhamnosus* GR-1 were maintained in the same media at -80°C. The *L. plantarum* 299v strain was identified by polymerase chain reaction (PCR) using an Eppendorf Mastercycler® PCR machine (Eppendorf, Hamburg, Germany).

Frozen stocks of both *L. plantarum* 299v and *L. rhamnosus* GR-1 were resuscitated by propagating in MRS broth, as described above. Isolated colonies were streaked onto MRS agar to prepare a sub-culture. Single colonies were used to inoculate 3 ml of MRS broth, and in turn used to inoculate 500 mL MRS broth. After growth, the cells were centrifuged at 1600 *x g* for 20 minutes. The supernatant was completely removed, and the cells washed twice with cold sterile phosphate buffered saline (PBS). The pellet was resuspended in 25 mL 10% sterile skim milk (Nestlé, Markham, ON) to a concentration of 3.0×10^{10} colony forming units (CFU)/mL. Aliquots of 100 µL of *L. rhamnosus* GR-1 in skim milk, or skim milk alone as a placebo control, were placed in sterile 1.5 mL Eppendorf tubes to create individual daily treatments. These were stored at -20°C until use.

Bacterial cell numbers were confirmed by serial dilution and bacteriological plate count. In addition, aliquots were subjected to freeze-thaws to reconfirm the bacterial numbers present and to ensure the placebo was not contaminated.

Table 1. Probiotic strains and preferred growth media used in animal studies

	Strain	Source	Non-selective growth media	Selective growth media
L. plantarum	299v	GoodBelly probiotic drink isolate (NextFoods)	MRS broth/agar	LPSM
L. rhamnosus	GR-1	Distal urethral clinical isolate	MRS broth/agar	MRS FA

MRS: de Man, Rogosa, Sharpe *Lactobacillus* culture medium

LPSM: *Lactobacillus plantarum* selective medium[184]

MRS FA: de Man, Rogosa Sharpe *Lactobacillus* culture medium with the addition of 32 µg/mL fusidic acid (FA)

2.1.2 Animals

The animal experiments were approved by the Animal Use Subcommittee of Western University and a copy of the the Animal Use Protocol is provided in the appendix. Procedures adhered to the guidelines of the Canadian Council on Animal Care (Ottawa, ON). The experiments were performed on male Sprague-Dawley rats weighing between 175-225 grams (body weight was determined immediately prior to commencing the study). Animals were randomly assigned treatment groups as outlined in table 2. Animals were fed a standard chow diet throughout the studies.

2.1.3 Coronary artery ligation model for heart failure in the rat

Heart failure was induced using a sustained coronary artery ligation (CAL) model. Rats were anesthetized by intraperitoneal injection with pentobarbital sodium (5 mg/kg body weight), intubated, and artificially ventilated using a rodent respirator (model 683, Harvard Apparatus). To induce myocardial infarction in the LV, the left main coronary artery was ligated using a silk suture. For sham operation, the suture was placed in the exact fashion, then was removed without any tying. All animals were housed singly per cage after surgery. The animals sustained ligation for either 4 or 6 weeks without any reperfusion of the coronary artery, then were euthanized for further analysis. The surgery was performed by Dr. Karmazyn's staff, and the analyses performed for each trial are listed in table 3.

2.1.4 Probiotic administration

Probiotic administration was provided immediately following the CAL or sham surgery. Rats were provided 1 dose/day of probiotic in their drinking water for *ad libitum* consumption for either 4 or 6 weeks. The water was changed daily. The specific surgeries and probiotic treatments are listed in table 2. Based on typical daily water consumption, it was estimated that the average probiotic dose in the active treatment group was 1.5×10^9 CFU/day.

Table 2. Surgery and treatment assignments for animal studies

	Group #	**Surgery**	**Treatment**
4 week pilot study (n=5/group)	1	CAL	Water
	2	CAL	*L. plantarum* 299v
	3	CAL	*L. rhamnosus* GR-1
6 week study (n=10/group)	1	Sham	Water
	2	Sham	*L. rhamnosus* GR-1
	3	CAL	Water
	4	CAL	*L. rhamnosus* GR-1
	5	CAL	Sterile skim milk
	6	CAL	4 weeks *L. rhamnosus* GR-1 + 2 weeks sterile skim milk

CAL: coronary artery ligation

Table 3. Analyses performed for animal studies

Study	Parameters measured
4 week pilot study (n=5/group)	Left ventricular hypertrophy, cardiac mechanical function, bacterial cultivation of digesta
6 week study (n=10/group)	Left ventricular hypertrophy, cardiac mechanical function, left ventricular internal dimensions, hemodynamics, blood cytokine and adipokine concentrations, bacterial cultivation of digesta, microbial profile analysis of cecum digesta

2.1.5 Echocardiography

Echocardiography evaluations were performed on each animal prior to CAL surgery and probiotic treatment (week 0), and every 2 weeks thereafter until sacrifice. Rats were

anesthetized with 2% isoflurane, placed in supine position on a heated platform. Images were analyzed using the Vevo 770 Protocol-Based Measurements software (VisualSonics, Canada) and calculations for the dimensions of the LV diameter were taken using M-Mode 2 dimensional echocardiography images. Doppler measurements were taken to determine peak early diastolic filling velocity (E wave), peak late diastolic filling velocity (A wave), and E/A ratios. The echocardiography evaluations were performed by Dr. Karmazyn's staff. The same reader conducted all evaluations blindly.

2.1.6 Hemodynamic measurement

Hemodynamic measurement was taken immediately prior to sacrifice, 6 weeks after CAL or sham surgery. Rats were anesthetized with pentobarbital sodium (50 mg/kg body weight), and an anterior thoracotomy was performed. The LV was catheterized once via the right carotid artery using a 2.0F P-V Mikro-Tip catheter (Millar Instruments, USA), and the left ventricular systolic and diastolic pressures were measured. The hemodynamic measurement was performed by Dr. Karmazyn's staff.

2.1.7 Blood collection and chemokine/cytokine analysis

Immediately following hemodynamic measurements, 4 mL of blood was collected by terminal bleeding directly from the heart and kept on ice in Vacutainer™ 10.8 mg K2 EDTA blood collection tubes (BD) until processing. Blood samples were centrifuged at 1000 *x g* for 10 minutes and plasma was stored at -80°C. The following nine cytokines were chosen from The MILLIPLEX® MAP Rat Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore, Billerica, MA, USA) for blood cytokine analysis: Fractalkine, GRO/KC, IFN-γ, IL-1α, IL-6, MCP-1α, MIP-1α, RANTES, and TNF-α. Blood plasma samples were thawed to room temperature and the assay and analysis was performed following the manufacturers protocol for the Bio-Plex 200® system (Bio-Rad, Mississauga, ON).

2.1.8 Heart weight measurement and tissue processing

At sacrifice, the hearts were removed from the animals, images were obtained, and the whole heart and dissected LV were weighed. Fifty to one hundred milligrams of tissue

from the LV (non-ischemic region) of each heart from the six week-long study was collected and stored at -80°C for later ANP gene expression.

2.1.9 ANP gene expression analysis of left ventricular tissue

RNA was collected from the non-ischemic region of left ventricular tissue using QIAzol Reagent (QIAGEN, Mississauga, ON) as per the manufacturer's instructions. Reverse transcription (RT) of RNA into cDNA for real-time quantitative polymerase chain reaction (qPCR) analysis of ANP gene expression was performed using M-MLV Reverse Transcriptase (Invitrogen, Burlington, ON) as per the manufacturer's protocol. The real-time qPCR reactions were performed using SYBR Green Master Mix (Applied Biosystems, USA), and gene products were quantified using a DNA Engine Opticon 2 thermal cycler. PCR conditions and primer sequences are summarized in table 4. The housekeeping gene, 18S rRNA, was measured and quantified to normalize gene expression levels using the standard curve method. The analysis was performed by Dr. Karmazyn's staff.

Table 4. Gene primer sequences and cycle conditions used for real-time quantitative PCR analysis of heart tissue

Gene	**Forward Primer Sequence (5'-3')**	**Reverse Primers sequence (5'-3')**
ANP	CTGCTAGACCACCTGGAGGA	AAGCTGTTGCAGCCTAGTCC
18S rRNA	GTAATCCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Cycle conditions	40 x (30 seconds @ 95°C , 25 seconds @ 60°C, 30 seconds @ 72°C)	

ANP: atrial natriuretic peptide

2.1.10 Cecum digesta sample collection and bacterial cultivation

After sacrifice, the cecum and colon were removed immediately. Three grams of cecum and colon digesta were collected and frozen at -80°C until further use, or immediately used for cultivation experiments. Fresh cecum and colon digesta (0.3 g each) were resuspended in 1 mL sterile PBS. Serial dilutions (10^{-3}-10^{-8}) were made and 10 µL of each was drop-plated in triplicate. The selective growth media listed in table 1 were used to isolate colonies of both *L. rhamnosus* GR-1 and *L. plantarum* 299v. For isolating *L. rhamnosus* GR-1, 32 µg/mL fusidic acid (FA) (Sigma, Mississauga, ON) was filter sterilized and added to sterile MRS agar. For isolating *L. plantarum* 299v, *Lactobacillus plantarum* Selective Media (LPSM) was used[184]. This medium has the same MRS formulation except for the replacement of dextrose with sorbitol, and the addition of 0.02g/L bromocresol purple, and later 0.02 g/L filter sterilized ciprofloxacin to the sterile agar medium. In the presence of bromocresol purple, the production of acid from sorbitol by *L. plantarum* 299v turns the originally purple media yellow. MRS agar, MRS FA and LPSM plates were incubated at 37°C anaerobically for 48 hours and colonies were enumerated.

2.1.11 Cecum microbiota analysis

Both the colon and cecum samples were cultivated on selective media immediately following sacrifice. Once the colonies were isolated and enumerated it became apparent that both sites had similar bacterial abundance. It has been reported that nearly all endogenous metabolic activity by gut bacteria in the rat occurs in the cecum and colon only[185]. Based on the levels of short chain fatty acids detected, the gut microbiota of the cecum is more robust in fermenting and metabolism than the colon[185]. As metabolite levels are indicative of the function and activity of the endogenous bacteria, this suggests that the majority of gut microbial activity in the rat is housed in the cecum. Based on this, and the similar results from the cultivation of cecum and colon digesta, it was decided to consolidate the next-generation sequencing 16S rRNA gene analysis by investigating the microbiota of cecum digesta only.

Cecum digesta samples from the six week study were thawed to room temperature and DNA was extracted using the QIAamp DNA stool mini stool kit (QIAGEN) according to the manufacturer's protocol with the following additional steps: approximately 0.2 grams of digesta was suspended in the lysis buffer with a 90 second bead beating step prior to 95°C lysis. For optimal DNA yield, all centrifuge times were doubled and the final incubation with the Buffer AE elution buffer was increased to 2 minutes. DNA from each sample was immediately quantified using a Qubit® 2.0 fluorometer (Life Technologies, Burlington, ON), and stored at -80°C.

2.1.12 Amplification of bacterial DNA

The V6 hypervariable region of the 16S rRNA gene was amplified from DNA template extracted from each cecum digesta sample (refer to table 5 for primer sequences and cycle conditions). Each left forward 5' primer (V6LT) was tagged with a unique barcode sequence at the 3' end. This barcode enabled identification of the samples after the sequencing. The right reverse primer (V6RT) contained an Ion Torrent adapter sequence on the 5' end.

Forty µL PCR reactions were prepared using 5 µL of DNA template per reaction, or 5 µL of nuclease-free water as negative control. Each PCR reaction contained 1.5 mM $MgCl_2$ 0.8 µM each primer, 4 µL 10X PCR buffer (Invitrogen), 0.2 mM dNTPs, 0.05U GO *Taq* polymerase (Invitrogen), and 0.1 µL 5% bovine serum albumin (Sigma). PCR was performed as described using an Eppendorf Mastercycler® PCR machine (Eppendorf).

Amplification products were quantified using Qubit® fluorometer (Life Technologies) to determine DNA concentration, and equimolar quantities were pooled and purified using the QIAquick PCR purification kit (Invitrogen). V6 16S rRNA next-generation sequencing using the Ion Torrent platform (Life Technologies) was then performed at the London Regional Genomics Center, Robarts Research Institute, Western University, as per the Center's standard operating procedure.

Table 5. Primer sequences and cycle conditions used for PCR amplification of DNA extracted from cecum digesta samples.

16S rRNA gene	**Primer sequence (5'-3')**
V6LT	CCATCTCATCCCTGCGTGTCTCCGACTCAG
V6RT	AC(A or G)ACACGAGCTGACGAC
PCR cycle conditions	1 X (2 minutes @ 95°C) 25 X (1 minute @ 95°C, 1 minute @ 55°C, 1 minute @ 72°C)

V6LT: Left forward V6 primer

V6RT: Right reverse V6 primer

2.1.13 Sequence Analysis

Raw sequence data was filtered, processed, and analyzed using a modified version of a data analysis pipeline developed by Dr. Gregory Gloor (Department of Biochemistry, Western University)[186]. Custom Perl scripts were used to assign barcoded reads to each individual sample. For quality control, each bar-coded sequence required a minimum 500 reads. Reads were discarded that weren't within 70 – 90 base-pairs in length between the right and left primers. To avoid erroneous taxonomic assignment that is intrinsic to Ion Torrent sequencing in cases of low abundance reads, sequences present at less than 0.5% abundance were not kept for analysis. Sequences present at 0.5% abundance and greater were clustered by similarity to a seed sequence at 97% identity using Uclust version 3.0.617[187]. The most abundant sequence in each cluster was designated as a representative operational taxonomic unit (OTU). Taxonomic assignments were made using Seqmatch from the Ribosomal Database Project[188]. Seqmatch data was parsed and matched using the Greengenes database[189]. For multiple top matches with equal scores, the highest common taxonomy was assigned to a given OTU. Classification was assigned at either the family or genus level if the sequence alignment was less than a 95% match. Taxonomic assignments were then arranged and presented using QIIME for 16S rRNA analysis[190] and microbial communities from each sample were compared using weighted UniFrac beta-diversity analysis[191].

2.2 The effect of probiotic administration on hypertrophic cardiomyocytes cultured *in vitro*

To assess the effect of probiotic administration on cultured cardiomyocytes, co-culture assays were developed using neonatal rat ventricular cardiomyocytes (NVCM). Primary NVCM cultures were isolated from 1-3 day old neonatal Sprague-Dawley rat pups by lab staff of Dr. Karmazyn as previously described[43] and in accordance with the guideline of the Canadian Council on Animal Care (Ottawa, ON). For experiments, NVCM isolated from the same litter were pooled to represent one biological replicate (one "n" value). I developed assays to evaluate the effect of probiotic treatment on hypertrophic NVCM, using cell surface area and gene expression measurements as indices of hypertrophy. The specific procedures for each assay are described below.

2.2.1 Neonatal rat ventricular cardiomyocyte culture

NVCM were plated on Primaria™ (Falcon) culture dishes at optimal concentrations for each co-culture experiment: 3×10^4 cells to allow room for growth and visualization for cell surface area measurements and 6×10^4 cells to ensure sufficient RNA production for gene expression experiments. Two mL of warm culture medium (table 6) was added to each dish and the cells were maintained at 37°C 5% CO_2. After 48 hours, cells were washed with warm PBS-ABC (table 7) and the culture media was changed daily thereafter. All reagents were tissue culture grade and filter sterilized or autoclaved. The NVCM cell culture medium was adjusted to a pH of 7.10. Cells were maintained for either 3 days for cell surface area experiments, or 5 days for gene expression experiments, before commencing each experiment.

Table 6. Culture medium reagents for NVCM cell culture

Chemical Name	Manufacturer/ Catalogue #	Concentration
DMEMF12 + HEPES	Gibco 11330-032	1L
$NaHCO_3$	Sigma/S6014	30 mM
Fetal Calf Serum	Gibco 12483-020	10%
Transferrin	Sigma/T-0665	10 mg/L
Insulin	Sigma/I-5500	10 mg/L
Penicillin/Streptomycin	Gibco/15140-122	1%
Bromodeoxyuridine	Sigma/B-5002	0.1 mM
Linoleic Acid	Sigma/L-1012	5 mg/L
Bovine Serum Albumin (Fraction V)	Gibco/15260-037	2 g/L
MEM Non-essential Amino Acids	Sigma/A-4403	1%
Vitamin x 100	Gibco/11120-052	0.1%
Sodium Selenite	Sigma	10 µg/L
Pyruvic acid	Sigma	3mM
L-Ascorbic Acid		100 uM

Table 7. PBS solutions for NVCM cell culture

Reagent	**Chemical**	**Concentration**
PBS A	NaCl	10 g/L
	KCl	0.250 g/L
	$Na_2HPO_4 \cdot 7H_2O$	2.71 g/L
	KH_2PO_4	0.250 g/L
PBS B	$CaCl_2 \cdot 2H_2O$	1.32 g/L
PBS C	$MgCl_2 \cdot 6H_2O$	2.13 g/L
PBS ABC[i]	PBS A	80%
	PBS B	10%
	PBS C	10%

PBS: phosphate buffered saline

[i]PBS ABC was prepared fresh from PBS A, PBS B, PBS C stocks at the beginning of each NVCM cell culture experiment.

2.2.2 Probiotic cultures and conditions

Five probiotic strains were used for NVCM co-culture experiments: *Lactobacillus rhamnosus* GR-1, *Lactobacillus rhamnosus* CMPG10200, *Lactobacillus reuteri* RC-14, *Lactobacillus plantarum* 299v, and *Streptococcus salivarius* K12.

Lactobacillus rhamnosus CMPG10200 is a mutant *L. rhamnosus* GR-1 strain (details are provided in the next section). *L. reuteri* RC-14 is a vaginal clinical isolate, and *S. salivarius* K12 is a probiotic strain used for oral health in the probiotic lozenge BLIS K12® (BLIS Technologies, Dunedin, Otago, New Zealand). *S. salivarius* K12 was grown in Todd Hewitt broth/agar.

The strains were resuscitated from -80°C freezer stocks kept at the Canadian Centre for Human Microbiome and Probiotic Research (London, Ontario, Canada) and propagated to second sub-cultures as described in the previous section. All strains were grown to 10^9 CFU/mL, anaerobically using the GasPak™ system (BD) at 37°C.

2.2.3 Msp1 knock out *L. rhamnosus* GR-1 strain CMPG10200

The major secreted protein (Msp1) produced by *L. rhamnosus* GR-1 is homologous to p75[192], the protein produced by *L. rhamnosus* GG that has shown to have anti-apoptotic activity in intestinal cells[136] and confers protection against I/R in the heart[179]. The Msp1 knock out mutant strain was kindly provided by Dr. Sarah Lebeer (Department of Bioengineering, University of Antwerp, Belgium). The strain was constructed by insertational mutagenesis[192]. Briefly, an internal fragment of the *msp1* gene was amplified by PCR and cloned into pCRII-TOPO vector. (Invitrogen). The vector was digested with *Eco*RI and further ligated into an erythromycin-resistant vector. *E. coli* TOP10F was transformed using this ligation product and the suicide vector was then electroporated to *L. rhamnosus* GR-1. *L. rhamnosus* CMPG10200 was grown in MRS broth/agar with the addition of 5 µg/mL erythromycin.

The Msp1 protein is a cell wall hydrolase that is necessary for daughter cell separation[192]. This protein was of interest because in previous studies it was shown to protect against ischemic injury in the heart and prevented stress-induced cellular apoptosis in intestinal

epithelial cells[136,178,179,193]. Prior to receiving the strain, the absence of Msp1 knock-out was confirmed by Western blot using anti-Msp1 antiserum. Microscopic examination of the mutant also confirmed the absence of Msp1: While the mutant is able to grow to the same density as the wild-type (WT), there is a clear defect of cell wall separation visualized by bright-field microscopy (figure 1).

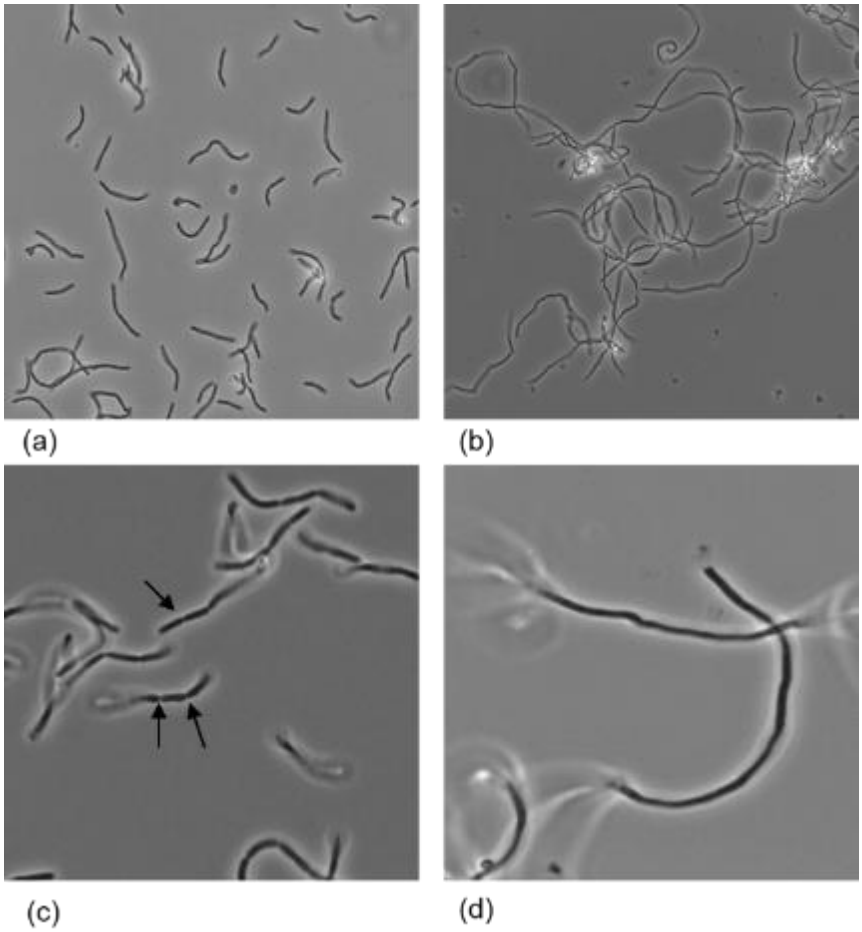

Figure 1: Phase-contrast images of WT GR-1 and CMPG10200 strains

(a) WT GR-1 (40x); **(b)** CMPG10200 (40x); **(c)** WT GR-1 (100x). Arrows indicate WT sites of cell septa; **(d)** CMPG10200 (100x)

2.2.4 Probiotic treatment preparation

The probiotic treatments designed for the NVCM/probiotic co-culture experiments are listed in table 8. Following resuscitation and propagation, 10 mL of the appropriate broth media was inoculated from a single isolated colony of each probiotic strain from an agar plate, and the culture was grown anaerobically at 37°C as described above. The broth culture (10^9 CFU/mL) was used as the standard probiotic treatment for each strain. All cultures were serially diluted and drop-plated onto the appropriate agar media to confirm concentration.

To create the probiotic-conditioned media (PCM) with probiotic cells removed, 1 mL of the broth culture was placed in a clean, sterile, microcentrifuge tube and centrifuged at 12,000 *x g* at 4°C for 5 minutes. The supernatant was carefully collected without disturbing the bacterial pellet and transferred to a new, sterile microcentrifuge tube. This centrifugation step was repeated, and the supernatant was transferred to a new sterile microcentrifuge tube, to be used as the PCM treatment. Samples of the PCM treatments were plated onto MRS agar to ensure no probiotic cells could be detected by cultivation.

Aliquots of the PCM were also either heat denatured (HD) by incubation at 80°C for 30 minutes or incubated in a 1:1 trypsin-EDTA (Gibco, Burlington, ON) solution at 37°C for 20 minutes. The trypsin-PCM (Tryp PCM) solution was then treated with a 0.05% trypsin soybean inhibitor *Glycine max* (Sigma) to eliminate trypsin activity on the NVCM culture. For filtered PCM treatments, the probiotic PCM was filtered using Centricon Plus-20 centrifugal filters according to manufacturer's instructions (Millipore). Both the filtrate and retentate were collected and applied as a treatment.

The original bacterial pellet from the PCM treatment preparation was saved, washed with ice cold sterile PBS, and centrifuged at 12,000 *x g* at 4°C. The supernatant was discarded and the pellet was resuspended in 1 mL of sterile PBS. The pellet was then vortexed thoroughly and the suspension was used as the live probiotic cell treatment, with PCM removed. Aliquots of these cells were heat-killed (HK) by incubation at 80°C for 30 minutes. These cells were plated onto MRS agar to ensure no live probiotic cells were present.

All volumes of probiotic treatments used in the co-culture experiments were 50 µL, unless otherwise stated. The average concentration of all probiotic cultures used in each experiment was 10^9 CFU/mL.

Table 8. Probiotic treatments for NVCM co-culture studies.

Probiotic Strain	Treatment
L. rhamnosus GR-1	Standard probiotic treatment **(GR-1)**
	Live cells suspended in PBS **(GR-1 cells)**
	Heat-killed cells suspended in PBS **(HK GR-1 cells)**
	PCM **(GR-1 PCM)**
	Heat-denatured PCM **(HD PCM)**
	Centrifugal filtered PCM **(PCM Filtrate/Retentate)**
	Trypsinized PCM **(Tryp PCM)**
L. rhamnosus CMPG10200	Standard probiotic treatment **(CMPG10200)**
	Live cells suspended in PBS **(CMPG10200 cells)**
	PCM **(CMPG10200 PCM)**
L. plantarum 299v	Standard probiotic treatment **(299v)**
L. reuteri RC-14	Standard probiotic treatment **(RC-14)**
S. salivarius K12	Standard probiotic treatment **(K12)**

2.2.5 Induction of hypertrophy and probiotic administration

To induce hypertrophy in NVCM, the α1-adrenergic receptor agonist phenylephrine (PE) was applied to the cells. Following 3 days of culture for cell surface area experiments and 5 days for gene expression experiments, 10 μM PE was added to each dish. After gently swirling to distribute the PE, a probiotic treatment was immediately added to the NVCM in triplicate. The dishes were gently swirled again, and incubated at 37°C, 5% CO_2 for 24 hours. After the 24 hour co-culture, the medium was removed from each dish and replaced with PBS. Media containing probiotic cells were spread-plated to determine viability after the 24 hour co-culture.

2.2.6 Cell surface area measurement and analysis

The physical effect of probiotic administration on hypertrophic NVCM was evaluated by taking cell surface area measurements. Cells were viewed and images were obtained using phase contrast microscopy with the Nikon Eclipse TE2000 inverted microscope (Nikon Instruments Inc., USA). Surface area measurements of 50 random cells from at least 5 fields of view per dish (20x objective) were conducted blindly using the calibrated NIS-Elements BR software (Nikon Instruments Inc., USA). The average cell surface area per dish was used to calculate a total average cell surface area per treatment. The fold change in surface area was determined by dividing the treatment average by the control average. All experiments were repeated independently at least 5 times.

2.2.7 Gene expression of hypertrophic markers in NVCM co-cultured with probiotics

In order to confirm that the change in cell surface area was due to an attenuation of hypertrophy, the gene expression of the hypertrophic markers ANP and α-skeletal actin (aSKA) were measured after a 24 hour treatment period. The analysis was performed using the real-time qPCR technique, and all experiments were repeated independently 3 times.

2.2.8 RNA extraction and purification

Following the 24 hour treatment period, the medium was aspirated off each dish and stored in sterile a microcentrifuge tube at 4°C for later bacterial enumeration. 1 mL of TRIzol (Invitrogen) was added to each dish. The cells were scraped thoroughly off of each dish using a plastic cell scraper and the TRIzol mixture was transferred to an RNase-free 1.5 mL microcentrifuge tube, vortexed, and incubated at room temperature for 10 minutes. Two hundred µL of chloroform was added to each tube, vortexed vigorously, and incubated at room temperature for 10 minutes. The tubes were then centrifuged at 16,000 *x g* for 15 minutes at 4°C. 500 µL of the upper aqueous phase containing RNA was carefully transferred into a new RNase-free microcentrifuge tube. 500 µL of 100% ethanol was added to each tube containing the aqueous RNA and the tubes were vortexed for 20 seconds. 500 µL of the ethanol-RNA mixture was transferred to a PureLink® RNA spin column (Life Technologies). The column was centrifuged at 12,000 *x g* for 15 seconds, and the flow through was discarded. This was repeated until all of the ethanol-RNA mixture was passed through the column. Following the PureLink® RNA mini kit protocol for binding, washing, and elution (Life Technologies), RNA was recovered and quantified using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). Purity of RNA was quantified using 260/280 and 260/230 ratios. RNA quality cut-off was set at >1.75 and >1.8 for 260/280 and 260/230, respectively. RNA quality was also confirmed by viewing products via electrophoresis in a 1% agarose gel using 1x TAE stained with ethidium bromide, and viewed under UV light using AlphaImager (Alpha Innotech Corporation, USA).

RNA samples falling under the quality limits underwent an ethanol precipitation protocol as follows: 3 µL of sodium acetate and 90 µL of 100% ethanol were added to each RNA sample for overnight precipitation at -20°C. The next day, RNA samples were centrifuged at 16,000 *x g* for 20 minutes at 4°C. The supernatant was discarded and the pellet containing crude RNA was resuspended in ice-cold 70% nuclease-free ethanol, vortexed, and centrifuged at 16,000 *x g* for 20 minutes at 4°C. This step was repeated once more. All the ethanol was removed from each tube using a fine pipette tip, and the tubes were left open for 1 minute to evaporate the last of the ethanol. The crude RNA was

then resuspended in 15 µL nuclease-free water, and quality was assessed using the NanoDrop 1000 spectrophotometer, as described above. RNA samples were stored at -20°C until further processing.

2.2.9 Reverse transcription

Samples of RNA ranging from 300 ng – 2 µg were used as a template for RT PCR. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Canada). The master mix and cycle conditions for RT are listed in table 9.

Ten µL of the RT master mix was combined and mixed with 10 uL of RNA sample (or 10 uL of nuclease-free water for the negative control) in an RNAase-free 96-well plate, creating a 20 µL reaction. The PCR reaction was carried out in an Eppendorf Mastercycler® PCR machine (Eppendorf). cDNA concentration was measured on all negative controls and 3 random cDNA samples using a Qubit® 2.0 fluorometer (Life Technologies) to confirm successful RT. The cDNA was then diluted into 340 µL of nuclease-free water and stored at -20°C until further use.

Table 9. Master Mix components for reverse transcription

Component	**Volume/reaction (µL)**
10x RT Buffer	2.0
25x dNTP Mix (100mM)	0.8
10x RT Random Primers	2.0
Multiscribe™ Reverse Transcriptase	1.0
Nuclease-free water	4.2
Final Volume	10
PCR conditions	10 minutes @ 25°C 120 minutes @ 37°C 5 minutes @ 85°C

RT: Reverse transcription

dNTP: deoxyribonucleotide triphosphate

2.2.10 Quantitative real-time PCR

qPCR was used to evaluate the gene expression of the hypertrophic markers atrial natriuretic peptide (ANP) and α-skeletal actin (aSKA), relative to a housekeeping gene, in this case 18S rRNA. ANP, aSKA and 18S rRNA primer sequences were designed using the Primer-BLAST tool from the National Center for Biotechnology Information (NCBI)[194]. The sequences are listed in table 10.

The primers were stored in a stock solution containing 800 nM of both forward and reverse primer. Prior to running the cDNA samples, the primers were validated using serial dilutions of a positive control cDNA sample. Five µL of each serial dilution was combined in triplicate with 10 µL Power SYBR Green Master Mix (Life Technologies), and 5 µL of the 800 nM primer stock solution. The PCR reaction was then carried out in a 384-well reaction plate using the 7900 HT Sequence Detection System (SDS) and primer efficiencies were determined using SDS 2.3 Sequencing Software (Applied Biosystems, Life Technologies). The efficiencies for 18S rRNA, ANP and aSKA respectively were 97.24, 97.50 and 98.03%. The PCR products were then viewed via electrophoresis in a 3% agarose gel using 1x TAE stained with ethidium bromide, and viewed under UV light using AlphaImager (Alpha Innotech Corporation, Santa Clara, CA, USA) (figure 2).

After primer validation, cDNA samples were used as template for qPCR reactions. 5 µL of each cDNA sample was combined in triplicate with 10 µL Power SYBR Green Master Mix (Life Technologies), and 5 µL of 800 nM primer stock solution. Each reaction was carried out in triplicate for all 3 primers in a 384-well reaction plate using the same machine and method as above.

2.2.11 Real-time quantitative PCR data analysis

Analysis was carried out using RQ Manager 1.2 Data Analyzer (Applied Biosystems, Life Technologies). Relative gene expression of aSKA and ANP was determined using 18S rRNA as the endogenous control. Gene expression in a treatment cDNA sample

measured in terms of relative quantification (RQ), using the comparative threshold cycle ($C_{t)}$ method. The C_t of the PCR reaction in which aSKA or ANP was detected was compared to the C_t of detection for 18S rRNA. The total difference in C_t over 40 cycles of PCR is then used to determine the RQ of a sample. RQ represents the fold change in gene expression compared to a calibrator (untreated control cDNA sample). The RQ of the calibrator = 1. The standard deviation (SD) of C_t values was used to determine the quality of the technical replicates in each sample. If the SD was over 0.25, the RQ value for that sample was considered unreliable and was not used in the data analysis.

Table 10. Genes and primer sequences used for quantitative real-time PCR.

Gene	Primer Sequence
aSKA (NM_019212.2)	Forward: 5'-CAGAGTCAGAGCAGCAGAAACT-3' Reverse: 5'-GTTGTCACACACAAGAGCGG-3' Product size: 71 base pairs (BP)
ANP (NM_012612.2)	Forward: 5'-CCCTCCGATAGATCTGCCCT-3' Reverse: 5'-TTCGGTACCGGAAGCTGTTG-3' Product size: 148 BP
18S rRNA (NR_046237.1)	Forward: 5'-GTAACCCGTTGAACCCCATT-3' Reverse: 5'-CCATCCAATCGGTAGTAGCG-3' Product size: 148 BP
PCR cycle conditions	40x (10 minutes @ 95°C, 15 seconds @ 60°C)

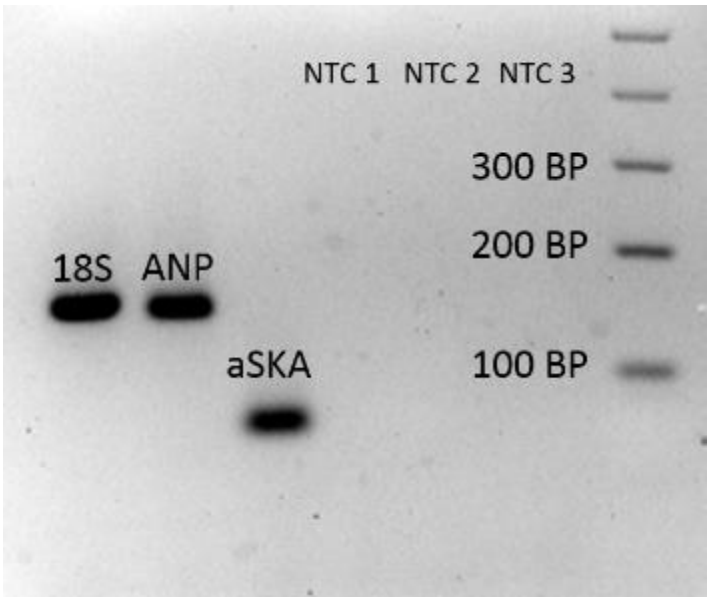

Figure 2. PCR products from primer validation for real-time qPCR

2.3 Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5 and statistical analysis reports for all NVCM experiments are provided in the appendix.

Data from CAL animal studies were reported as means ± standard error. Echocardiographic data were analyzed using 2-way analysis of variance (ANOVA) with repeated measures and a post hoc Tukey test and all other data were analyzed using a 1-way ANOVA followed by a post hoc Tukey test. Differences were considered significant when $P < 0.05$. Statistical analysis for the next-generation sequencing data was performed using Uclust[187], QIIME[190], UniFrac[191], and a modified version of a data analysis pipeline published by Dr. Gregory Gloor[186,195].

For NVCM experiments, technical triplicates for each experiment were performed on at least 3 biological replicates. Data reported are means of biological replicates ± standard deviation. Differences between means of the treatments were compared using a 1-way ANOVA followed by a post hoc Tukey test, or by a Student's *t*-test. Differences were considered significant when $P < 0.05$.

Chapter 3 : Results

3 Results

3.1 Probiotic administration attenuates heart failure in the rat

In both the 4 week pilot study and the 6 week study, probiotic administration attenuated several parameters of HF after MI induced by CAL surgery. Overall, none of the surgeries or treatments exerted any effect of body weight growth throughout the trials. In terms of the outcome of HF, animals receiving *L. rhamnosus* GR-1 for 4 weeks, followed by a 2 week treatment cessation period had the identical outcome as animals receiving *L. rhamnosus* GR-1 for the entire 6 week trial. Herein, the data corresponding to "GR-1" represents to the 6 week treatment group only. There was also no difference in outcome in animals on the control skim milk treatment versus the control water treatment. Because there was no sham group on skim milk treatment, it was decided to use the water control group for the data analysis. Herein, the data corresponding to "water" represents the control group for either sham or CAL operated animals. The outcomes on specific indices for HF are described in detail in following sections.

3.1.1 Probiotic administration attenuates cardiac hypertrophy

Cardiac hypertrophy was measured by comparing the LV weight (LVW) to total body weight (BW) for each animal. An increase in the LVW/BW ratio indicates left ventricular hypertrophy. In both studies, CAL significantly increased LVW ($P < 0.05$). Figures 3 and 4 show that this was significantly attenuated by both *L. rhamnosus* GR-1 and *L. plantarum* 299v administration ($P < 0.05$).

In the 6 week-long study, hypertrophy was also measured by ANP gene expression in the LV. CAL significantly increased ANP expression, indicating a hypertrophic response in the LV, 6 weeks post-surgery ($P < 0.05$). This response was nearly normalized with *L. rhamnosus* GR-1 administration (figure 4).

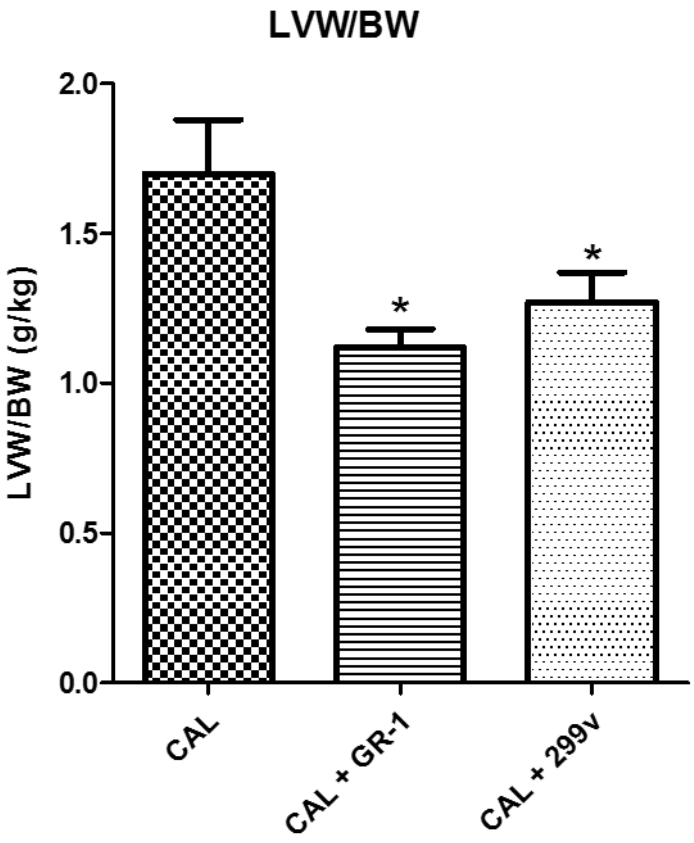

Figure 3. LVW/BW ratios in animals subjected to 4 weeks of CAL

The left ventricle of each animal was dissected and weighed at the end the study period. The LVW/BW ratio was significantly reduced by GR-1 and 299v treatment. Error bars indicate ± SEM (n=5). *$P < 0.05$ compared to CAL.

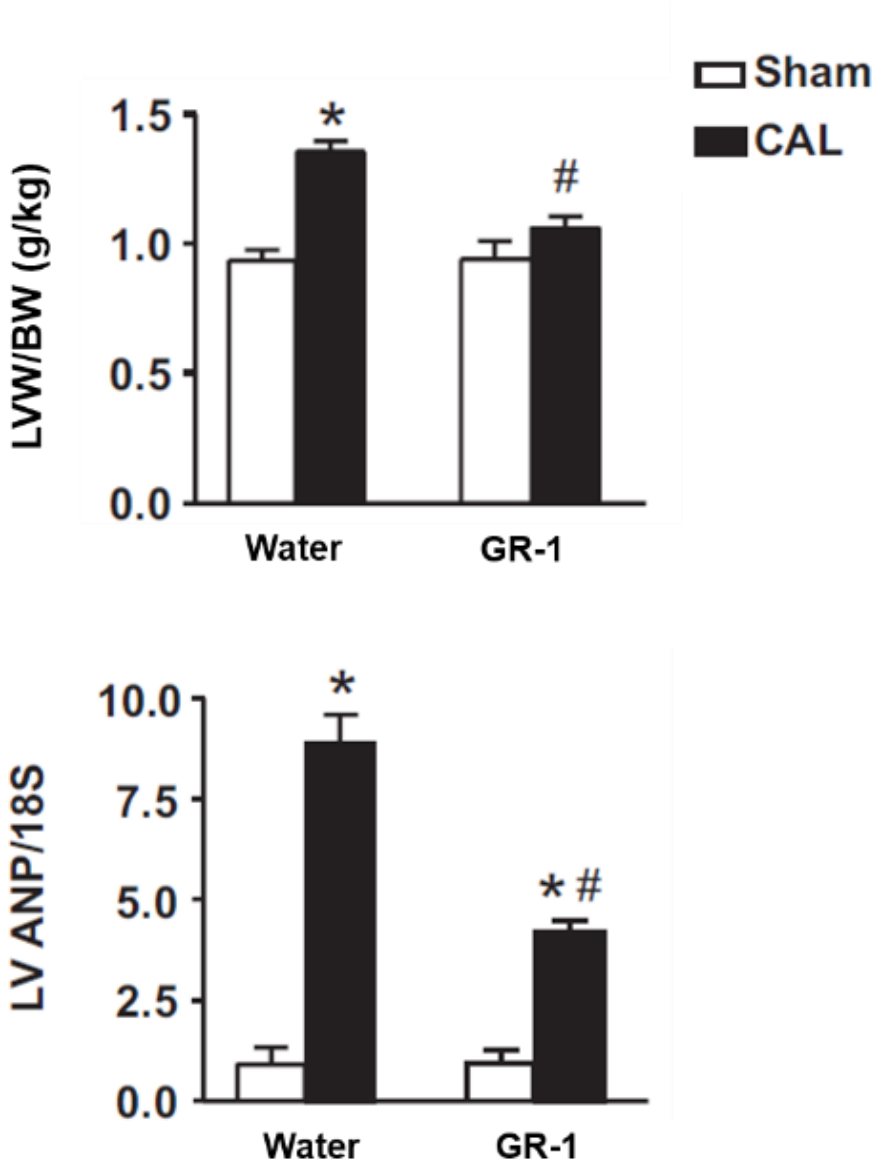

Figure 4. LVW/BW ratios and LV ANP gene expression in animals subjected to CAL or sham surgery for 6 weeks

The left ventricle of each animal was dissected, weighed, and processed for qPCR at the end the study period. Gene expression of ANP was normalized to the 18S rRNA housekeeping gene and is reported as a ratio to 18S rRNA expression. Error bars indicate ± SEM (n=10). * $P < 0.05$ compared to the sham group; $^{\#}P < 0.05$ compared to CAL + water.

3.1.2 Probiotic administration attenuates LV dysfunction post-CAL

Cardiac mechanical function was assessed by serial echocardiography. Animals were analyzed immediately prior to surgery and probiotic treatment, and every two weeks thereafter. Figures 5 and 6 show data from LV echocardiographic analyses performed over the 4 week pilot study and the 6 week study respectively. CAL induced a significant decrease in ejection fraction (EF) and fractional shortening (FS) and a significant increase in early wave to atrial wave (E/A) ratio over the duration of both studies ($P < 0.05$). *L. rhamnosus* GR-1 and *L. plantarum* 299v administration resulted in near-normalization of these parameters. The left ventricular internal diameters (LVID) were also assessed in the 6 week-long study. Representative echocardiography images and the LVID systolic (LVIDs) and LVID diastolic (LVIDd) are shown in figure 7. CAL significantly increased LVID over 6 weeks ($P < 0.05$), although this was significantly attenuated with *L. rhamnosus* GR-1 administration.

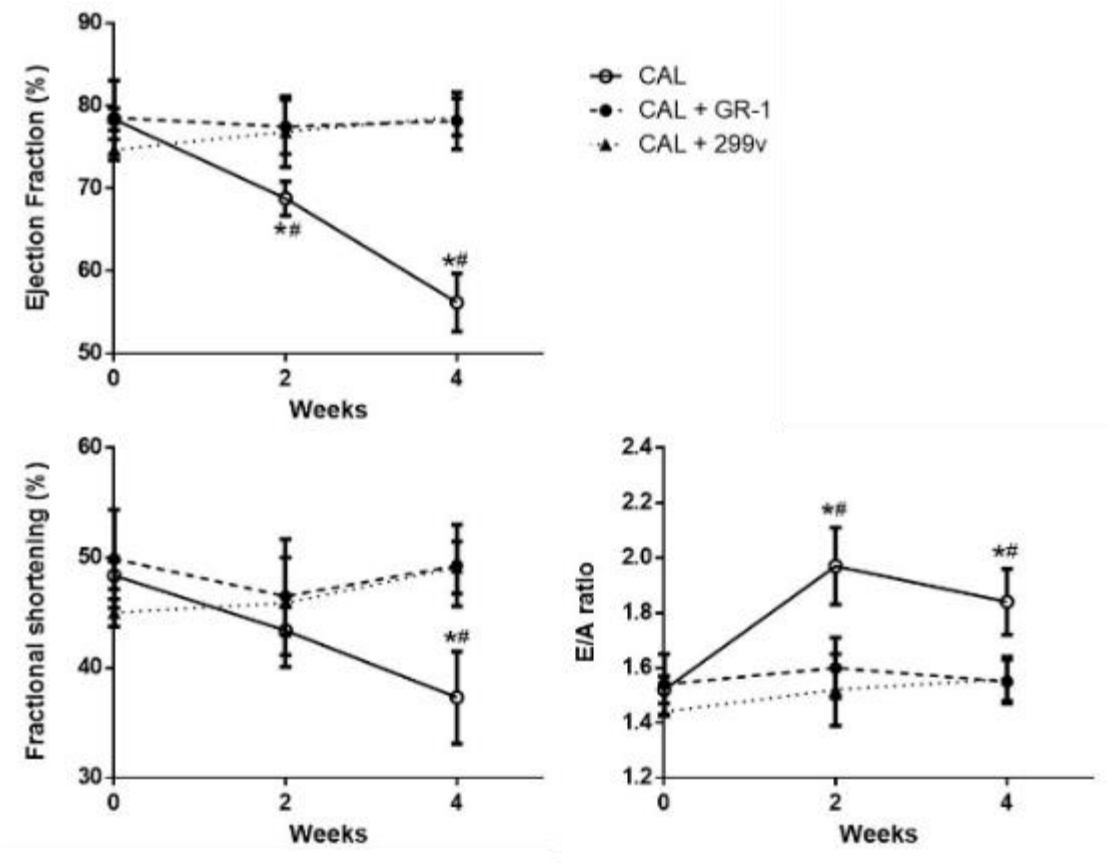

Figure 5. Echocardiography data collected every 2 weeks for 4 weeks total of sustained CAL

GR-1 and 299v treatment attenuated the CAL-induced decrease in EF and FS and CAL-induced increase in E/A ratio. Error bars indicate ± SEM (n=5). * $P < 0.05$ from CAL + GR-1 and CAL + 299v; $^{\#}P < 0.05$ compared to week zero.

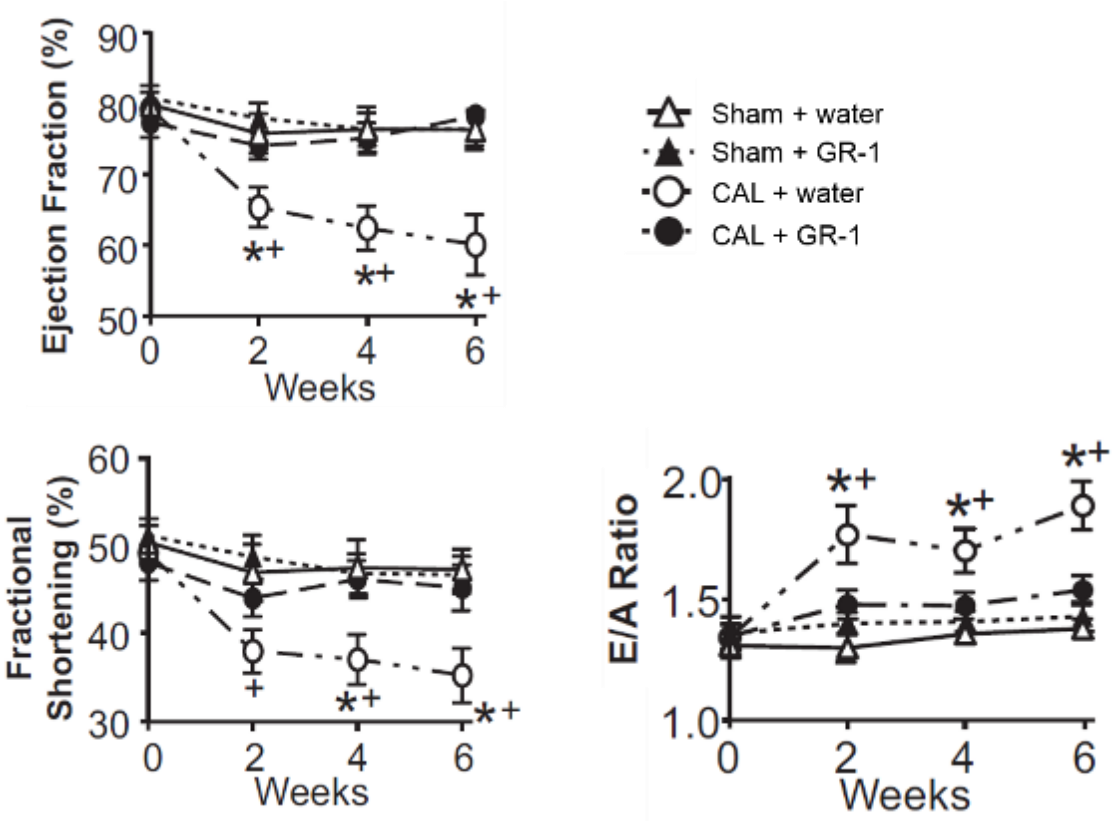

Figure 6. Echocardiography data collected every 2 weeks for 6 weeks total of sustained CAL or sham surgery

GR-1 administration significantly attenuated the CAL-induced reduction in ejection fraction and fractional shortening and increase in E/A ratio in animals over the 6 week trial period. Error bars indicate ± SEM (n=10). * $P < 0.05$ compared to the CAL + GR-1, sham + GR-1, and sham + water groups; $^{+}P < 0.05$ from week zero (n=10).

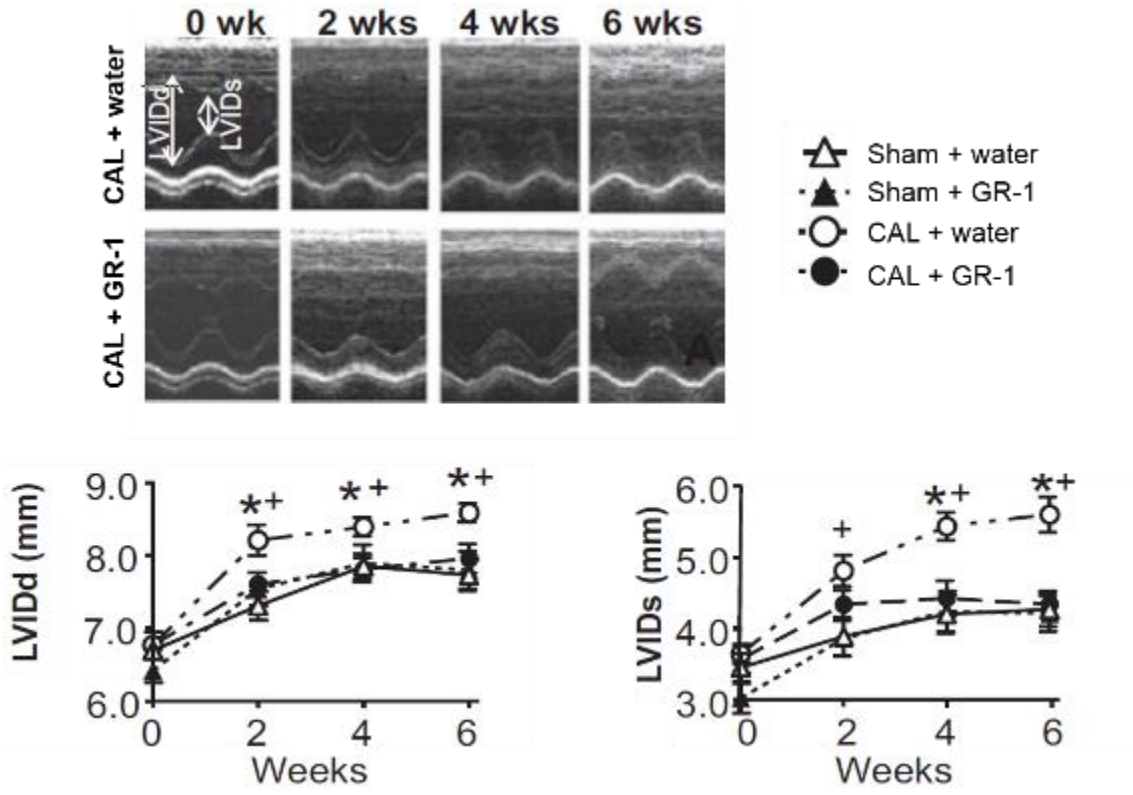

Figure 7. Representative images and corresponding quantified diastolic and systolic left ventricular internal diameters from echocardiography evaluations

GR-1 treatment significantly attenuated the CAL-induced increase in LVID over the 6 week trial period. Error bars indicate ± SEM (n=10). $*P < 0.05$ compared to the CAL + GR-1, sham + GR-1, and sham + water groups; $^{+}P < 0.05$ from week zero.

3.1.3 Probiotic administration attenuates hemodynamic dysfunction function post-CAL

At the end of the 6 week study, animals were subjected to catheter-based hemodynamic analyses and the data is summarized in figure 8. The hemodynamic data indicates systolic and diastolic dysfunction were induced by CAL. The left ventricular end systolic pressure (LVSP) significantly decreased with CAL ($P < 0.05$), while the left ventricular end diastolic pressure (LVEDP) significantly increased ($P < 0.05$). These abnormalities were significantly improved with *L. rhamnosus* GR-1 treatment ($P < 0.05$). Similarly, there was a significant reduction in cardiac output and stroke volume after 6 weeks of sustained CAL that was attenuated with *L. rhamnosus* GR-1 treatment ($P < 0.05$).

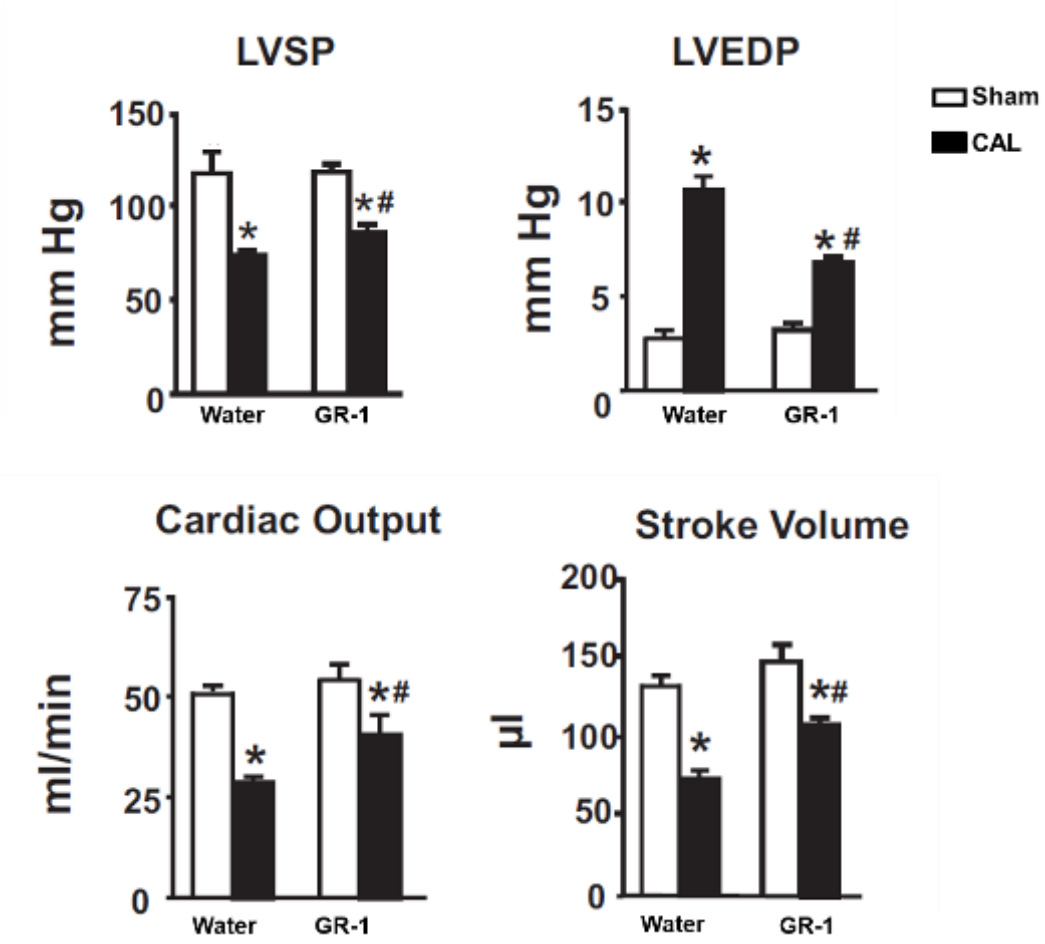

Figure 8. Hemodynamic parameters assessed by cardiac catheterization after 6 weeks of sustained CAL

CAL induced abnormalities in left ventricular end systolic and diastolic pressure (LVSP, LVEDP), cardiac output, and stroke volume after the 6 week trial period. These abnormalities were significantly improved with GR-1 treatment. Error bars indicate ± SEM (n=10). *$P < 0.05$ compared to respective sham group. $^{\#}P < 0.05$ compared to CAL + water group.

3.2 Probiotic administration has no effect on serum cytokine levels

To determine whether or not probiotic administration alters the levels of inflammatory factors involved in the cardiac remodeling process, serum samples were assayed for levels of 9 cytokines after the 6 week CAL period. Any individual sample within a group where a cytokine was not detected was excluded from the analysis. TNF-α values for all samples fell below the threshold detection limit and therefore the data are not reported. For all the other detectable values, there were no significant changes in concentration across all the groups (figure 9).

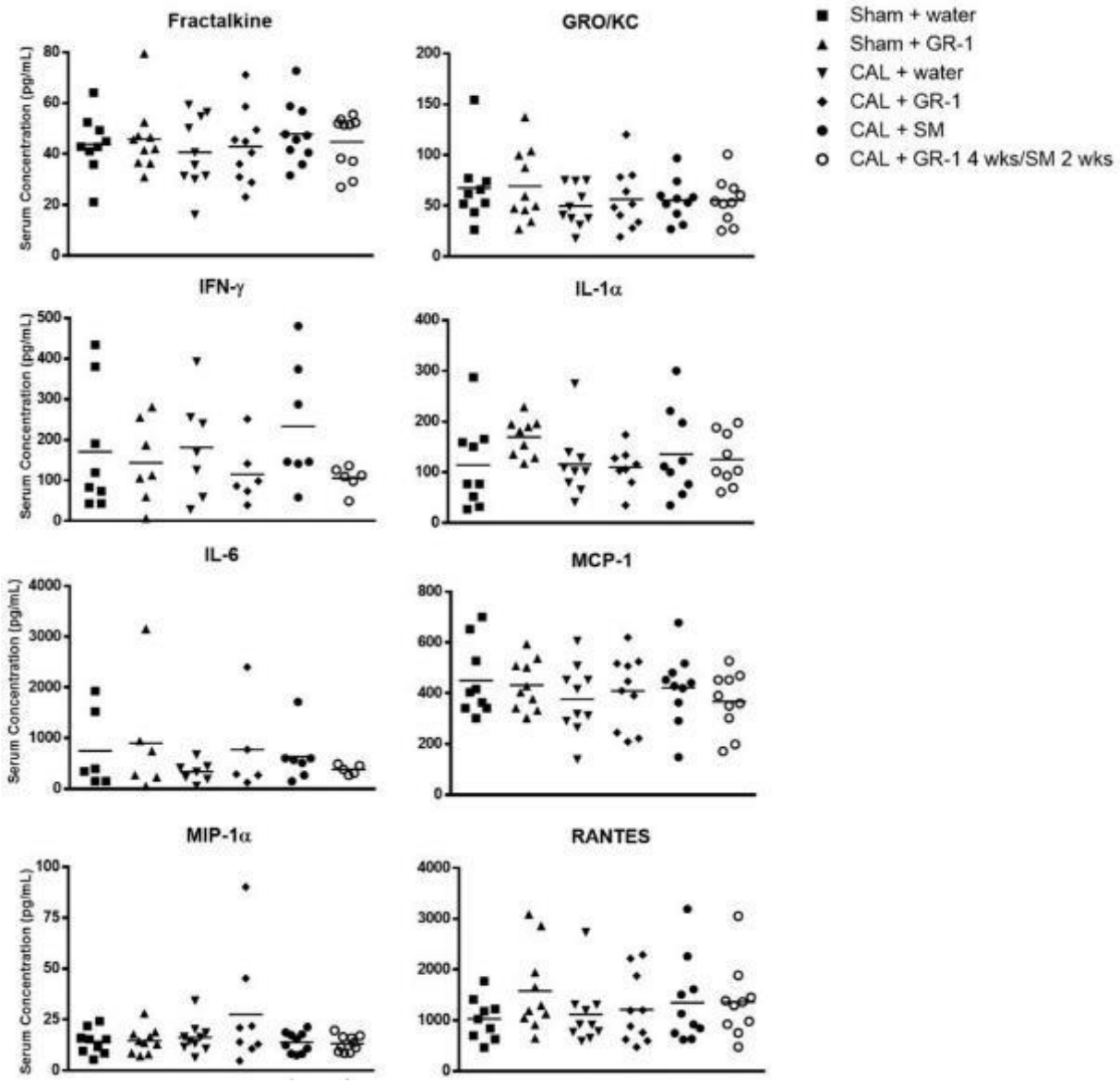

Figure 9. Concentration of 8 pro-inflammatory cytokines detected in plasma of animals taken 6 weeks post-CAL or sham surgery

There was no significant difference in concentration of any of the cytokines across all treatment groups (n=6-10)

3.3 Probiotic administration improves CAL-induced increase in plasma leptin to adiponectin concentration ratio

Two adipokines, leptin and adiponectin, were assayed for serum concentration following 6 weeks of CAL. These adipokines are considered to have antagonistic function against each other, and are associated with chronic HF[47,52,196]. CAL significantly increased serum leptin levels ($P < 0.05$) but not serum adiponectin. There was a two-fold increase in leptin, when normalized to adiponectin. These CAL-induced changes were significantly attenuated with *L. rhamnosus* GR-1 administration ($P < 0.05$). These data are summarized in figure 10.

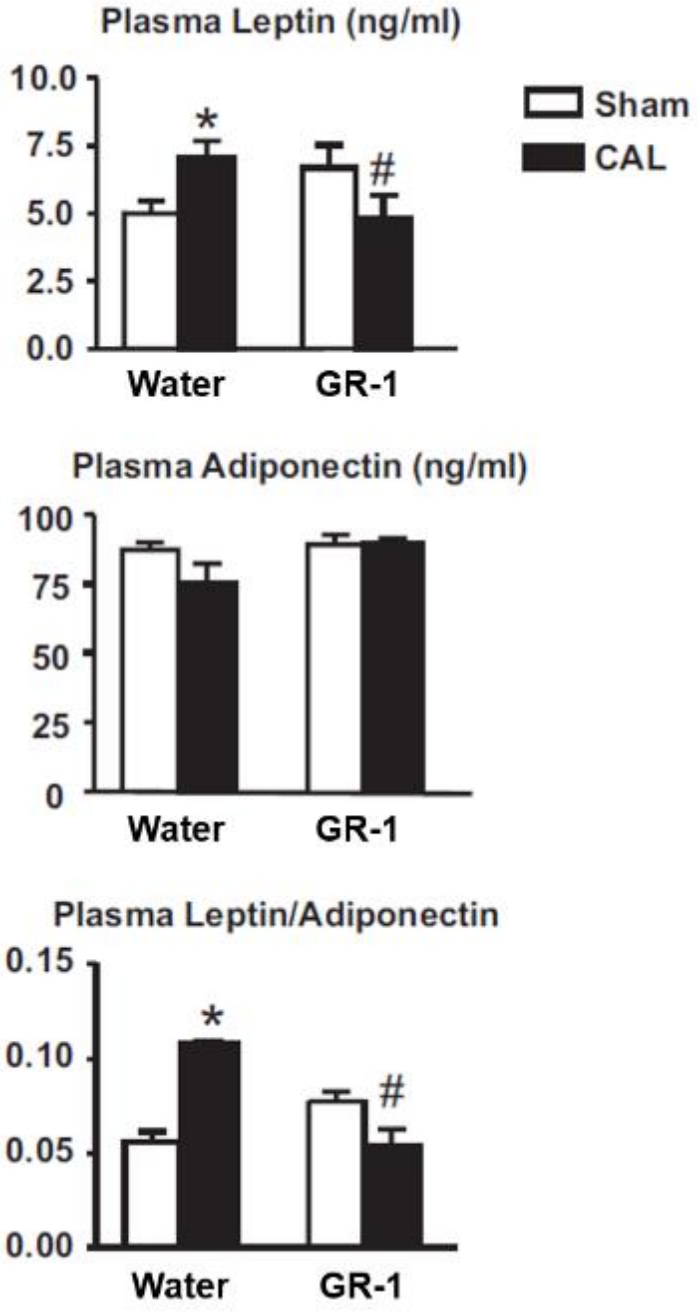

Figure 10. Plasma leptin and adiponectin levels 6 weeks post-CAL and sham surgery

GR-1 treatment significantly attenuated the CAL-induced increase in plasma leptin levels and plasma leptin/adiponectin ratio. Error bars indicate ± SEM (n=8). *$P < 0.05$ compared to sham + water; $^{\#}P < 0.05$ compared to CAL + water.

3.4 Probiotic lactobacilli were found viable in the cecum and colon digesta

L. rhamnosus GR-1 and *L. plantarum* 299v were readily cultivated from fresh cecum and colon digesta samples on selective MRS agar (table 11 and table 12). The successful cultivation of *L. plantarum* 299v was indicated by the purple LPSM agar turning bright yellow due to sorbitol fermentation by *L. plantarum* 299v. Neither *L. rhamnosus* GR-1 nor *L. plantarum* 299v were successfully cultivated on selective media from digesta samples of rats in milk or water controls. Representative images of isolated colonies are displayed in figure 11.

Table 11. Cultivation of probiotic colonies on selective agar media from cecum and colon digesta samples collected 4 weeks post-CAL. The data represents the average CFU/mL in each group (n=10)

	Cecum (CFU/mL)		**Colon (CFU/mL)**	
Group	MRS FA	LSPM	MRS FA	LPSM
CAL	Not detected	Not detected	Not detected	Not detected
GR-1	6.59×10^8	Not detected	1.52×10^9	Not detected
299v	Not detected	5.77×10^5	Not detected	5.49×10^5

Table 12. Cultivation of probiotic colonies on selective agar media from cecum and colon digesta samples collected 6 weeks post-CAL or sham surgery. The data represents the average CFU/mL in each group (n=10)

Group	**Cecum (CFU/mL)**	**Colon (CFU/mL)**
1 (Sham + water)	Not detected	Not detected
2 (Sham + GR-1)	8.71×10^7	8.81×10^7
3 (CAL + water)	Not detected	Not detected
4 (CAL + GR-1)	6.73×10^7	5.67×10^7
5 (CAL + skim milk)	Not detected	Not detected
6 (CAL + GR-1 4wks/milk 2wks)	7.56×10^4	6.58×10^4

Figure 11. Representative images of agar plates inoculated with cecum digesta from animals from the 4 week pilot study

Top: Purple LPSM plate inoculated with cecum digesta from the CAL only group showing no cultivation of bacteria **Middle**: The originally purple LPSM plate turned yellow after inoculation by cecum digesta from the 299v group and produced isolated colonies. **Bottom**: Isolated colonies on MRS FA plate inoculated with cecum digesta from the GR-1 group.

3.5 Probiotic administration does not affect the microbial composition of cecum digesta

Sequencing of the V6 region of the 16S rRNA gene generated 2,254,397 total usable reads from 60 cecum digesta samples collected from the animals in the 6 week study. Three samples did not run successfully and were excluded from the analysis (1 each from group 2, 3, and 5). Using a minimum of 0.5% abundance threshold for any sample and a ≥97% sequence identity, there were a total 242 distinct organizational taxonomic units (OTU) groupings (figure 12). GR-1 was not detected in any of the 60 individual samples. A heat map displaying the 50 most abundant OTUs detected in the cecum digesta samples was generated and is displayed in figure 13. Community level variance was evaluated using the ANOSIM nonparametric statistical method in QIIME. There was no significant variance in microbial composition across the 6 treatment groups (R=0.1308). Although OTU 0 appears to have variable abundance, one-way ANOVA testing indicates that the variation is not significant ($P > 0.05$). Overall, there was no distinct grouping of community compositions in any of the 60 samples, with respect to probiotic administration. Weighted β-diversity UniFrac analysis-generated Principal Coordinate Analysis (PCoA) plots were generated to display the dissimilarities in community compositions of each sample (figure 14). There was no distinct grouping cecum digesta samples from rats that received CAL surgery compared to sham, or rats that received *L. rhamnosus* GR-1 compared the placebo treatment. This indicates that the both the CAL surgery and probiotic treatment did not induce significant changes to the microbial composition of the gut after 6 weeks of treatment.

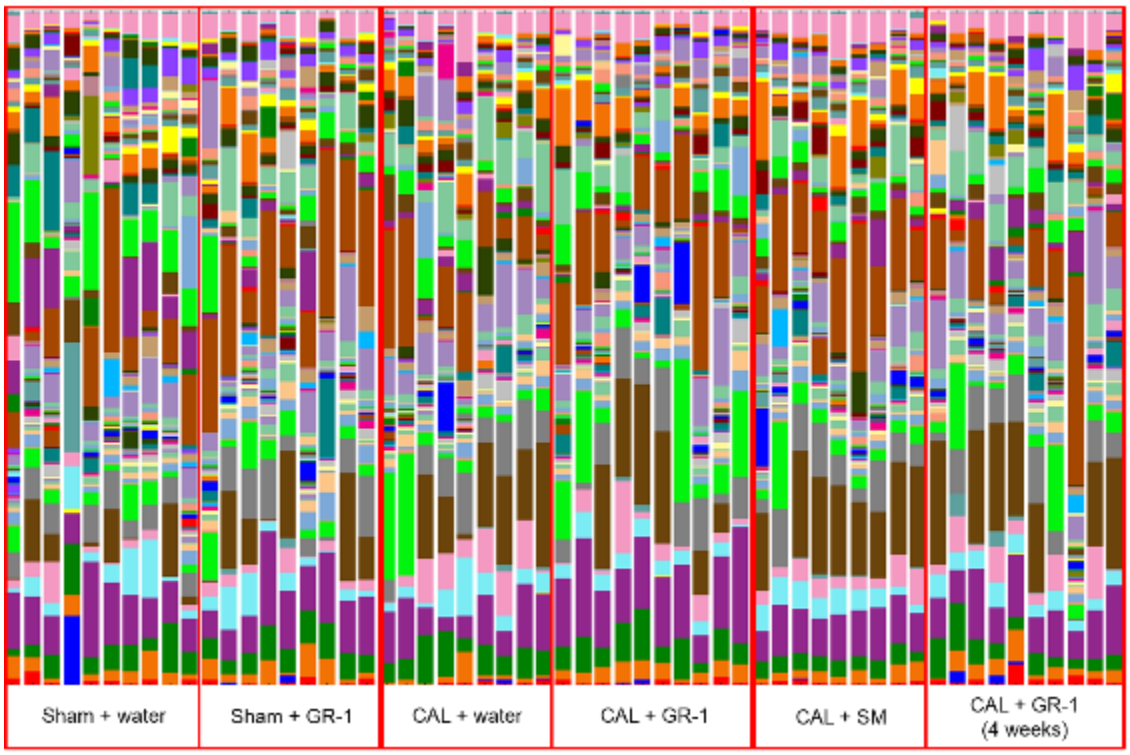

Figure 12. Bar plot displaying the microbial composition of cecum digesta

Each vertical bar represents the microbial profile of one individual animal (n=57). Each colour represents a specific OTU detected in the digesta sample by 16S rRNA next-generation sequencing (see colour legend). The amount of each colour in the vertical bar represents the relative abundance of the OTU.

Legend to accompany figure 10 bar plot. Each OTU was assigned taxonomy based on BLAST (NCBI) and Greengenes gene sequence alignment. Alignments with less that 97% identity were classified no higher than the genus level.

Legend (continued) to accompany figure 10 bar plot. Each OTU was assigned taxonomy based on BLAST (NCBI) and Greengenes gene sequence alignment. Alignments with less that 97% identity were classified no higher than the genus level.

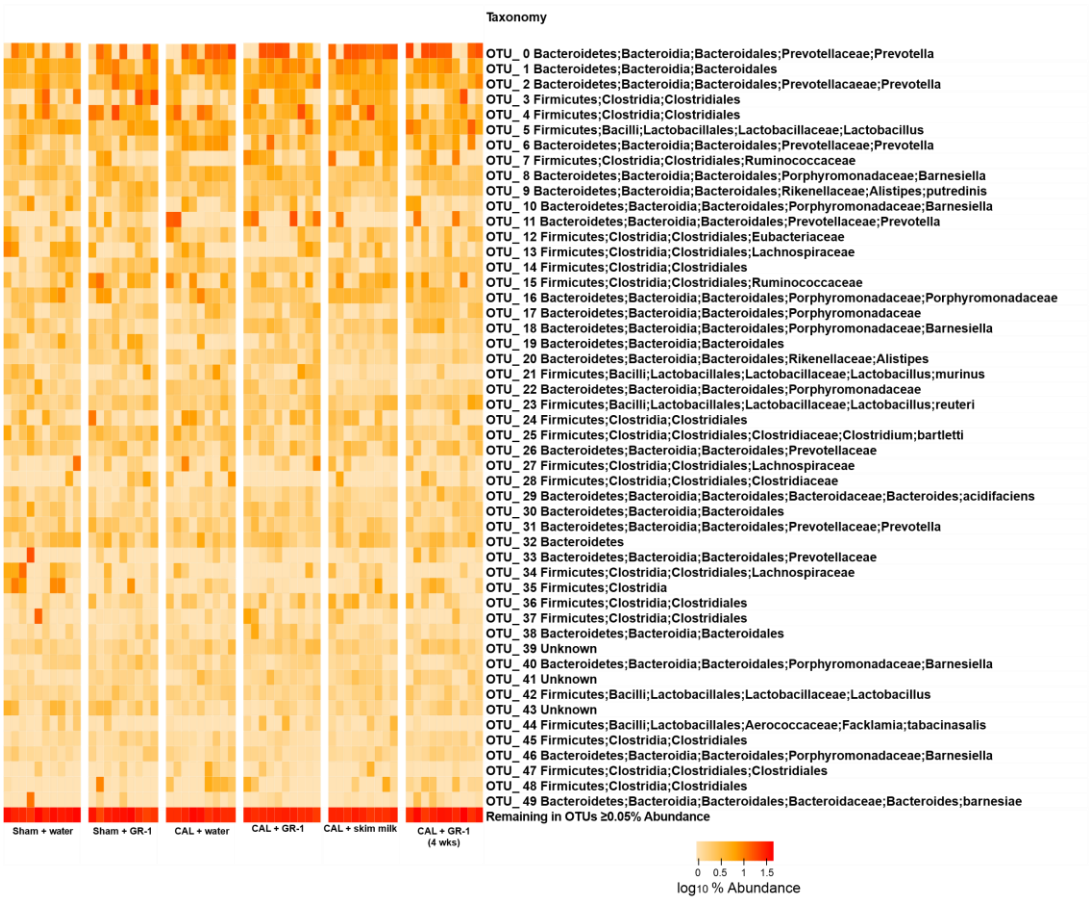

Figure 13. Heat map displaying the 50 most abundant OTUs detected in cecum digesta samples using 16S rRNA next-generation sequencing

Each vertical bar represents the cecum digesta microbiota of one individual animal (n=57).

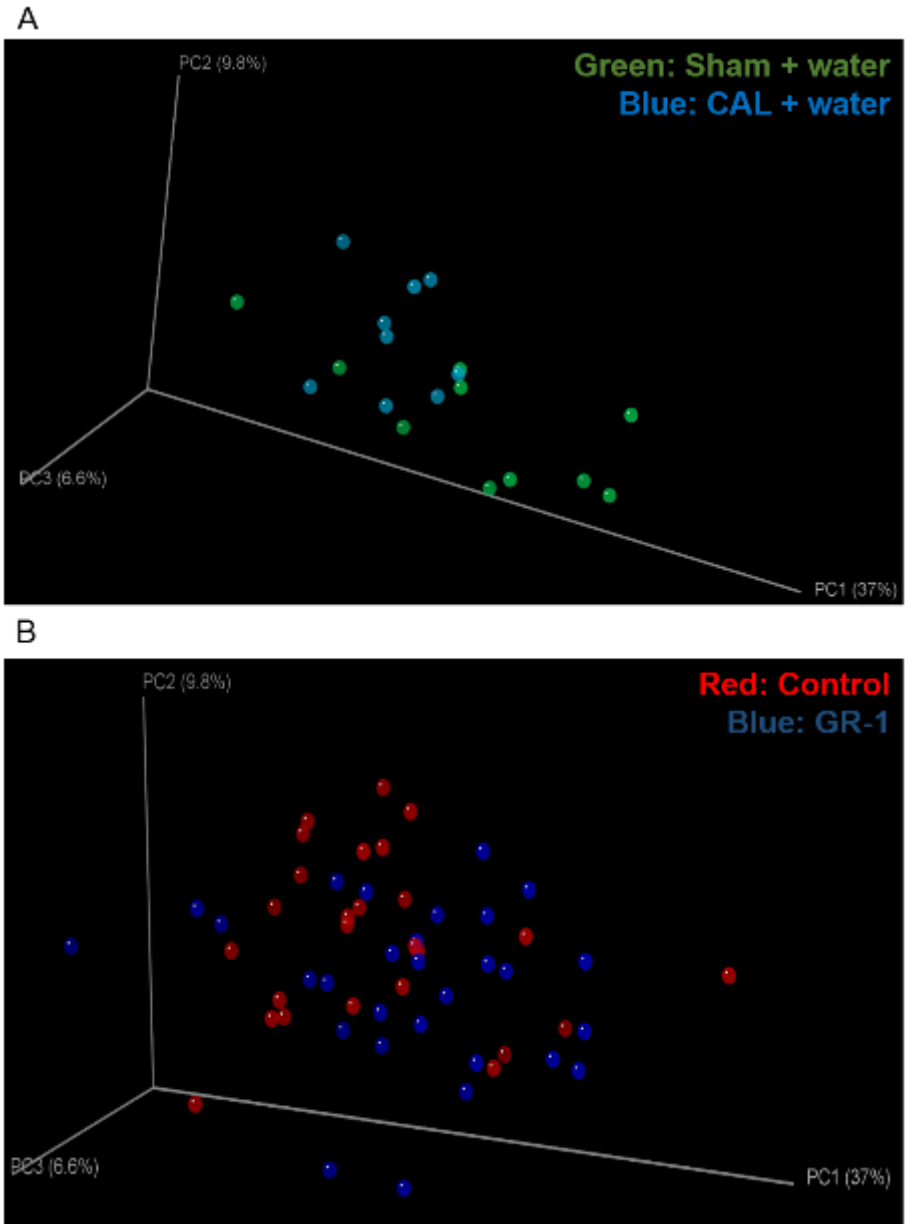

Figure 14. Weighted β-diversity UniFrac analysis-generated PCoA plots

The plots display dissimilarities in microbial community compositions of each sample. Samples with highly dissimilar microbial compositions are spaced far apart from each other. **A:** PCoA plot of communities from animals receiving sham surgery with skim milk treatment compared to animals receiving CAL surgery with skim milk treatment. **B:** The comparison of communities from animals on GR-1 treatment versus control treatment (skim milk or water).

3.6 Probiotics co-cultured with neonatal rat ventricular cardiomyocytes (NVCM) attenuate phenylephrine-induced hypertrophy

It was hypothesized that probiotics will provide a direct beneficial effect to cardiomyocytes under conditions that simulate HF. To test this, probiotic treatments were applied to NVCM culture following the administration of PE – a hypertrophic agent. To assess the outcome of the probiotic NVCM co-culture, the cell surface area of the cell and gene expression of hypertrophic markers was measured after 24 hours of treatment. Except for the average cell surface area data across all biological replicates, expressed in μm^2, the treatment-induced changes are reported as the fold change compared to the control. NVCM isolated from different litters may inherently grow at slightly different rates and sizes and therefore the fold change in surface area and gene expression was calculated to account for those differences. Fold change calculations are the ratio of treated to untreated measurements and the values represent one biological replicate.

3.6.1 PE administration induces hypertrophy in NVCM

In order to determine the hypertrophic response to PE administration, and to rule out that MRS broth affects the growth of cultured NVCM, 50 µL of sterile MRS broth was added NVCM culture with or without PE. Twenty four hour PE exposure induced an average 1.41-fold increase in NVCM surface area, compared to untreated control cells. PE with MRS broth induced a 1.35 fold increase in cell surface area. The difference in cell surface area compared to both of these treatments was statistically significant ($P < 0.05$), but difference between the two PE treatments without MRS broth was not significant ($P = 0.52$). Without PE, The average fold change in surface area of cells cultured with MRS broth compared untreated cells after 24 hours was 1.02. A student's *t*-test, was performed to determine the difference between MRS broth-treated and untreated cells. The difference was not significant ($P = 0.75$).

The gene expression of the hypertrophic markers ANP and aSKA was evaluated to confirm a hypertrophic response in NVCM exposed to PE. The administration of PE

induced a 1.91-fold and 1.51-fold increase in gene expression of ANP and aSKA, respectively. The administration of PE with 50 µL of sterile MRS broth induced a 2.01-fold increase in gene expression of ANP, and a 2.10-fold of aSKA. The difference in ANP expression with both PE and PE + MRS broth compared to untreated cells was statistically significant ($P < 0.05$), but the difference between the two PE treatments was not statistically significant. There was no statistically significant difference in expression of aSKA between PE and untreated cells ($P = 0.0921$), likely because of the high standard deviation in the PE group. There was, however, a significant difference in aSKA expression between MRS broth and PE + MRS broth ($P < 0.01$).

The administration of MRS broth without PE produced a 0.96 and 0.79-fold change in gene expression of ANP and aSKA, respectively, over 24 hours, compared to untreated cells. A student's *t*-test was performed to determine the difference in gene expression between untreated and MRS broth-treated NVCM. The expression of ANP and aSKA were both found to be not significantly different ($P = 0.1160$ and $P = 0.1909$, respectively).

These results confirm that PE administration induced hypertrophy over 24 hours in NVCM. The presence of sterile MRS broth has no effect on the growth of NVCM.

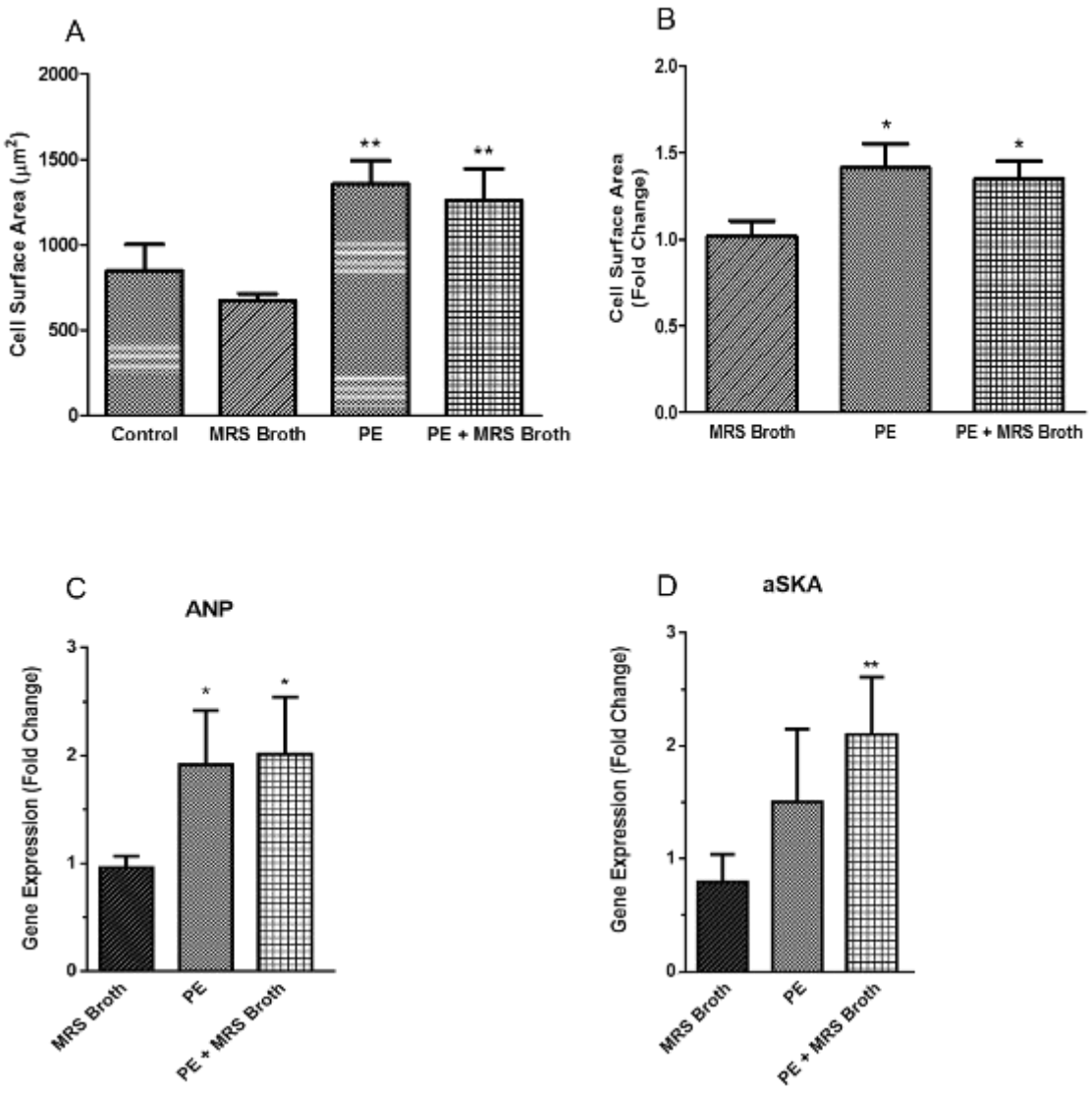

Figure 15. PE induces hypertrophy in NVCM

A: The NVCM surface area following 24 hour MRS broth culture with or without the administration of PE (n=5). **B**: The fold change in NVCM surface area compared to untreated (control) cells, following 24 hour MRS broth culture with or without the administration of PE (n=5). **C, D**: The fold change in gene expression of ANP and aSKA, respectively in NVCM (n=3). *$P < 0.05$ and **$P < 0.01$ compared to control and MRS broth. Error bars indicate the standard deviation.

3.6.2 *L. rhamnosus* GR-1, *L. plantarum* 299v, and *S. salivarius* K12 co-cultured with NVCM did not affect viability of NVCM

NVCM co-cultured with *L. rhamnosus* GR-1, *L. plantarum* 299v, and *S. salivarius* K12 had normal cell morphology after 24 hours, as indicated by the representative micrographs in figure 16. In addition, the cells were spontaneously beating, an indication (however not a prerequisite) of NVCM viability. The probiotics were also successfully isolated from the culture media 24 hours after co-culture with NVCM, indicating that they were not susceptible to the penicillin/streptomycin antibiotic solution in the NVCM culture medium.

The majority of NVCM co-cultured with *L. reuteri* RC-14 did not survive the 24 hour treatment period, as evidenced by loss of cell attachment and abnormal morphology (figure 16). A scarce number of NVCM cultured with *L. reuteri* RC-14 were beating after the 24 hour treatment period. The *L. reuteri* RC-14 cells also did not survive the treatment period, as they were unsuccessfully isolated from the culture medium.

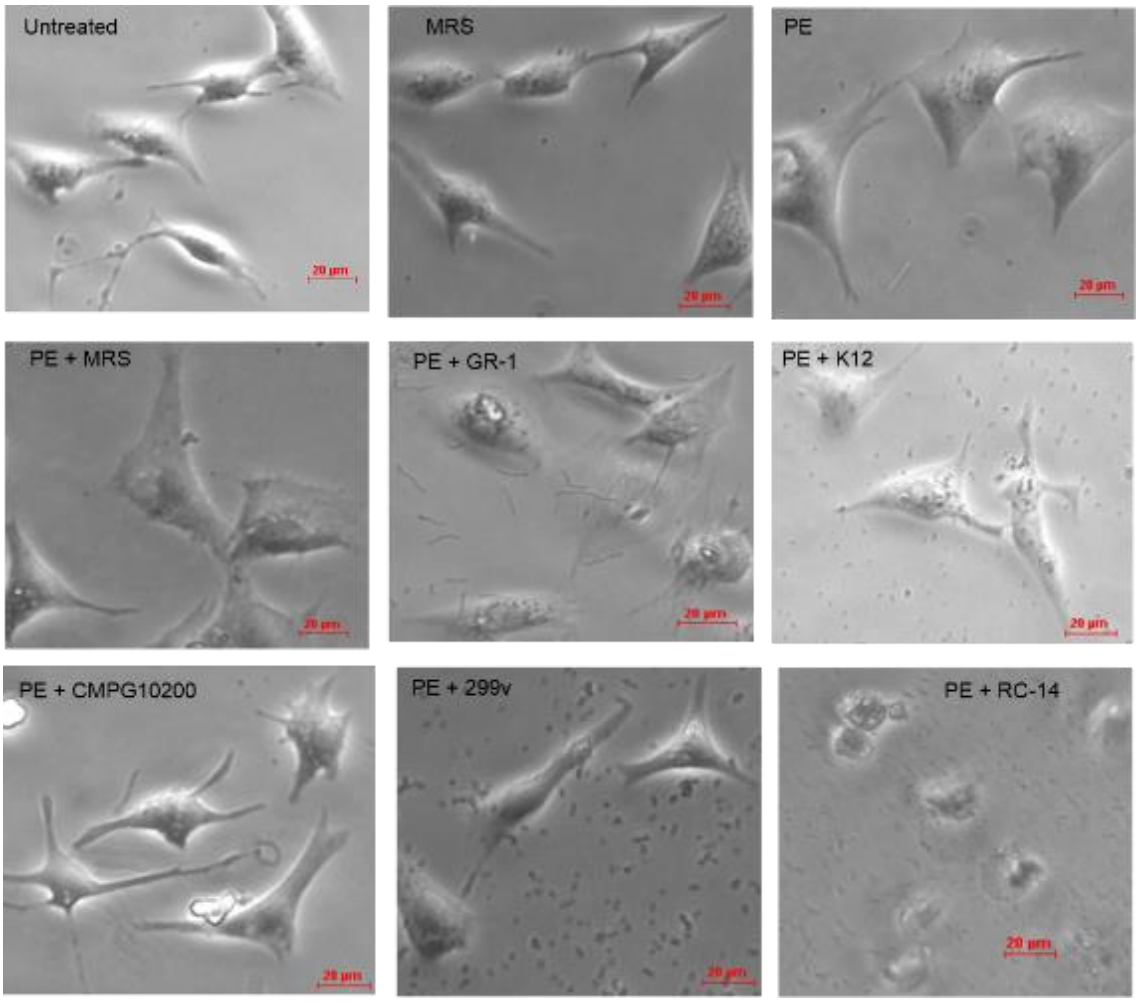

Figure 16. Representative micrographs illustrating NVCM exposed to PE alone or with probiotics

The control NVCM were either untreated, or co-cultured with MRS broth. Original magnification = 20×

3.6.3 *L. rhamnosus* GR-1 administration inhibits PE-induced hypertrophy in NVCM

When *L. rhamnosus* GR-1 was co-cultured with NVCM immediately following PE administration, there was no increase in NVCM surface area compared to untreated cells. The inhibition of hypertrophy was confirmed by the gene expression of ANP and aSKA relative to untreated cells. The expression of both hypertrophic markers was significantly reduced when NVCM cells exposed to PE were co-cultured with *L. rhamnosus* GR-1 ($P < 0.05$).

To confirm the presumption the live probiotic cells are required to confer an anti-hypertrophic effect, *L. rhamnosus* GR-1 cells were HK by incubation at 80°C for 30 minutes. HK GR-1 cells and live GR-1 cells had the same average cell surface area, however, when the data was normalized by calculating the fold change in cell surface area, there was a loss of anti-hypertrophic activity in HK GR-1 cells. The difference in gene expression of ANP in PE-treated NVCM without HK GR-1 cells and PE-treated NVCM with HK GR-1 cells was not statistically significant. For both ANP and aSKA, there was a slight increase in gene expression with HK GR-1 cells, compared to live *L. rhamnosus* GR-1. The increase, however, was not statistically significant. ($P = 0.35$ and 0.36 for ANP and aSKA respectively). These data are summarized in figure 17.

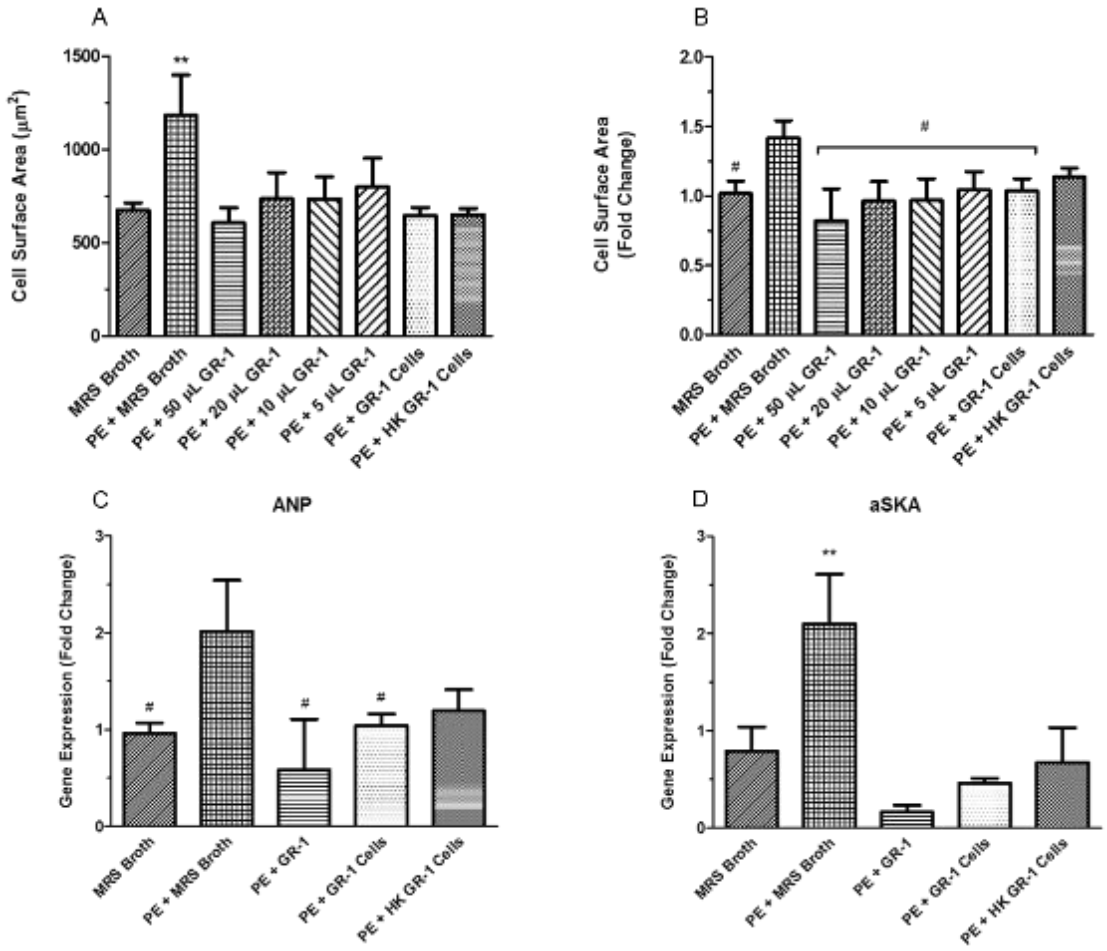

Figure 17. *L. rhamnosus* GR-1 inhibits PE-induced hypertrophy in NVCM

A: The NVCM surface area following 24 hour co-culture with GR-1 (n=5). **B**: The fold change in NVCM surface area compared to untreated (control) cells, following 24 hour co-culture with GR-1 (n=5). **C, D**: The fold change in gene expression of ANP and aSKA in NVCM (n=3). Unless otherwise stated, all volumes of GR-1 treatments were 50 µL (1 x 10^9 CFU/mL stock culture). *$P < 0.05$ and **$P < 0.01$ compared to all other treatments. #$P < 0.05$ compared to PE + MRS broth. Error bars indicate the standard deviation.

3.6.4 Inhibition of the PE-induced hypertrophy in NVCM is not probiotic strain specific

The attenuation of PE-induced hypertrophy by probiotics was not limited to the *L. rhamnosus* GR-1 strain. Based on the cell surface area and gene expression of ANP and aSKA after 24-hour co-culture, *L. plantarum* 299v and *S. salivarius* K12 inhibited the PE-induced hypertrophy in NVCM. There was a significant decrease in ANP and aSKA gene expression in NVCM co-cultured with *L. plantarum* 299v compared to the control ($P < 0.05$). These data are summarized in figure 18.

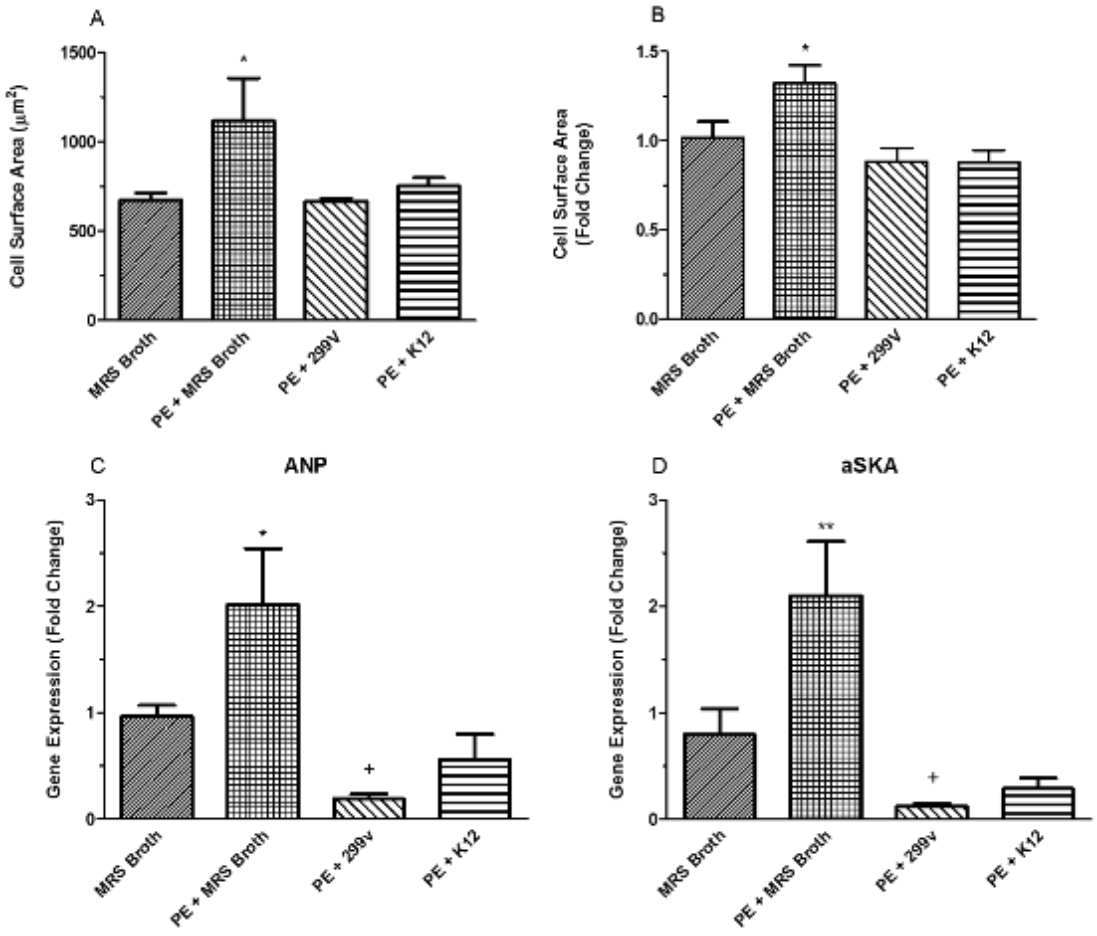

Figure 18. *L. plantarum* 299v and *S salivarius* K12 inhibits PE-induced hypertrophy in NVCM

A: The NVCM surface area following 24 hour co-culture with 299v and K12 (n=5). **B**: The fold change in NVCM surface area compared to untreated cells, following 24 hour co-culture with 299v and K12 (n=5). **C, D**: The fold change in gene expression of ANP and aSKA compared to untreated NVCM after 24 hour co-culture with 299v and K12 (n=3). All volumes of 299v and K12 treatments were 50 µL (1 x 10^9 CFU/mL stock culture). *$P < 0.05$ and **$P < 0.01$ compared to all other treatments. $^{+}P < 0.05$ compared to MRS broth. Error bars indicate the standard deviation.

3.6.5 Msp1 is not required for inhibiting PE-induced increase in NVCM surface area

Msp1 produced by *Lactobacillus rhamnosus* was of interest because it has shown to protect against ischemic injury and stress induced apoptosis in the heart and small intestine. We obtained a mutant *msp1* knock out *L. rhamnosus* GR-1 strain to test whether or not this protein is beneficial to NVCM. When CMPG10200 was co-cultured with NVCM immediately following PE administration, there was a similar inhibition of PE-induced increase in NVCM surface area as seen with the WT *L. rhamnosus* GR-1 strain (figure 19). This suggests that the protein Msp1 is not required for preventing cell surface area increase in NVCM exposed to PE, and therefore, gene expression analysis using this strain was not pursued.

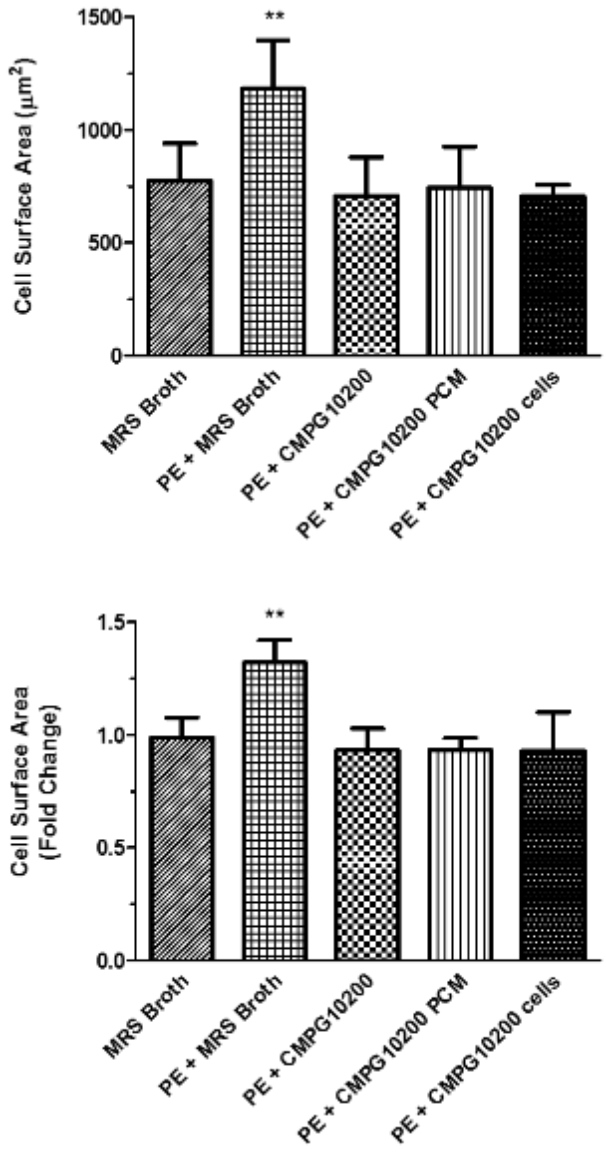

Figure 19. *L. rhamnosus* GR-1 Msp1 knock out strain CMPG10200 inhibits PE-induced hypertrophy in NVCM

Top: The NVCM surface area following 24 hour co-culture with CMPG10200 (n=5). **Bottom**: The fold change in NVCM surface area compared to untreated cells, following 24 hour co-culture CMPG10200 (n=5). All volumes of CMPG10200 treatments were 50 µL (1 x 10^9 CFU/mL stock culture). $^{**}P < 0.01$ compared to all other treatments. Error bars indicate the standard deviation.

3.6.6 PE-induced hypertrophy in NVCM is attenuated by PCM treatment alone

When NVCM were treated with GR-1 PCM void of any live bacteria, the PE-induced increase in cell surface area and gene expression of ANP and aSKA was significantly attenuated, similar to treatments with live probiotic cells ($P < 0.05$). I hypothesized that PCM contains a soluble protein that could directly prevent PE-induced increase in cell surface area. Various assays were performed to test this hypothesis, and the results are summarized in figure 20. First, Attempts were made to elucidate the size of the potential anti-hypertrophic factor by centrifugally filtering the PCM. While the overall surface area of cells indicates that both the filtrate and retentate retained an anti-hypertrophic effect, the fold-change in cell surface area indicates that this effect was lost.

Second, the proteins were destabilized by heat denaturing the PCM by a 30 minute incubation at 80°C and by trypsin treatment. Heat denaturation resulted in a loss of anti-hypertrophic activity of the PCM, as indicated by the fold change in NVCM surface area and ANP gene expression. In NVCM co-cultured trypsin-treated PCM, the attenuation of PE-induced increase in ANP gene expression was lost. There was an increase in PE-induced aSKA gene expression with HD and trypsin-treated PCM compared to regular PCM, however the difference was not significant. Heat denatured PCM significantly attenuated the PE-induced increase in aSKA expression ($P < 0.05$).

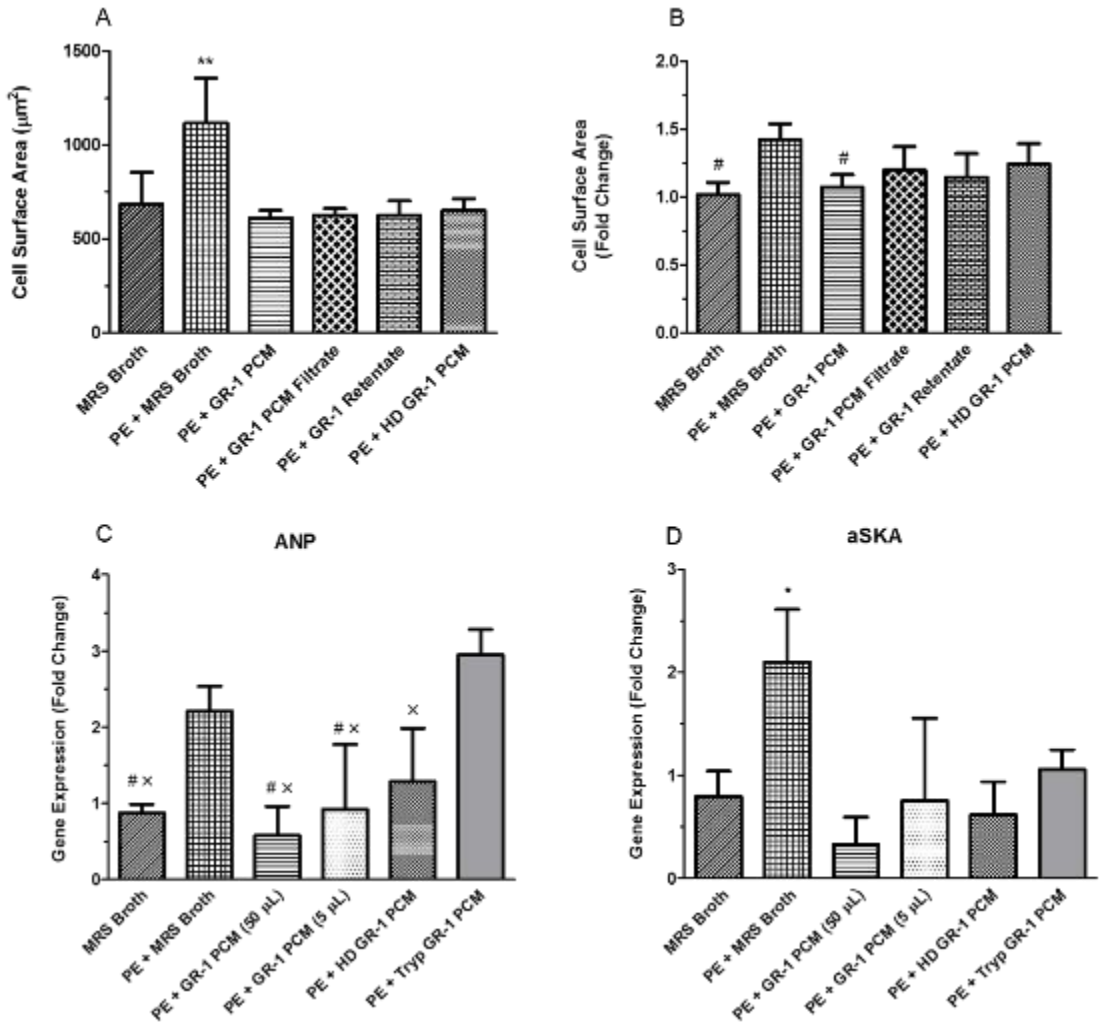

Figure 20. PCM inhibits the PE-induced hypertrophy in NVCM

A. NVCM surface area following 24 hour co-culture with various PCM treatments (n=5). **B**: The fold change in NVCM surface area compared to untreated cells, following 24 hour co-culture with various PCM treatments (n=5). **C, D**: The fold change in gene expression of ANP and aSKA compared to untreated NVCM after 24 hour co-culture with various PCM treatments (n=3). Unless otherwise stated, all PCM volumes were 50 μL. *$P < 0.05$ and **$P < 0.01$ compared to all other treatments. $^{\#}P < 0.05$ compared to PE + MRS broth. $^{\times}P < 0.05$ compared to PE + Tryp GR-1 PCM. Error bars indicate the standard deviation.

Chapter 4 : Discussion

4 Discussion

This thesis describes novel applications of probiotic lactobacilli to cardiovascular health. Using an animal model and *in vitro* experimentation, *Lactobacillus rhamnosus* GR-1, a probiotic known to transit the intestinal tract and confer benefits there and in the urogenital tract, was found to induce improved modeling of heart tissue following ligation injury simulate a heart attack. These results are the first to report that probiotic administration can attenuate cardiac hypertrophy and HF in a rat model, and can protect against hypertrophy in cardiomyocyte culture. This work adds to those studies already described in the existing literature showing that probiotic administration can confer cardiovascular health benefits via: (i) reduction of hypertension through production of ACE-inhibitory peptides in the gut[175-177], (ii) reduction of serum cholesterol by strains expressing BSH[174,197] or by active reduction and excretion of cholesterol [169,170] and (iii) their protection against cardiac ischemia/reperfusion injury[179,180].

With the incidence of CVD worldwide reaching alarming rates, there is a need for novel prevention and treatment strategies. The five-year 50% mortality rate for HF patients indicates that early detection and treatment is critical to improving outcomes. Previous to the research for this thesis, the use of probiotics as a treatment for HF has never been investigated. The present research suggests a unique application for probiotics and sheds light on a potential novel mechanism of action of probiotics.

4.1 The salutary effects of probiotic administration in the CAL-induced rat model of heart failure

In a preliminary 4 week-long pilot study, the benefit of two probiotic strains on the outcome of heart failure in rats was evaluated using the CAL model for MI-induced HF in rats. The CAL model differs from the I/R surgery used previously to examine *L. plantarum* 299v, in that there is no release of the infarction-inducing ligature throughout the duration of the study. Considering that both *L. plantarum* 299v and *L. rhamnosus* GR-1 demonstrated identical benefit to rats in terms of echocardiography imaging, the salutary effect of this probiotic therapy is likely not strain or species specific. This is an interesting point, considering the extremely different ecological environments from

which each of these strains was isolated. *L. plantarum* 299v is a strain isolated from the human gastrointestinal tract and is marketed as a treatment to relieve symptoms of irritable bowel syndrome[128]. *L. rhamnosus* GR-1 is a distal urethral strain that has the ability to reduce urogenital pathogens and is used to treat bacterial vaginosis[134,183,198] and prevent UTI[134,199-201] as well as enhance immunity in HIV patients[202,203]. The ability of these two strains to improve outcomes of HF speaks to the universality of some of the health benefits conferred by probiotics and their ability to affect organs throughout the entire body[204,205].

To follow-up on the pilot study, a 6 week-long study was performed including sham-operated and placebo control groups using *L. rhamnosus* GR-1 alone. To determine the effect of treatment cessation, one group of animals received *L. rhamnosus* GR-1 for the initial 4 weeks post-CAL surgery and then were placed on a placebo treatment for the remaining 2 weeks. Identical beneficial effects of *L. rhamnosus* GR-1 were evident in this group as for the group on *L. rhamnosus* GR-1 treatment for the entire 6 week period. This suggests that the probiotic induced an effect early after surgery. Since this study did not include *L. rhamnosus* GR-1 treatment for only the final few weeks of the trial, it is difficult to estimate an exact timeframe. Although MI-induced damage to the myocardium cannot be entirely prevented, the subsequent compensatory response of cardiac remodeling and maladaptive hypertrophy begins as early as 2-3 days after injury[30]. Preventing the progression of this response is key for preventing the progression of HF[16,30]. Probiotic *L. plantarum* (WCFS1 as distinct from 299v) can induce differential expression of gene reporters within an hour after ingestion[206], thus it is reasonable to imagine an effect within days for GR-1.

The ability to improve cardiac remodeling and ventricular dysfunction in patients with HF, likewise, is a treatment of great interest. Using the same model for CAL-induced HF in the rat as this study, it has been demonstrated that ginseng, administered in drinking water to rats 4 weeks post-surgery, can reverse established CAL-induced hypertrophy[207]. Unlike the probiotic mechanism examined herein, ginseng has been reported to act by upregulating the expression of MMP-2 and MMP-9[208]. This is particularly interesting as it indicates more than one way to improve heart function. Although, to verify that these

effects are not somehow rat specific, it would be important to test the concept in another animal and in humans.

4.1.1 The effect of probiotic therapy on cardiac function, hemodynamics and cardiac hypertrophy in the CAL model for heart failure

Echocardiography and cardiac catheterization, widely used in the clinic to track HF, were used to monitor the effect of CAL surgery. With the added benefit of *ex vivo* analysis of blood and heart tissue following euthanasia of the animals at the end point of the studies, this provided novel insight into the effect of probiotic therapy on the outcome of HF and the potential clinical applications.

Echocardiography is an essential diagnostic tool for diagnosing and tracking HF. This non-invasive method is used to image physical abnormalities of the heart as well as overall cardiac mechanical function. Indices such as FS, EF, and E/A ratio, all of which were analyzed here, are especially important in detecting LV dysfunction. EF values indicate the amount of blood that is pumped out of the ventricle with each contraction. Healthy values range between 55 and 60%, whereas HF patients can have EF under 40%[209]. A low EF can result in inadequate blood circulation for the physiological needs of the body. Half of HF patients have reduced EF, while the others are patients with preserved EF, also known as diastolic LV dysfunction[210]. The latter experience normal systolic contraction, but abnormal ventricular filling during diastolic contraction (ventricular relaxation).While the outcome of HF with preserved EF is similar to that in patients with reduced EF, patients with the former risk misdiagnosis and lack of proper treatment due to the inability to detect diastolic LV dysfunction[210]. Accordingly, other parameters, such as FS and the E/A ratio are important for tracking HF.

Both FS and E/A ratio are a measure of LV function. FS is a ratio of diastolic dimension of the ventricle compared to systolic. Decreased FS suggests either diastolic dysfunction (the LV does not properly relax), or systolic dysfunction (the LV does not contract enough). The E/A ratio is obtained by tracking the velocity of blood flow across the mitral valve into the LV. The early (E) wave represents passive filling during ventricular

diastole, while the atrial or after (A) wave represents active filling with atrial systole. In healthy patients, the E wave is slightly greater than the A wave, however in HF, the A wave is greater than the E wave, indicating diastolic dysfunction[211]. The E/A ratio, therefore, is useful for detecting diastolic dysfunction where EF may still be preserved.

Serial echocardiography performed throughout these studies indicated that *L. rhamnosus* GR-1 and *L. plantarum* 299v administration not only attenuated the deterioration in cardiac mechanical function caused by CAL surgery, but essentially normalized the parameters that were assessed. Remarkably, animals on probiotics experienced no significant changes in EF, FS, or E/A ratio compared to sham operated animals over the duration of the studies. As demonstrated in the echocardiography data, the consequences of CAL-induced myocardial infarction in terms of these parameter manifested within two weeks post-surgery. This supports the notion that the salutary effects of probiotic administration on these animals occur early after CAL surgery and are maintained even as therapy is withdrawn.

Abnormalities in the hemodynamic properties of the heart are a strong risk factor and diagnostic marker for HF. Following myocardial infarction, the damage sustained to the myocardium sets off compensatory changes that result in an increase in blood volume and pressure within the LV. At the same time, infarction also compromises the contractility of the LV, which results in a decreased output and increased overload of blood in the LV with each rhythmic cycle. These pathologies perpetuate the hemodynamic abnormalities of the LV, and become a major complication in HF patients. To monitor hemodynamic properties, catheterization is often required. Results from this minimally invasive technique are usually compared to echocardiograms and are used as a diagnostic tool for vascular and hemodynamic abnormalities. LVEDP is a reflection of intravascular volume and pressure that affects ventricular performance, and is an important parameter for tracking HF[212]. Elevated LVEDP is common following MI and may result in clinical manifestation of HF, however, due to several other confounding factors, it is a poor independent predictor of HF[212].

The catheter-based hemodynamic assessment on animals after 6 weeks of sustained CAL indicate that the surgery induced several hemodynamic abnormalities in the LV. Similar to EF, the cardiac output and stroke volume was significantly reduced in animals with CAL, indicating an insufficient volume of blood cycling out of the heart. The LVEDP was also increased, confirming a manifestation of HF induced by CAL surgery. All of these pathologies were significantly attenuated with *L. rhamnosus* GR-1 administration, but not normalized. This is likely because the infarction of the LV causes irreversible damage to the heart that results in an unpreventable increase in LVEDP, regardless of any treatment administered post-infarction.

Rather than evaluate the direct damage sustained to the LV by CAL surgery, this CAL model was designed to assess the compensatory response that initiates and progresses with HF. Because there was no release of the ligation or reperfusion of the coronary artery, the CAL surgery induced cardiac hypertrophy that is a hallmark feature of HF and occurs secondary to myocardial infarction. This hypertrophic response is one of the key therapeutic targets for HF patients, as the initially adaptive hypertrophy eventually becomes maladaptive, and often coincides with impaired EF, cardiac output, and increased LVEDP. The compromised cardiac mechanical function in HF may be considered a direct consequence and symptom of the maladaptive compensatory response that occurs after MI. The evidence of improved cardiac mechanical function and LVEDP in CAL animals receiving *L. rhamnosus* GR-1 treatment suggests that the underlying mechanism lies in an attenuation of the maladaptive compensatory response to MI. Indeed, upon assessing cardiac hypertrophy, *L. rhamnosus* GR-1 significantly attenuated the CAL-induced increase in LVW and ANP gene expression. In the four week study, a reduction in cardiac hypertrophy by *L. plantarum* 299v was also determined by LVW/BW assessment. This impressive outcome suggests a novel application for probiotics. Related studies have explored the use of probiotics as a protective treatment prior to a major cardiac event such as MI, while these results indicate that probiotics are also effective in improving the recovery following such an event.

Clinically, these findings have major implications. The immediate intervention strategies for reducing the severity of ischemia during MI have improved mortality in recent years,

yet the risk of developing HF still remains high[17]. The recovery process that precedes and persists throughout the stages of HF is a critical therapeutic target post-MI. The parameters analyzed in these studies are among some of the key factors for diagnosing HF, as well as therapeutic targets. The current treatment strategies rely on β-blockers and ACE-inhibitors to reduce contractility, slow the heart rate, and reduce blood pressure[210]. While these drugs can improve the symptoms of HF, there still remains a therapy to be developed that will effectively prevent HF. Newly diagnosed HF patients unfortunately face the reality of long-term medication in order to manage the disease. HF is most common in adults over 65 years old[213] and often these patients are already taking medication for other chronic conditions. The impact of chronic medication plays a significant role in patients' lives. First, the cost of HF medication in addition to other medications, is substantial. A study assessing the cost of HF medication to outpatients in the US found that medication for HF alone cost $340 per month[213]. For patients with HF and other non-cardiovascular comorbidities such as chronic obstructive pulmonary disease and diabetes, medication costs $600 per month[213]. The cost of medication for patients with severe heart failure was significantly higher than those with mild heart failure, indicating that as the disease progresses, more medication is required[213]. For patients without access to health insurance and lacking personal financial resources, these costs stand as a strong deterrent for medication compliance. Prescribed dosing may be altered or stopped altogether to decrease cost. Lack of sufficient medication can exacerbate the progression of HF and eventually result in hospitalization and further economic burden. Based on the suggested retail price of a product containing one of the probiotic strains tested here and assuming the dosing would remain the same then the cost would be less than $40 per month. While it is unlikely that probiotics will entirely replace current treatment regimens for heart failure, combination therapy could improve the financing.

Probiotics also provide additional benefits that cannot be offered by standard HF medications. In addition to high cost, the chronic use of HF medications such as β-blockers and ACE-inhibitors, or any pharmacological agent, brings risk of adverse side effects. The most common side effects of both β-blockers and ACE-inhibitors are cough, dizziness, hypotension, and bradycardia[214,215]. Impaired renal function is a risk with

ACE-inhibitor medication that increases proportionally to dose. This side effects are often managed by additional medication which altogether complicates the issue of costs and risks associated with chronic medication. Again, non-compliance may become a serious issue for patients unable to cope with the requirements of chronic medication. The use of a probiotic, with very low occurrence of adverse effects and several systemic nutritional and health benefits, in addition to or as a replacement for pharmaceutical drugs, is worthy of consideration for preventing progression of HF while minimizing adverse effects of chronic medication. The ability of *L. rhamnosus* GR-1 and *L. plantarum* 299v to prevent the CAL-induced HF is an exciting finding. Neither strain has been evaluated for ACE-inhibitory activity, however other *L. rhamnosus* and *L. plantarum* strains have both been reported to produce ACE-inhibitory peptides and have potential in antihypertensive therapy[177,216].

4.1.2 The effect of probiotic administration on adipokine signaling

The role of adipokine signaling in cardiovascular health is of great interest as a potential therapeutic target, as clinical and animal studies suggest that leptin production and circulation is upregulated in CVD[47,52,57,59,196]. Leptin, a 16 kDA peptide, is widely known for the important role it plays in energy metabolism and food intake. Receptors in the hypothalamus interact with leptin to cause an overall feeling of satiety, resulting in food intake inhibition and an increase energy expenditure[56]. Mutations in the leptin gene, *ob*, cause obesity in mice, where knock out animals are three times as heavy and have a five-fold increase in body fat content compared to wildtype[217]. Similar to insulin, complications in leptin signaling have become a major issue with obesity. Insulin resistance caused by obesity drives excessive leptin production, which can also lead to leptin resistance[53]. The role of the gut microbiome in adipokine signaling has generated interest, due to the advances in next-generation sequencing that enable the evaluation of community-level differences in microbiota composition with a given state of disease[218]. 16S rRNA sequencing of the gut microbiota of leptin-deficient mice has revealed a major reduction of the Bacteriodetes phyla and increase in Firmicutes[219], similar to the gut microbial profile of obese individuals[67,86]. The role the constituent bacteria in each phyla

plays in the pathogenesis of obesity is not well understood, and attempts to identify particular species directly involved in obesity are ongoing.

One theory relates to the emerging concept of the microbiome-gut-brain axis. There is indirect and direct evidence that bidirectional signaling mediated by the gut microbiota occurs between the gut and the brain. Altered composition of the gut microbiota has been clinically reported in patients with autism and short-term improvement of symptoms are reported with antibiotic treatment[88,96]. Psychiatric symptoms of stress and anxiety trigger flare-ups in irritable bowel syndrome and inflammatory bowel disease patients[220,221]. Animal studies on the gut-brain axis have provided direct evidence for a role of the gut microbiota. Germ-free animals have lower corticosterone levels in response to stress and display less stress and anxiety-like behavior than animals colonized with specific pathogens. The colonization of commensal bacteria in the germ-free animals resulted in a normalization of these behaviours[222]. The possibility exists that similar gut-derived influences on the brain may involve adipocytes and leptin signaling in the hypothalamus. A way to investigate this would involve monitoring the leptin levels and food consumption of germ-free, specific pathogen, and/or probiotic-colonized mice with and without mutations of the leptin gene, *ob*. Depending on the composition or manipulation of the gut microbiota composition, there may or may not be a difference in leptin-related food consumption and energy metabolism.

In addition to metabolism, leptin is involved in cardiovascular health. It is considered a deleterious hormone in HF[223]. High levels of circulating leptin are common in HF patients, and leptin activity exacerbates inflammation and hypertrophy[52,57,59,196]. While the cardiac pathologies associated with leptin are often tied to obesity, the deleterious effect of leptin in HF can also occur independent of obesity[224]. This is likely because leptin is not exclusively produced by adipose tissue, but also by the heart[56]. The study performed by Lam *et al*.[180] revealed that pre-administration of *L. plantarum* 299v prior to I/R in the heart significantly reduced circulating leptin levels and ischemic injury to the heart. The cardioprotection conferred by *L. plantarum* 299v against ischemic injury was abolished when leptin was administered to the rats prior to the surgery. This indicates a

pivotal role of leptin in the mechanism of action of *L. plantarum* 299v against ischemic injury.

In the present CAL model for HF, it is apparent that *L. rhamnosus* GR-1 also conferred an anti-leptin effect. As expected, blood analysis indicated a significant increase in absolute plasma leptin concentration after 6 weeks of sustained CAL. This was entirely blocked with *L. rhamnosus* GR-1 administration, resulting in positive outcomes in cardiac function. Theoretically, a significant decrease in leptin should cause a reduction in satiety and increase in food consumption. Probiotics have reduced circulating leptin levels in other models of disease, but it is rarely without any associated change in body weight or adiposity[225-227]. There was no observed weight gain or change in eating habits in animals with reduced plasma leptin in this study.

We surprisingly saw a trend that *L. rhamnosus* GR-1 administration to sham-operated animals caused an increase in absolute plasma leptin, to the same level as CAL-operated animals without *L. rhamnosus* GR-1. As there was no indication of under-eating in these animals, nor any significant weight loss, presumably this increase in leptin did not result in any adverse metabolic outcomes. This result makes it difficult to speculate on the role of leptin in this model, however clinical studies indicate that the concentration of leptin relative to adiponectin is a more important marker for CVD than the absolute plasma leptin concentration [223,228]. The substantial increase in plasma leptin to adiponectin ratio in CAL-operated animals was significantly attenuated by *L. rhamnosus* GR-1 administration. The was still an increase in the leptin to adiponectin ratio in sham-operated animals administered *L. rhamnosus* GR-1 compared to the control, however this difference was not statistically significant. It is possible that there is a healthy range of activity of these two adipokines that is influenced or managed by *L. rhamnosus* GR-1. Physiologically, the relationship between leptin and adiponectin is somewhat mutually exclusive; when one is upregulated, the other is downregulated, and the two generally have opposing systemic effects. However, considering the complexity of the diseases these adipokines are associated with, including HF, it is difficult to ascertain the cause and effect relationship between *L. rhamnosus* GR-1 and leptin activity. This study indicates that the attenuation of HF by probiotic administration was in part mediated by

reduction of leptin activity, however it cannot be determined from this data whether leptin levels were reduced because of the improved parameters of cardiac function and remodeling, or whether it was the reduction of circulating leptin by probiotics that helped improve the cardiac abnormalities. Future studies could include exogenous administration of leptin along with probiotics, or the use of leptin deficient animals.

4.2 Exploring the mechanisms responsible for the attenuation of HF by *L. rhamnosus* GR-1

The CAL animal studies were designed to investigate whether probiotics attenuated HF, and if so, whether it was via modulation of the existing gut microbiota and/or inflammatory signaling. In addition, mechanistic *in vitro* studies were performed on primary cardiomyocytes isolated from the neonatal rat heart.

4.2.1 The role of cytokines

The initial inflammatory response to MI is a pre-requisite for the healing process of phagocytosis and scar formation to take place, however, chronic inflammation can be detrimental as it causes additional cell death and promotes cardiac remodeling[24]. HF is often associated with chronic inflammation in which pro-inflammatory cytokine parameters are elevated in patients[222]. IL-6, TNF-α, fractalkine are among the several of the pro-inflammatory cytokines that positively correlate to severity of MI, impaired survival post-MI, as well as poor short-term and long-term clinical outcomes[229].

Reducing inflammation in HF patients is important for managing disease progression. Animal experiments have suggested that anti-inflammatory therapies might be beneficial in HF, however translating these findings to a clinical setting has been widely unsuccessful, as there is likely no one common inflammatory pathway involved[230]. While the use of probiotics as an anti-inflammatory therapy for HF has not been explored, there are many studies showing that certain probiotic strains, including *L. rhamnosus* GR-1, confer anti-inflammatory effects[231-233]. In the gut epithelium, probiotics interact with toll-like receptors and transcription factors that regulate widespread inflammatory responses, resulting in benefits for gastrointestinal diseases such as inflammatory bowel disease and necrotizing enterocolitis[137,138] and also for distal inflammatory diseases such as

rheumatoid arthritis[139]. Genetically engineering probiotics for the production of anti-inflammatory factors, such as IL-10, is a strategy for colonic inflammation that is currently under clinical investigation[29].

It was hypothesized that probiotics could improve outcomes of HF in the CAL study by reducing systemic inflammation. At the completion of the 6 week-long study, the blood levels of nine pro-inflammatory cytokines (fractalkine, GRO/KC, IFN-γ, IL-1α, IL-6, MCP-1α, MIP-1α, RANTES, and TNF-α) were analyzed. No change in blood cytokine levels was found with respect to the CAL surgery, nor was there any influence of *L. rhamnosus* GR-1 treatment on serum cytokine levels. This was a surprising result, considering that all other indices of HF measured were positively correlated the CAL surgery. However, the findings corroborate with those published by Lam *et al.*[180] who reported that *L. plantarum* 299v administration did not affect the blood levels of 22 cytokines measured in their I/R model. A limitation in that study was that these blood-borne factors were analyzed only at the end-point. Blood sample collection from rats can be troublesome and invasive, and it is possible that the associated stress will influence other parameters under investigation. It is recommended to not to take blood samples more than once every two weeks, and many popular methods, such as retro-orbital bleeding, require anaesthetization[234]. For these reasons, we decided to only collect blood samples by terminal bleeding at sacrifice. Without collecting blood samples before surgery, immediately post-surgery, or mid-trial, the serum cytokine analysis is limited to only end-point data, and it cannot be concluded that neither CAL surgery nor probiotics influence inflammation in this model.

4.2.2 The role of the gut microbiota

The gut microbiome is a rich ecosystem that plays a critical role in our health. Beginning from the early postnatal days of colonization, the gut microbiome develops into a synergistic organ in which a specific equilibrium is required for optimal health. The microbes of the gut perform a multitude of functions, including nutrient breakdown, vitamin and metabolite production for absorption across the intestine[68], as well as immune system activation and modulation[82].

The core gut microbiota develops rapidly after birth and is generally considered stable throughout adulthood, however perturbations such as those brought on by environmental stress, disease, altered diet, can drastically alter the core gut microbial profile[82]. Dysbiosis has an impact on disease and has been associated with a wide range of conditions, from *C. difficile* infections[70] to autism[88]. Common therapies for restoring microbial equilibrium involve the use of nonspecific antibiotics, however the inability of these drugs to discriminate between 'good' and 'bad' bacteria emphasizes the need for alternative therapies. Some animal studies have demonstrated that probiotic therapy can restore a dysbiotic gut microbiota associated with obesity[159], yet other studies, including human clinical trials, have shown that probiotic administration results in neither probiotic colonization nor any over dramatic changes in species composition of the gut[160].

In the 6 week-long CAL study, we performed 16S rRNA next-generation sequencing on gut digesta samples for with 2 main objectives: (i) to determine whether or not the gut microbial profile of healthy animals differs from those with HF, and (ii) to determine whether or not the effects of probiotic administration on the outcome of HF is conferred by an alteration of the gut microbial composition.

Among the 242 OTUs identified in the 16s rRNA next-generation sequencing analysis, *L. rhamnosus* GR-1 was not detected in cecum digesta samples. In order to maximize accuracy and avoid erroneous OTU assignment, the cut-off for detection and taxonomic assignment for each sample was set at a minimum 0.5% species abundance. The fact that *L. rhamnosus* GR-1 was not detected in this analysis indicates that it represents less than 0.5% of the total species present in the cecum. Considering the overall diversity and richness of the gut microbiota (10^{14} organisms), the daily dose of 10^{9} CFU of *L. rhamnosus* GR-1 would account for only 0.001% of the total bacteria present, therefore we were not surprised that GR-1 was not detected using the Ion Torrent platform. It is apparent that *L. rhamnosus* GR-1 cause a significant change in the composition of the cecum microbiota, at the community level. It is possible that the phenotype observed with probiotic administration occurs due to a direct interaction between the probiotics and the heart. While bacterial translocation across the epithelial border may occur in patients with compromised gut barrier permeability[235], it is not thought to have occurred in this model.

Despite the cardiac injury sustained by CAL, the rats were otherwise in good health without any symptoms of gastrointestinal dysfunction. Instead, it is more likely that the probiotics produce one or more soluble factors, such as a small protein or metabolite that crossed the gut epithelial barrier and entered the blood circulation. Upon reaching the heart, or via factors that affect the heart, this factor(s) could potentially be responsible for the attenuation of cardiac remodeling, hypertrophy, and HF in the rat.

4.2.3 The direct interaction of probiotics with cardiomyocytes *in vitro*

To further investigate the mechanisms responsible for the attenuation of HF by *L. rhamnosus* GR-1 and *L. plantarum* 299v, co-culture experiments were designed using neonatal rat ventricular cardiomyocytes (NVCM). These terminally differentiated primary cells are often used for *in vitro* HF models because of their ideal response to pharmacological manipulation[1]. It was hypothesized that the addition of live probiotic cells to NVCM culture would confer cardiac benefits. The initial simple assays were performed to determine NVCM viability after exposure to live probiotic cells. Cell lines that are physiologically accustomed to bacterial exposure, such as gut epithelial cells, gingival cells, skin cells, or vaginal cells, may be tolerant to bacterial co-culture *in vitro*. However, in culturing cells derived from otherwise "sterile" organs, great measures are taken to avoid bacterial exposure. In the case of NVCM, these cells are not conventionally exposed to bacteria. To prevent contamination, antibiotics were added to the culture media. We were initially unsure, therefore if either the NVCM or probiotics would survive the co-culture conditions, as reports of similar co-culture techniques using NVCM are scarce in the literature. The heart is considered a "sterile" organ, in which encounters with bacterial species can result in serious infections. We were impressed to find that the addition of 5×10^7 CFU of *L. rhamnosus* GR-1 to NVCM had no adverse effects on cell viability or function. In fact, with the daily replacement of cell culture media and *L. rhamnosus* GR-1 cells, spontaneously beating NVCM were maintained for as long as seven days, which is the recommended duration of culture for these primary cells[1]. The inclusion of antibiotics in the cell culture media also seemingly did not affect *L. rhamnosus* GR-1 cell viability, as its cells were cultivated from the media after 24

hours. These encouraging preliminary assays seemed to support the hypothesis that probiotics provide a direct benefit to cardiomyocytes, independent of the gut.

To employ a model relevant to HF, PE was to induce hypertrophy in NVCM. This α-adrenergic receptor agonist is an agent commonly used in models of cardiac hypertrophy[35,236,237]. The effect of PE on NVCM was evaluated by calculating the cell surface area and gene expression of the hypertrophic markers ANP and aSKA 24 hours after PE exposure. To closely mimic the design of the CAL studies, each probiotic treatment was administered to NVCM immediately following PE exposure. Physical analysis on the change in cell surface area indicates that *L. rhamnosus* GR-1, *L. plantarum* 299v, and *S. salivarius* K12 administration blocks PE-induced hypertrophy in NVCM over 24 hours, and normal NVCM morphology and function is maintained. This phenotype was seen for a range of concentrations of *L. rhamnosus* GR-1, suggesting a strong potency of the treatment. The anti-hypertrophic activity of *L. rhamnosus* GR-1 was confirmed by a significant attenuation of the PE-increased gene expression of both ANP and aSKA. This supported the hypothesis that the cardiac benefits conferred by probiotics *in vivo* and *in vitro* is not strain specific. Interestingly, NVCM co-cultured with *Lactobacillus reuteri* RC-14, a vaginal isolate, generally did not survive the assays. Attempts to isolate *L. reuteri* RC-14 from the culture media after the 24 hour co-culture were unsuccessful, indicating that the co-culture conditions were not viable for *L. reuteri* RC-14 either. This showed that not all probiotics have the same mechanism of action or confer the same health benefits. It is possible that the *L. reuteri* RC-14 co-culture is non-viable because it produces hydrogen peroxide in culture[134].

It was presumed that live probiotic cells are required to confer salutary cardiac benefits. Dead *L. rhamnosus* GR-1 or *L. plantarum* 299v cells were not administered in the CAL studies, however in NVCM co-culture experiments, HK *L. rhamnosus* GR-1 cells were administered. *L. rhamnosus* GR-1 cells were removed from the PCM, washed, and incubated at 80°C. After unsuccessfully cultivating these cells on MRS agar, they were determined non-viable. A loss of anti-hypertrophic activity was noted, as determined by the fold change in cell surface area and ANP gene expression. However, the difference in the NVCM hypertrophy of live GR-1 cells compared to HK GR-1 cells was not

statistically significant for all parameters measured. One possible explanation is that *L. rhamnosus* GR-1 formed a heat-stable biofilm and thus undetectable live organisms were still present. Another is that anti-hypertrophic factors produced by *L. rhamnosus* GR-1 were present despite the heat treatment. Further experiments are warranted to explain the results.

It is apparent that probiotic strains can provide direct protection to cardiomyocytes against hypertrophy. While cardiomyocytes will not encounter such high densities of live probiotic cells *in vivo*, soluble factors produced by the lactobacilli may cross the gut epithelial barrier into the blood and reach the heart.

There is evidence that *L. rhamnosus* GG produces a soluble protein with anti-apoptotic activity that is directly protective against I/R-associated injury to the heart[178,179]. We were able to obtain a knock out mutant strain for this protein in *L. rhamnosus* GR-1 CMPG10200. Its absence did not affect the outcome on the NVCM surface area, as CMPG10200 conferred an anti-hypertrophic effect identical to the wild type *L. rhamnosus* GR-1. Based on these results, it was decided not to continue analysis on this mutant, but to analyze the effect of NVCM co-culture with PCM, void of live probiotic cells.

The *L. rhamnosus* GR-1 supernatant has been studied in other settings, and found to contain some biosurfactant[238,239]. In the present studies, when lactobacilli whole cells were completely removed, the PCM conferred a similar effect against PE-induced hypertrophy in terms of cell surface area and gene expression of ANP and aSKA, albeit to a lesser magnitude than the whole cell preparation. This indicated that *L. rhamnosus* GR-1 produces a factor that prevented PE-induced hypertrophy in NVCM. It was hypothesized that the responsible factor was a protein, and treated the PCM with trypsin, with the result that the effects on surface area and ANP gene expression were completely lost. While this effect was not as marked in aSKA, there was a slight increase in gene expression compared to regular PCM.

The role of proteins derived from *Lactobacillus rhamnosus* GR-1in preventing hypertrophy was further examined by centrifugal filtration based on molecular size

exclusion. Filtration of the PCM through 20 nm pore size filter did not result in a significant difference in the average NVCM surface area compared to untreated cells, however the fold change in surface area indicated that anti-hypertrophic activity was lost in both the filtrate and retentate. This suggested that there is may be more than one protein responsible for attenuating hypertrophy which cannot be effectively separated by size. It was hoped to elucidate the heat sensitivity of these potential proteins by incubating the PCM at a high temperature, typically heat stable proteins are smaller in mass. Trypsin acts indiscriminately on proteins by cleaving peptide chains, but heat denaturing a solution may result in variable outcomes, depending on the nature of the proteins present. Large proteins are typically heat sensitive and denature at lower temperatures than smaller ones. Small, heat stable proteins (less than 20 kDa) have the potential to maintain function after heat incubation and are reported to be produced by various lactobacilli strains[240,241]. In these experiments, heat denatured PCM did not significantly attenuate the PE-induced increase in cell surface area (fold change) or increase in ANP gene expression, indicating a loss in anti-hypertrophic activity. This may be due to the destabilization of the protein(s) by the heat treatment. There was no difference in gene expression of aSKA with heat denatured PCM compared to regular PCM. These mixed results might suggest that the PCM affects ANP expression differently than aSKA, or that is more than one protein involved.

There was a difference in the magnitude of the change of gene expression in ANP compared to aSKA, depending on the nature of the PCM treatment. Both of these hypertrophic markers are expressed during prenatal cardiac development and then are downregulated after birth[242,243]. Baseline expression of ANP and aSKA in NVCM is higher than adult cardiomyocytes and gene expression can be induced in both cell types by hormonal and hemodynamic stimuli as well as pharmacological hypertrophic agents[242]. The signaling mechanisms and pathways for ANP and aSKA differ from one another, and they are not equally as responsive to some stimuli. As discussed earlier, ANP is a vasodilator that becomes active in response to hypertension[242]. aSKA is involved in muscle formation and is associated with cellular growth[243]. Hypertrophic phenotypes, therefore, may feature activated ANP expression, while aSKA is not induced[243]. The difference lies in the nature of the stimulus used to induce hypertrophy.

While PE has shown to induce both ANP and aSKA expression in several models of cardiac hypertrophy[207,237,244], other factors may affect the complex and diverse signal transduction pathways. In the present studies, it is possible that PCM interferes with the signaling pathway for PE-induced aSKA gene expression, while ANP is unaffected. Additional assays can be used to confirm hypertrophy, such as cellular leucine incorporation as a marker for protein synthesis. To continue these mechanistic studies, a comprehensive functional analysis of the *L. rhamnosus* GR-1-derived PCM should first be performed in order to understand exactly what factors are present in the PCM.

The main limitation to the design of the NVCM co-culture experiments was the availability of NVCM. These primary cells were kindly harvested and provided by the Karmazyn lab, however Dr. Karmazyn's own projects understandably took precedence over this one. The inability to pass and store these cells, as they are already terminally differentiated and do not divide, meant that the use of NVCM for these co-culture experiments was entirely dependent on the availability of fresh cells provided with each harvest. The best attempts were made to match the treatments for cell surface area experiments to the gene expression experiments in order to confirm the anti-hypertrophic activity of probiotics. Given the limited availability of cells, this was not always achieved. With better access to NVCM, future co-culture experiments and treatments should include dose and time-dependent trials, and additional manipulation of the PCM. In spite of the limitations, these *in vitro* NVCM co-culture experiments provided the foundation for future research into exploring the mechanism behind the attenuation of HF by probiotics. The next step for this model would be to test the ability of lactobacilli factors to cross an epithelial barrier using a transwell apparatus. These experiments will be required in order demonstrate that the anti-hypertrophic factors derived from *L. rhamnosus* GR-1 are able to pass through the gut and access the heart.

As schematic diagram of the potential mechanism of action of the probiotic *in vivo* is displayed in figure. This diagram summarizes the hypotheses that were tested in this thesis project and the remaining questions that are left to be answered.

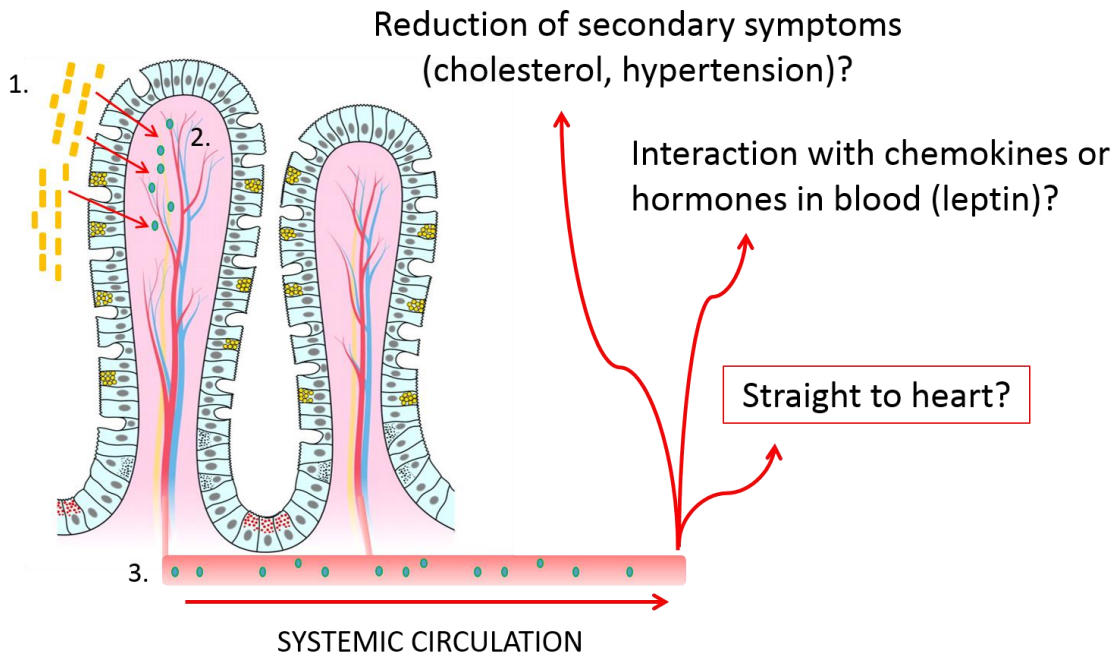

Figure 21: Schematic diagram depicting the potential mechanism of action of probiotics at the gut epithelium.

1. Probiotic cells arrive at the intestinal epithelium and produce a soluble factor through metabolic processes. 2. The soluble factor is transported across the epithelial barrier via active or passive transport, and enters blood circulation. 3. The portal venous system transports the soluble probiotic products via the liver to the heart. Along the way, there is potential interaction with cholesterol and bile acids, and cytokines and hormones in the blood. The probiotic products then may arrive at the heart via the vena cava and provide a direct therapeutic benefit to the heart.

4.3 Clinical implications and the potential for probiotic therapy for heart failure patients

Probiotics represent one of the fastest growing consumer items on the functional food and nutraceutical market today[245]. While researchers and regulatory agencies across the world emphasize the importance of validating efficacy claims made by manufacturers, there unfortunately still exists a multitude of products with unsubstantiated claims and misleading applications for use. In order to ensure consumer confidence, rigorous clinical trials are required before a probiotic product can gain approval for therapeutic use. To date, Health Canada has approved only one probiotic product with cardiovascular health claims. This product, Cardioviva™, contains 2 billion CFU of encapsulated *Lactobacillus reuteri* NCIMB 30242 and has been clinically proven to lower LDL-cholesterol levels by 11.6% in hypercholesterolemic adults[246]. This product represents decades of research and development into validating the activity of bile salt hydrolase and cholesterol sequestration by lactobacilli that has been widely reported in the literature.

Another benefit of probiotic therapy is that cases of adverse effects are very rare. For both Cardioviva™ and GoodBelly containing *Lactobacillus plantarum* 299v, the potential side effects are gas and bloating within the initial days of consumption. *L. rhamnosus* GR-1 has been notified and accepted by the American Food & Drug Administration and has been used extensively for the maintenance of a healthy vaginal microbiota and for lowering the risk of bacterial vaginosis and recurrent urinary tract infections without side effects [122,134,183,231]. With the safety of probiotic use established, future research into the mechanism of action, strain specificity, and dose and time-course regimes should be prioritized.

A plethora of probiotic products are available to consumers and it is imperative to thoroughly characterize the benefits of administration for HF before recommending particular products in a clinical setting. The data presented in this thesis hopefully represents a seminal point in therapy for HF. Given the relatively safe track record of probiotics[247] it should be relatively easy to test this concept in clinical settings and verify that these outcomes are not rat specific. With the extreme unlikelihood of any adverse drug interactions, it is possible that probiotics can be used in addition to the current drugs

available for HF. Furthermore, the evidence that sterile PCM can confer anti-hypertrophic effects may provide a natural supplement route, albeit without any health claims. Whether or not probiotics have an additive effect to conventional HF medication remains to be seen. Such combination testing would be interesting to conduct in an animal model.

4.4 Conclusions

Several conclusions can be made from the results of the studies presented in this thesis, all of which should be considered in the wider context of novel approaches to treating HF:

(1) Oral probiotic administration immediately following myocardial infarction reduces the severity of HF in the rat. This is based on the measurement of several parameters that are similarly used as indices of HF in a clinical setting. The administration of two different species of probiotic *Lactobacillus* exerted identical cardiac benefits, to a drug-like effect. This implies that probiotic administration post-myocardial infarction may be a novel treatment strategy for HF.

(2) The salutary effect of oral probiotic administration on the heart occurs independent of the composition of the gut microbiota. This is based on the 16S rRNA gene next-generation sequencing analysis in the CAL study and the co-culture experiments evaluating the direct interaction of probiotic cells with NVCM. These findings imply that probiotics function as they pass through the intestine and produce effects that directly affect the heart.

(3) *Lactobacillus rhamnosus* GR-1 produces one or many soluble factors in broth culture that independently confers an anti-hypertrophic benefit to cultured NVCM. This suggests that the attenuation of HF by probiotic administration might occur primarily through a reduction of cardiac hypertrophy and ventricular remodeling and based on *in vitro* experiments, it is likely that this anti-hypertrophic factor is a protein.

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Appendices

Appendix 1. Copyright agreement from the American Heart Association for the manuscript published in *Circulation: Heart Failure*

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Appendix 2: Animal use protocol approval from the Animal Use Subcommittee of the University Council on Animal Care

AUP Number: 2013-031
PI Name: Karmazyn, Morris
AUP Title: Heart Failure
Approval Date: 06/13/2013

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Heart Failure" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2013-031::1

1. This AUP number must be indicated when ordering animals for this project.
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Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

Appendix 3. Statistical analysis results for NVCM experiments

ANOVA results: Controls

Cell size (μm^2)

	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	Control vs GR-1	240.4	3.736	No	ns	-34.81 to 515.7
18	Control vs MRS Broth	173.3	2.692	No	ns	-102.0 to 448.5
19	Control vs PE	-509.7	9.466	Yes	****	-740.0 to -279.4
20	Control vs PE + MRS Broth	-413.4	6.424	Yes	**	-688.7 to -138.2
21	GR-1 vs MRS Broth	-67.17	0.8521	No	ns	-404.3 to 269.9
22	GR-1 vs PE	-750.2	10.64	Yes	****	-1052 to -448.6
23	GR-1 vs PE + MRS Broth	-653.9	8.296	Yes	****	-991.0 to -316.8
24	MRS Broth vs PE	-683.0	9.688	Yes	****	-984.5 to -381.5
25	MRS Broth vs PE + MRS Broth	-586.7	7.444	Yes	****	-923.8 to -249.6
26	PE vs PE + MRS Broth	96.29	1.366	No	ns	-205.2 to 397.8

Cell size (Fold change)

	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	GR-1 vs MRS Broth	0.008463	0.1204	No	ns	-0.2957 to 0.3126
18	GR-1 vs PE	-0.3859	6.138	Yes	**	-0.6579 to -0.1139
19	GR-1 vs PE + MRS Broth	-0.3231	4.598	Yes	*	-0.6272 to -0.01901
20	MRS Broth vs PE	-0.3943	6.273	Yes	**	-0.6663 to -0.1223
21	MRS Broth vs PE + MRS Broth	-0.3316	4.718	Yes	*	-0.6357 to -0.02747
22	PE vs PE + MRS Broth	0.06274	0.9980	No	ns	-0.2093 to 0.3348

ANOVA results: CMPG10200

Cell size (μm²)

	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	MRS Broth vs PE + MRS Broth	-406.8	6.262	Yes	****	-672.8 to -140.7
18	MRS Broth vs PE + CMPG10200	67.76	1.212	No	ns	-161.2 to 296.8
19	MRS Broth vs PE + CMPG10200 PCM	32.91	0.5886	No	ns	-196.1 to 261.9
20	MRS Broth vs PE + CMPG10200 cells	69.86	1.250	No	ns	-159.1 to 298.8
21	PE + MRS Broth vs PE + CMPG10200	474.5	6.416	Yes	****	171.6 to 777.5
22	PE + MRS Broth vs PE + CMPG10200 PCM	439.7	5.945	Yes	**	136.8 to 742.6
23	PE + MRS Broth vs PE + CMPG10200 cells	476.6	6.445	Yes	****	173.7 to 779.6
24	PE + CMPG10200 vs PE + CMPG10200 PCM	-34.86	0.5269	No	ns	-305.8 to 236.1
25	PE + CMPG10200 vs PE + CMPG10200 cells	2.094	0.03166	No	ns	-268.8 to 273.0
26	PE + CMPG10200 PCM vs PE + CMPG10200 cells	36.95	0.5586	No	ns	-234.0 to 307.9

Cell size (Fold change)

	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	MRS Broth vs PE + MRS Broth	-0.3338	5.508	Yes	**	-0.5903 to -0.07731
18	MRS Broth vs PE + CMPG10200	0.05528	0.9851	No	ns	-0.1822 to 0.2927
19	MRS Broth vs PE + CMPG10200 PCM	0.05323	0.9486	No	ns	-0.1842 to 0.2907
20	MRS Broth vs PE + CMPG10200 cells	0.05581	0.9946	No	ns	-0.1816 to 0.2933
21	PE + MRS Broth vs PE + CMPG10200	0.3891	7.596	Yes	****	0.1723 to 0.6058
22	PE + MRS Broth vs PE + CMPG10200 PCM	0.3870	7.556	Yes	****	0.1702 to 0.6038
23	PE + MRS Broth vs PE + CMPG10200 cells	0.3896	7.606	Yes	****	0.1728 to 0.6064
24	PE + CMPG10200 vs PE + CMPG10200 PCM	-0.002048	0.04470	No	ns	-0.1959 to 0.1918
25	PE + CMPG10200 vs PE + CMPG10200 cells	0.0005332	0.01164	No	ns	-0.1933 to 0.1944
26	PE + CMPG10200 PCM vs PE + CMPG10200 cells	0.002581	0.05634	No	ns	-0.1913 to 0.1965

ANOVA results: 299v & K12

Cell size (μm²)

	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	MRS Broth vs PE + MRS Broth	-443.0	6.422	Yes	**	-747.0 to -139.0
18	MRS Broth vs PE + GR-1	42.22	0.6122	No	ns	-261.8 to 346.2
19	MRS Broth vs PE + K12	-80.20	1.040	No	ns	-420.1 to 259.7
20	MRS Broth vs PE + 299V	6.778	0.08789	No	ns	-333.1 to 346.6
21	PE + MRS Broth vs PE + GR-1	485.2	8.123	Yes	****	222.0 to 748.5
22	PE + MRS Broth vs PE + K12	362.8	5.260	Yes	*	58.82 to 666.8
23	PE + MRS Broth vs PE + 299V	449.8	6.521	Yes	**	145.8 to 753.8
24	PE + GR-1 vs PE + K12	-122.4	1.775	No	ns	-426.4 to 181.6
25	PE + GR-1 vs PE + 299V	-35.45	0.5139	No	ns	-339.4 to 268.5
26	PE + K12 vs PE + 299V	86.98	1.128	No	ns	-252.9 to 426.8

Cell size (Fold change)

	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	MRS Broth vs PE + MRS Broth	-0.3025	4.830	Yes	*	-0.5815 to -0.02360
18	MRS Broth vs PE + GR-1	0.1260	2.103	No	ns	-0.1407 to 0.3927
19	MRS Broth vs PE + K12	0.1403	2.095	No	ns	-0.1579 to 0.4385
20	MRS Broth vs PE + 299V	0.1350	2.016	No	ns	-0.1632 to 0.4332
21	PE + MRS Broth vs PE + GR-1	0.4285	7.789	Yes	****	0.1835 to 0.6735
22	PE + MRS Broth vs PE + K12	0.4428	7.070	Yes	**	0.1639 to 0.7218
23	PE + MRS Broth vs PE + 299V	0.4376	6.986	Yes	**	0.1586 to 0.7165
24	PE + GR-1 vs PE + K12	0.01432	0.2390	No	ns	-0.2524 to 0.2810
25	PE + GR-1 vs PE + 299V	0.009054	0.1512	No	ns	-0.2577 to 0.2758
26	PE + K12 vs PE + 299V	-0.005261	0.07857	No	ns	-0.3035 to 0.2929

ANP

	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	MRS Broth vs PE + MRS Broth	-1.049	6.990	Yes	**	-1.698 to -0.3995
18	MRS Broth vs PE + 299v	0.7725	5.149	Yes	*	0.1234 to 1.422
19	MRS Broth vs PE + K12	0.4055	2.920	No	ns	-0.1954 to 1.007
20	PE + MRS Broth vs PE + 299v	1.821	11.36	Yes	****	1.127 to 2.515
21	PE + MRS Broth vs PE + K12	1.454	9.693	Yes	****	0.8050 to 2.103
22	PE + 299v vs PE + K12	-0.3670	2.446	No	ns	-1.016 to 0.2822

aSKA

	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	MRS Broth vs PE + MRS Broth	-1.307	8.968	Yes	****	-1.938 to -0.6763
18	MRS Broth vs PE + 299v	0.6749	4.631	Yes	*	0.04429 to 1.305
19	MRS Broth vs PE + K12	0.5083	3.767	No	ns	-0.07555 to 1.092
20	PE + MRS Broth vs PE + 299v	1.982	12.72	Yes	****	1.308 to 2.656
21	PE + MRS Broth vs PE + K12	1.815	12.46	Yes	****	1.185 to 2.446
22	PE + 299v vs PE + K12	-0.1666	1.143	No	ns	-0.7972 to 0.4640

ANOVA results: GR-1

Cell size (μm²)

16	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	MRS Broth vs PE + MRS Broth	-433.1	7.985	Yes	****	-667.0 to -199.1
18	MRS Broth vs PE + GR-1 PCM	73.47	1.113	No	ns	-211.3 to 358.2
19	MRS Broth vs PE + HD GR-1 PCM	31.37	0.5784	No	ns	-202.6 to 265.3
20	MRS Broth vs PE + GR-1 PCM Filtrate	57.47	1.060	No	ns	-176.5 to 291.4
21	MRS Broth vs PE + GR-1 Retentate	57.73	0.9796	No	ns	-196.5 to 311.9
22	PE + MRS Broth vs PE + GR-1 PCM	506.5	6.730	Yes	****	181.9 to 831.2
23	PE + MRS Broth vs PE + HD GR-1 PCM	464.4	7.126	Yes	****	183.3 to 745.6
24	PE + MRS Broth vs PE + GR-1 PCM Filtrate	490.5	7.526	Yes	****	209.4 to 771.7
25	PE + MRS Broth vs PE + GR-1 Retentate	490.8	7.099	Yes	****	192.6 to 789.0
26	PE + GR-1 PCM vs PE + HD GR-1 PCM	-42.10	0.5594	No	ns	-366.8 to 282.6
27	PE + GR-1 PCM vs PE + GR-1 PCM Filtrate	-16.00	0.2125	No	ns	-340.7 to 308.7
28	PE + GR-1 PCM vs PE + GR-1 Retentate	-15.74	0.2000	No	ns	-355.3 to 323.8
29	PE + HD GR-1 PCM vs PE + GR-1 PCM Filtrate	26.11	0.4005	No	ns	-255.1 to 307.3
30	PE + HD GR-1 PCM vs PE + GR-1 Retentate	26.36	0.3813	No	ns	-271.9 to 324.6
31	PE + GR-1 PCM Filtrate vs PE + GR-1 Retentate	0.2534	0.003665	No	ns	-298.0 to 298.5

Cell size (Fold change)

16	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	MRS Broth vs PE + MRS Broth	-0.4055	5.745	Yes	**	-0.7193 to -0.09174
18	MRS Broth vs PE + GR-1 PCM	-0.05417	0.6645	No	ns	-0.4165 to 0.3082
19	MRS Broth vs PE + HD GR-1 PCM	-0.2226	3.053	No	ns	-0.5467 to 0.1015
20	MRS Broth vs PE + GR-1 PCM Filtrate	-0.1778	2.438	No	ns	-0.5019 to 0.1463
21	MRS Broth vs PE + GR-1 Retentate	-0.1278	1.676	No	ns	-0.4667 to 0.2112
22	PE + MRS Broth vs PE + GR-1 PCM	0.3514	4.977	Yes	*	0.03757 to 0.6652
23	PE + MRS Broth vs PE + HD GR-1 PCM	0.1829	3.026	No	ns	-0.08580 to 0.4516
24	PE + MRS Broth vs PE + GR-1 PCM Filtrate	0.2278	3.768	No	ns	-0.04095 to 0.4965
25	PE + MRS Broth vs PE + GR-1 Retentate	0.2778	4.310	No	ns	-0.008701 to 0.5642
26	PE + GR-1 PCM vs PE + HD GR-1 PCM	-0.1685	2.310	No	ns	-0.4925 to 0.1556
27	PE + GR-1 PCM vs PE + GR-1 PCM Filtrate	-0.1236	1.695	No	ns	-0.4477 to 0.2005
28	PE + GR-1 PCM vs PE + GR-1 Retentate	-0.07361	0.9654	No	ns	-0.4126 to 0.2653
29	PE + HD GR-1 PCM vs PE + GR-1 PCM Filtrate	0.04485	0.7103	No	ns	-0.2358 to 0.3255
30	PE + HD GR-1 PCM vs PE + GR-1 Retentate	0.09484	1.416	No	ns	-0.2029 to 0.3925
31	PE + GR-1 PCM Filtrate vs PE + GR-1 Retentate	0.04998	0.7463	No	ns	-0.2477 to 0.3477

ANOVA results: GR-1 (cont)

16	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	MRS Broth vs PE + MRS Broth	-1.339	4.968	Yes	*	-2.537 to -0.1410
18	MRS Broth vs PE + GR-1 PCM (50 µL)	0.2948	1.153	No	ns	-0.8415 to 1.431
19	MRS Broth vs PE + GR-1 PCM (5 µL)	-0.04742	0.1760	No	ns	-1.245 to 1.150
20	MRS Broth vs PE + HD GR-1 PCM	-0.4129	1.679	No	ns	-1.506 to 0.6805
21	MRS Broth vs PE + Tryp GR-1 PCM	-2.076	7.133	Yes	****	-3.370 to -0.7822
22	PE + MRS Broth vs PE + GR-1 PCM (50 µL)	1.634	6.390	Yes	**	0.4972 to 2.770
23	PE + MRS Broth vs PE + GR-1 PCM (5 µL)	1.291	4.792	Yes	*	0.09356 to 2.489
24	PE + MRS Broth vs PE + HD GR-1 PCM	0.9258	3.764	No	ns	-0.1676 to 2.019
25	PE + MRS Broth vs PE + Tryp GR-1 PCM	-0.7372	2.533	No	ns	-2.031 to 0.5565
26	PE + GR-1 PCM (50 µL) vs PE + GR-1 PCM (5 µL)	-0.3422	1.339	No	ns	-1.478 to 0.7941
27	PE + GR-1 PCM (50 µL) vs PE + HD GR-1 PCM	-0.7077	3.067	No	ns	-1.733 to 0.3180
28	PE + GR-1 PCM (50 µL) vs PE + Tryp GR-1 PCM	-2.371	8.519	Yes	****	-3.608 to -1.134
29	PE + GR-1 PCM (5 µL) vs PE + HD GR-1 PCM	-0.3655	1.486	No	ns	-1.459 to 0.7279
30	PE + GR-1 PCM (5 µL) vs PE + Tryp GR-1 PCM	-2.029	6.970	Yes	****	-3.322 to -0.7348
31	PE + HD GR-1 PCM vs PE + Tryp GR-1 PCM	-1.663	6.172	Yes	**	-2.861 to -0.4653

16	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	MRS Broth vs PE + MRS Broth	-1.307	5.970	Yes	**	-2.268 to -0.3457
18	MRS Broth vs PE + GR-1 PCM (50 µL)	0.4677	2.715	No	ns	-0.2886 to 1.224
19	MRS Broth vs PE + GR-1 PCM (5 µL)	0.04111	0.2028	No	ns	-0.8489 to 0.9311
20	MRS Broth vs PE + HD GR-1 PCM	0.1751	0.9463	No	ns	-0.6373 to 0.9875
21	MRS Broth vs PE + Tryp PCM	-0.2608	1.191	No	ns	-1.222 to 0.7005
22	PE + MRS Broth vs PE + GR-1 PCM (50 µL)	1.775	9.287	Yes	****	0.9356 to 2.614
23	PE + MRS Broth vs PE + GR-1 PCM (5 µL)	1.348	6.158	Yes	**	0.3868 to 2.309
24	PE + MRS Broth vs PE + HD GR-1 PCM	1.482	7.312	Yes	****	0.5921 to 2.372
25	PE + MRS Broth vs PE + Tryp PCM	1.046	4.470	Yes	*	0.01848 to 2.074
26	PE + GR-1 PCM (50 µL) vs PE + GR-1 PCM (5 µL)	-0.4266	2.477	No	ns	-1.183 to 0.3297
27	PE + GR-1 PCM (50 µL) vs PE + HD GR-1 PCM	-0.2926	1.937	No	ns	-0.9560 to 0.3707
28	PE + GR-1 PCM (50 µL) vs PE + Tryp PCM	-0.7285	3.813	No	ns	-1.568 to 0.1105
29	PE + GR-1 PCM (5 µL) vs PE + HD GR-1 PCM	0.1340	0.7241	No	ns	-0.6784 to 0.9464
30	PE + GR-1 PCM (5 µL) vs PE + Tryp PCM	-0.3019	1.379	No	ns	-1.263 to 0.6594
31	PE + HD GR-1 PCM vs PE + Tryp PCM	-0.4359	2.151	No	ns	-1.326 to 0.4541

ANOVA results: PCM

Cell size (μm²)

	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	PE + MRS Broth vs PE + 50 μL GR-1	577.9	9.763	Yes	****	303.9 to 851.9
18	PE + MRS Broth vs PE + 20 μL GR-1	448.2	7.573	Yes	****	174.2 to 722.2
19	PE + MRS Broth vs PE + 10 μL GR-1	448.8	7.582	Yes	****	174.7 to 722.8
20	PE + MRS Broth vs PE + 5 μL GR-1	384.5	6.497	Yes	**	110.5 to 658.5
21	PE + MRS Broth vs MRS Broth	509.2	7.270	Yes	****	185.0 to 833.4
22	PE + MRS Broth vs PE + GR-1 Cells	537.6	6.770	Yes	**	170.0 to 905.2
23	PE + MRS Broth vs PE + HK GR-1 Cells	533.0	7.610	Yes	****	208.8 to 857.2
24	PE + 50 μL GR-1 vs PE + 20 μL GR-1	-129.6	2.449	No	ns	-374.7 to 115.5
25	PE + 50 μL GR-1 vs PE + 10 μL GR-1	-129.1	2.439	No	ns	-374.2 to 116.0
26	PE + 50 μL GR-1 vs PE + 5 μL GR-1	-193.3	3.652	No	ns	-438.4 to 51.75
27	PE + 50 μL GR-1 vs MRS Broth	-68.69	1.059	No	ns	-368.9 to 231.5
28	PE + 50 μL GR-1 vs PE + GR-1 Cells	-40.27	0.5378	No	ns	-386.9 to 306.3
29	PE + 50 μL GR-1 vs PE + HK GR-1 Cells	-44.91	0.6926	No	ns	-345.1 to 255.3
30	PE + 20 μL GR-1 vs PE + 10 μL GR-1	0.5167	0.009759	No	ns	-244.6 to 245.6
31	PE + 20 μL GR-1 vs PE + 5 μL GR-1	-63.71	1.203	No	ns	-308.8 to 181.4
32	PE + 20 μL GR-1 vs MRS Broth	60.93	0.9398	No	ns	-239.2 to 361.1
33	PE + 20 μL GR-1 vs PE + GR-1 Cells	89.36	1.194	No	ns	-257.2 to 436.0
34	PE + 20 μL GR-1 vs PE + HK GR-1 Cells	84.72	1.307	No	ns	-215.4 to 384.9
35	PE + 10 μL GR-1 vs PE + 5 μL GR-1	-64.22	1.213	No	ns	-309.3 to 180.9
36	PE + 10 μL GR-1 vs MRS Broth	60.42	0.9318	No	ns	-239.7 to 360.6
37	PE + 10 μL GR-1 vs PE + GR-1 Cells	88.84	1.187	No	ns	-257.8 to 435.4
38	PE + 10 μL GR-1 vs PE + HK GR-1 Cells	84.21	1.299	No	ns	-216.0 to 384.4
39	PE + 5 μL GR-1 vs MRS Broth	124.6	1.922	No	ns	-175.5 to 424.8
40	PE + 5 μL GR-1 vs PE + GR-1 Cells	153.1	2.044	No	ns	-193.5 to 499.7
41	PE + 5 μL GR-1 vs PE + HK GR-1 Cells	148.4	2.289	No	ns	-151.7 to 448.6
42	MRS Broth vs PE + GR-1 Cells	28.43	0.3396	No	ns	-359.1 to 415.9
43	MRS Broth vs PE + HK GR-1 Cells	23.79	0.3177	No	ns	-322.8 to 370.4
44	PE + GR-1 Cells vs PE + HK GR-1 Cells	-4.639	0.05542	No	ns	-392.1 to 382.9

Cell size (Fold change)

	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	PE + MRS Broth vs PE + 50 μL GR-1	0.5945	9.290	Yes	****	0.3000 to 0.8891
18	PE + MRS Broth vs PE + 20 μL GR-1	0.4535	7.086	Yes	****	0.1590 to 0.7480
19	PE + MRS Broth vs PE + 10 μL GR-1	0.4476	6.994	Yes	****	0.1531 to 0.7422
20	PE + MRS Broth vs PE + 5 μL GR-1	0.3718	5.810	Yes	**	0.07728 to 0.6663
21	PE + MRS Broth vs MRS Broth	0.3971	5.145	Yes	*	0.04191 to 0.7523
22	PE + MRS Broth vs PE + GR-1 Cells	0.3805	4.930	Yes	*	0.02528 to 0.7357
23	PE + MRS Broth vs PE + HK GR-1 Cells	0.2797	3.623	No	ns	-0.07555 to 0.6349
24	PE + 50 μL GR-1 vs PE + 20 μL GR-1	-0.1411	2.312	No	ns	-0.4219 to 0.1398
25	PE + 50 μL GR-1 vs PE + 10 μL GR-1	-0.1469	2.407	No	ns	-0.4277 to 0.1339
26	PE + 50 μL GR-1 vs PE + 5 μL GR-1	-0.2227	3.650	No	ns	-0.5035 to 0.05808
27	PE + 50 μL GR-1 vs MRS Broth	-0.1974	2.642	No	ns	-0.5413 to 0.1465
28	PE + 50 μL GR-1 vs PE + GR-1 Cells	-0.2141	2.864	No	ns	-0.5580 to 0.1299
29	PE + 50 μL GR-1 vs PE + HK GR-1 Cells	-0.3149	4.213	No	ns	-0.6588 to 0.02905
30	PE + 20 μL GR-1 vs PE + 10 μL GR-1	-0.005837	0.09566	No	ns	-0.2867 to 0.2750
31	PE + 20 μL GR-1 vs PE + 5 μL GR-1	-0.08167	1.338	No	ns	-0.3625 to 0.1991
32	PE + 20 μL GR-1 vs MRS Broth	-0.05636	0.7541	No	ns	-0.4003 to 0.2876
33	PE + 20 μL GR-1 vs PE + GR-1 Cells	-0.07299	0.9766	No	ns	-0.4169 to 0.2709
34	PE + 20 μL GR-1 vs PE + HK GR-1 Cells	-0.1738	2.326	No	ns	-0.5177 to 0.1701
35	PE + 10 μL GR-1 vs PE + 5 μL GR-1	-0.07583	1.243	No	ns	-0.3566 to 0.2050
36	PE + 10 μL GR-1 vs MRS Broth	-0.05052	0.6760	No	ns	-0.3944 to 0.2934
37	PE + 10 μL GR-1 vs PE + GR-1 Cells	-0.06715	0.8985	No	ns	-0.4111 to 0.2768
38	PE + 10 μL GR-1 vs PE + HK GR-1 Cells	-0.1680	2.248	No	ns	-0.5119 to 0.1759
39	PE + 5 μL GR-1 vs MRS Broth	0.02531	0.3387	No	ns	-0.3186 to 0.3692
40	PE + 5 μL GR-1 vs PE + GR-1 Cells	0.008684	0.1162	No	ns	-0.3352 to 0.3526
41	PE + 5 μL GR-1 vs PE + HK GR-1 Cells	-0.09214	1.233	No	ns	-0.4361 to 0.2518
42	MRS Broth vs PE + GR-1 Cells	-0.01663	0.1927	No	ns	-0.4138 to 0.3805
43	MRS Broth vs PE + HK GR-1 Cells	-0.1175	1.361	No	ns	-0.5146 to 0.2797
44	PE + GR-1 Cells vs PE + HK GR-1 Cells	-0.1008	1.168	No	ns	-0.4980 to 0.2963

ANOVA results: PCM (cont)

ANP

	t test	
1	Table Analyzed	ANP analysis (GR-1 Cells)
2	Column E	PE + GR-1 Cells
3	vs	vs
4	Column F	PE + HK GR-1 Cells
5		
6	Unpaired t test	
7	P value	0.3549
8	P value summary	ns
9	Are means signif. different? (P < 0.05)	No
10	One- or two-tailed P value?	Two-tailed
11	t, df	t=1.045 df=4

	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	MRS Broth vs PE + MRS Broth	-1.049	5.420	Yes	*	-1.921 to -0.1765
18	MRS Broth vs PE + GR-1	0.3783	2.112	No	ns	-0.4292 to 1.186
19	MRS Broth vs PE + GR-1 Cells	-0.07739	0.4000	No	ns	-0.9496 to 0.7948
20	MRS Broth vs PE + HK GR-1 Cells	-0.2291	1.184	No	ns	-1.101 to 0.6431
21	PE + MRS Broth vs PE + GR-1	1.427	7.375	Yes	**	0.5547 to 2.299
22	PE + MRS Broth vs PE + GR-1 Cells	0.9712	4.696	Yes	*	0.03884 to 1.904
23	PE + MRS Broth vs PE + HK GR-1 Cells	0.8196	3.962	No	ns	-0.1128 to 1.752
24	PE + GR-1 vs PE + GR-1 Cells	-0.4557	2.355	No	ns	-1.328 to 0.4165
25	PE + GR-1 vs PE + HK GR-1 Cells	-0.6073	3.139	No	ns	-1.480 to 0.2648
26	PE + GR-1 Cells vs PE + HK GR-1 Cells	-0.1517	0.7333	No	ns	-1.084 to 0.7807

aSKA

	t test	
1	Table Analyzed	a-SKA analysis (GR-1 Cells)
2	Column E	PE + GR-1 Cells
3	vs	vs
4	Column F	PE + HK GR-1 Cells
5		
6	Unpaired t test	
7	P value	0.3588
8	P value summary	ns
9	Are means signif. different? (P < 0.05)	No
10	One- or two-tailed P value?	Two-tailed
11	t, df	t=0.9820 df=7

	Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
17	MRS Broth vs PE + MRS Broth	-1.307	8.037	Yes	****	-2.017 to -0.5968
18	MRS Broth vs PE + GR-1	0.6297	4.182	No	ns	-0.02778 to 1.287
19	MRS Broth vs PE + GR-1 Cells	0.3324	2.044	No	ns	-0.3777 to 1.043
20	MRS Broth vs PE + HK GR-1 Cells	0.1210	0.8805	No	ns	-0.4792 to 0.7212
21	PE + MRS Broth vs PE + GR-1	1.937	11.91	Yes	****	1.226 to 2.647
22	PE + MRS Broth vs PE + GR-1 Cells	1.639	9.430	Yes	****	0.8802 to 2.399
23	PE + MRS Broth vs PE + HK GR-1 Cells	1.428	9.485	Yes	****	0.7705 to 2.085
24	PE + GR-1 vs PE + GR-1 Cells	-0.2973	1.828	No	ns	-1.007 to 0.4129
25	PE + GR-1 vs PE + HK GR-1 Cells	-0.5087	3.701	No	ns	-1.109 to 0.09151
26	PE + GR-1 Cells vs PE + HK GR-1 Cells	-0.2114	1.404	No	ns	-0.8689 to 0.4461

Appendix 4. Peer-reviewed publications

Ettinger G, Burton JP, Reid G. If microbial ecosystem therapy can change your life, what's the problem? *Bioessays*. 2013; 35: 508-512

Prospects & Overviews

Recently in press

If microbial ecosystem therapy can change your life, what's the problem?

Grace Ettinger[1)2)], Jeremy P. Burton[1)3)] and Gregor Reid[1)2)3)]*

The increased incidence of morbidity and mortality due to *Clostridium difficile* infection, had led to the emergence of fecal microbial transplantation (FMT) as a highly successful treatment. From this, a 32 strain stool substitute has been derived, and successfully tested in a pilot human study. These approaches could revolutionize not only medical care of infectious diseases, but potentially many other conditions linked to the human microbiome. But a second revolution may be needed in order for regulatory agencies, society and medical practitioners to accept and utilize these interventions, monitor their long term effects, have a degree of control over their use, or at a minimum provide guidelines for donors and recipients.

modern life and medical science. First, its necessity illustrates the failures of treatments, in this case antibiotics, long accepted as standard medical practice and for which the search for alternatives has been met with apathy until recently. Second, it reflects the recent interest in the power of the microbiome and shows dramatically how complete transplantation of a set of well-established organisms in one person can result in benefits to another. Third, it, along with administration of a feces-based bulk microbiota [2], represents the most dramatic application of probiotics to date. Fourth, the process forces one to reflect on nature and history, leading to the realization that coprophagy has been practiced for centuries, often by animals extracting vitamins, minerals and proteins from the excrement, but also by the Bedouin who ate fresh, warm camel feces as a remedy for bacterial dysentery

Gan XT, **Ettinger G**, Huang CX, et al. Probiotic administration attenuates myocardial hypertrophy and heart failure following myocardial infarction in the rat. *Circ Heart Fail*. 2014; 7: 491

Original Article

Probiotic Administration Attenuates Myocardial Hypertrophy and Heart Failure After Myocardial Infarction in the Rat

Xiaohong Tracey Gan, MSc; Grace Ettinger, BSc; Cathy X. Huang, MD; Jeremy P. Burton, PhD; James V. Haist, BSc; Venkatesh Rajapurohitam, PhD; James E. Sidaway, PhD; Glynn Martin, BSc; Gregory B. Gloor, PhD; Jonathan R. Swann, PhD; Gregor Reid, PhD, MBA; Morris Karmazyn, PhD

Background—Probiotics are extensively used to promote gastrointestinal health, and emerging evidence suggests that their beneficial properties can extend beyond the local environment of the gut. Here, we determined whether oral probiotic administration can alter the progression of postinfarction heart failure.

Methods and Results—Rats were subjected to 6 weeks of sustained coronary artery occlusion and administered the probiotic *Lactobacillus rhamnosus* GR-1 or placebo in the drinking water ad libitum. Culture and 16s rRNA sequencing showed no evidence of GR-1 colonization or a significant shift in the composition of the cecal microbiome. However, animals administered GR-1 exhibited a significant attenuation of left ventricular hypertrophy based on tissue weight assessment and gene expression of atrial natriuretic peptide. Moreover, these animals demonstrated improved hemodynamic parameters reflecting both improved systolic and diastolic left ventricular function. Serial echocardiography revealed significantly improved left ventricular parameters throughout the 6-week follow-up period including a marked preservation of left ventricular ejection fraction and fractional shortening. Beneficial effects of GR-1 were still evident in those animals in which GR-1 was withdrawn at 4 weeks, suggesting persistence of the GR-1 effects after cessation of therapy. Investigation of mechanisms showed a significant increase in the leptin:adiponectin plasma concentration ratio in rats subjected to coronary ligation, which was abrogated by GR-1. Metabonomic analysis showed differences between sham control and coronary artery ligated hearts particularly with respect to preservation of myocardial taurine levels.

Conclusions—The study suggests that probiotics offer promise as a potential therapy for the attenuation of heart failure. (***Circ Heart Fail*. 2014;7:491-499.**)

Key Words: cardiomegaly ■ heart failure ■ microbiota ■ probiotics

Curriculum Vitae: Grace Ettinger

EDUCATION

2012-Present **Graduate Studies – Master of Science**
Department of Microbiology and Immunology
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Supervisor: Dr. Gregor Reid

2007-2012 **Undergraduate Studies – Honors Bachelor of Science**
Major in Biology
University of Guelph, Guelph, Ontario, Canada

2010-2011 **Ontario-Jiangsu Exchange Program – Exchange student**
Specialization in Mandarin Chinese
Jiangnan University, Wuxi, Jiangsu, China

AWARDS AND HONOURS

2012-2014 **Western Graduate Research Scholarship**
Value: $4500
Western University, London, Ontario, Canada

2013 **Department of Microbiology and Immunology Travel Award**
Value: $1000
Western University, London, Ontario, Canada

2010-2011 **Ontario-Jiangsu Student Exchange Program Scholarship**
Value: $2500

2007-2012 **Dean's Honour List**
University of Guelph, Guelph, Ontario, Canada

2007 **Entrance Scholarship Award**
Value: $2500
University of Guelph, Guelph, Ontario, Canada

RESEARCH AND WORK EXPERIENCE

2013-2014 **Teaching Assistant and Laboratory Demonstrator**
Biology 1200 – Department of Biology
Western University, London, Ontario Canada

2012 **Summer Student**
Canadian Research & Development Center for Probiotics
LHRI, London, Ontario, Canada

VOLUNTEER AND LEADERSHIP EXPERIENCE

2013-2014 **Vice-President: Communications**
Students and Fellows Association (SFA)
International Association for Probiotics and Prebiotics (ISAPP)

2012-2013 **Department Representative**
(Department of Microbiology & Immunology)
Society of Graduate Students Council
Western University, London, Ontario, Canada

2012-2013 **Department Liaison: Let's Talk Science**
(Department of Microbiology & Immunology)
Western University, London, Ontario, Canada

2011-2013 **Vice-President: Internal Liaison**
Rotaract Club of Guelph/University of Guelph
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PEER REVIEWED PUBLICATIONS

Ettinger G, Burton JP, Reid G. If microbial ecosystem therapy can change your life, what's the problem? *Bioessays*. 2013; 35: 508-512

Gan XT, **Ettinger G**, Huang CX, et al. Probiotic administration attenuates myocardial hypertrophy and heart failure following myocardial infarction in the rat. *Circ Heart Fail*. 2014; 7: 491

INVITED PODIUM PRESENTATIONS

March 2014 **Ettinger G,** Burton JP, Karmazyn M, Reid G. Probiotic therapy for heart failure: Investigating the anti-hypertrophic properties of

probiotics. London Health Research Day. London, Ontario, Canada

INVITED SEMIAR PRESENTATIONS

February 2014 **Ettinger G,** Reid G. My heart will go on: Probiotic therapy for heart failure. Talks on Fridays. LHRI. London, Ontario, Canada

POSTER PRESENTATIONS

June 2014 **Ettinger G,** Burton JP, Karmazyn M, Reid G. The potential anti-hypertrophic properties of probiotics. ISAPP-SFA annual conference. Aberdeen, Scotland, UK

June 2013 **Ettinger G,** Burton JP, Karmazyn M, Reid G. Improved outcomes of myocardial infarction and heart failure with probiotic treatment. Canadian Society of Microbiologists annual conference. Carleton University, Ottawa, Ontario, Canada.

June 2013 **Ettinger G,** Burton JP, Karmazyn M, Reid G. Probiotics for heart failure: Attenuated maladaptive hypertrophy and improved cardiac mechanical function with probiotic treatment**.** Probiotics, Prebiotics, and the Host Microbiome: The Science of Translation. ISAPP-SFA annual conference. New York Academy of Sciences, New York, New York, USA.

March 2013 **Ettinger G**, Burton JP, Karmazyn M, Reid G. Probiotics attenuate maladaptive hypertrophy and improve cardiac mechanical function after myocardial infarction in rats. London Health Sciences Research Day. London, Ontario, Canada.

LANGUAGES

Conversational Russian and Mandarin Chinese