Enzymatically active probiotic bacteria for topical and oral therapy

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ABSTRACT

A novel approach whereby one can use probiotic bacteria for the purpose of topical and oral therapy is presented. More specifically, a treatment modality using enzymatically active probiotic production of gaseous nitric oxide (*g*NO) for wound healing, antimicrobial, cosmetic, and dermatologic therapy is presented. In another aspect, a probiotic treatment modality for metabolic disease and metabolic syndrome using nitrate reductase (NiR) active probiotic bacteria is explored. In concurrence to these requirements, several *in-vitro* methods are designed and discussed in this report. For some of these studies the use of alginate microcapsules is explored. Results show that probiotic patches can be used for the production of *g*NO above therapeutic levels and for therapeutic durations and that probiotic *g*NO-producing patches are highly bacteriostatic, bactericidal, and fungicidal. Results show that the novel *g*NO-producing probiotic patch can be used to improve wound healing and increase the likelihood of wound closure in ischemic and infected full-thickness dermal wounds in a New Zealand White Rabbit model and that daily application of the patch is safe with respect to body weight, blood morphology, haematology, blood biochemistry, and methemoglobin levels. Also, results show that novel NiR-active probiotic bacteria can be selected for nitrate reductase (NiR) activity *in-vitro*, and can be microencapsulated or delivered free under simulated GI conditions, and in the presence of various food matrices while maintaining NiR activity, confirming the lab scale feasibility of the approach in delivering the probiotic orally for treating hypertension, inflammatory bowel disease, gastric ulcers, diabetes and

thrombosis. These findings may provide effective, safe, and less costly alternatives for delivering *g*NO topical and oral for therapy.

RÉSUMÉ

Une nouvelle approche selon laquelle on peut utiliser des bactéries probiotiques dans le but de la thérapie topique et de la thérapie par voie orale est présentée. Plus précisément, une modalité de traitement, utilisant des probiotiques à activité enzymatique produisant de l'oxyde nitrique gazeux (NO*g*) pour la cicatrisation des plaies, ainsi que comme thérapies antimicrobiennes, cosmétiques et dermatologiques, est présenté. Dans un autre aspect, une modalité de traitements probiotiques pour les maladies métaboliques et les syndromes métaboliques en utilisant du nitrate réductase (NiR) bactéries probiotiques actives est explorée. En accord à ces exigences, plusieurs méthodes in vitro sont conçus et discutés dans le présent rapport. Pour certaines de ces études l'utilisation de micro capsules d'alginate est explorée également. Les résultats montrent que les correctifs probiotiques peuvent être utilisés pour la production de NO*g* au-dessus des niveaux thérapeutiques et pour des durées thérapeutiques et que les patches de NO*g*-producteurs de probiotiques sont très bactériostatiques, bactéricide et fongicide. Les résultats montrent que les nouvelles patches de NO*g* producteurs de probiotiques peuvent être utilisés pour améliorer la cicatrisation et augmenter la probabilité de fermeture de la plaie dans les plaies de pleine épaisseur ischémique et infectés par voie cutanée dans un modèle néo-zélandais White Rabbit et que l'application quotidienne des patches est sécuritaire en proportion avec le poids du corps, la morphologie du sang, l'hématologie, biochimie sanguine, et les niveaux de méthémoglobine. En outre, les résultats montrent que les nouvelles NiR-bactéries probiotiques actives peuvent être sélectionnés pour la nitrate réductase (NiR) l'activité in vitro, et peut être micro encapsulées ou remis gratuitement

dans des conditions simulées GI, et en présence de diverses matrices alimentaires, tout en maintenant l'activité NiR, confirmant ainsi la faisabilité échelle du laboratoire de l'approche dans la réalisation des probiotiques par voie orale pour le traitement de l'hypertension, les maladies inflammatoires de l'intestin, les ulcères gastriques, le diabète et la thrombose. Ces résultats peuvent s'avérer efficaces, sûrs, et des solutions moins coûteuses pour offrir NO*g* topiques et oraux pour le traitement.

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PREFACE

In accordance with the thesis preparation and submission guidelines, I have taken the option of writing the experimental portion of this thesis in the form of original papers suitable for publication. This option is provided by **Section I-C** in the **Thesis Preparation and Submission Guidelines**, which reads as follows:

> As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research.

In this thesis, manuscripts of original papers are presented in **Chapters 3-6**. Each experiment based paper has its own Abstract, Introduction, Materials and Methods, Results, Discussion, and References. A common Abstract, Introduction, a final overall Conclusion, Summary, Clams to Original Contributions to Knowledge, and Recommendations are also included.

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CHAPTER 1: GENERAL INTRODUCTION

1.1. General Introduction

Since the discovery that endothelium derived relaxing factor (EDRF) is nitric oxide (NO) it has become evident that NO is a widely distributed and multifunctional cellular messenger[1], has a physiologically relevant endogenous metabolism (nitrate-nitrite-nitric oxide pathway)[2], and is utilized by the human innate and cellular immune systems as an antimicrobial agent[3, 4].

NO is synthesized in eukaryotes by a family of nitric oxide synthases (NOS) that catalyze the oxidation of L-arginine to L-citrulline. Endothelial NOS (eNOS) and neuronal NOS (nNOS) are responsible for constitutive synthesis of NO in endothelial and neural associated cells, whereas the inducible form (iNOS) is found in a wide variety of cells including epithelial, endothelial, and inflammatory cells. Importantly, the expression of iNOS is up regulated by cytokines, microbes or bacterial products[5]. NO is a transitory free radical responsible for the regulation of blood pressure, the control of platelet aggregation[6], protection against vascular injury caused by tissue deposition of immune complexes[7], and is used as a broad spectrum antimicrobial agent by both the innate and cell mediated immune systems[8, 9]. In particular, NO initiates a signal transduction pathway in the endothelium, regulating vascular tone and blood pressure, modulating of homeostasis and proliferation of vascular smooth muscle cells[10]. In leukocytes, NO signalling attenuates inflammatory responses through regulation of cytokine expression. Finally, elevated levels of NO, due to increased iNOS activity, increases collagen deposition, keratinocyte proliferation, and angiogenesis[11] during normal wound healing.

Nitric oxide can also be produced in the gastrointestinal tract (GIT). Inorganic nitrates and nitrites consumed in the diet are now believed to make up bio-inactive pools of NO, stored in blood, which have NO-like bioactivity and help mammals maintain homeostasis of a nitrogen cycle capable of producing NO[12]. These bio-inactive pools of NO can be used to generate NO when acted on by bacteria in the upper GIT and function as a protective agent against early inflammatory insults and a protector of a normal, intact mucosal barrier[13, 14].

Nitrate reductase (NiR) is an enzyme present in some commensal bacteria in the upper GI and catalyzes the reduction of dietary nitrates to nitrites which can then converted to NO under the acidic conditions of the stomach[15]. Under anaerobic conditions, oral bacteria with NiR activity can generate nitrite anions from inorganic nitrates present in either saliva or ingested food. The nitrite will become NO once it reaches the stomach through dismutation, NO will enter the blood stream through the mesenteric circulation, and finally be converted back to nitrate in blood. Nitrate can then be either eliminated in the urine, or be recycled to the saliva where it is concentrated and becomes available for further NO generation by bacteria in the upper GI, completing the so called entero-salivary recirculation of nitrate[16]. In the GIT, NO can function to protect against inflammatory insults and can act to protect the intact mucosal barrier[17, 18]. Other physiological reducers of nitrite to NO in the human body include xanthine oxidoreductase, ascorbate, and polyphenolic acids. The generated NO is thought to cause vasodilatation and help maintain normal blood pressure. In addition, NO reacts with free cysteine to form nitrosothiols, which inhibit collagen- and ADP-mediated platelet aggregation.

While the production of NO by GIT bacteria is important to maintain homeostasis of NO in blood and gut, so too, the production of NO on the surface of skin is important for homeostasis of skin, topical antimicrobial activity, and integral for the wound healing process. The reduction of nitrate to nitrite, and its subsequent dismutation to NO, appears to be an innate antimicrobial process protecting skin as is true within the gastrointestinal tract (GIT)[15, 19-21]. It is believed that NO is generated on the surface of human skin and that nitrate in sweat is reduced to nitrite by bacterial NiR in anaerobic niches, with the resulting nitrite dismutated to NO[22]. Skin surface NO generation is unaffected by arginine analogs and NO production is increased by topical acidification of the skin surface. In addition, patients on long-term antibiotics show significantly reduced NO production on skin. Thus, it is believed that NO generation on skin is dependent on bacterial nitrate reduction to nitrite and subsequent reduction by acidification. The nitric oxide produced is believed to have a physiologic role in the inhibition of infection by improving cutaneous T-cell function, keratinocyte differentiation, and skin blood flow[23].

Artificial cell microencapsulation is a technique used to encapsulate biologically active materials in specialized ultra-thin semi-permeable polymer membranes[24, 25]. It is a valuable tool for delivery and protection of biologic materials, as microencapsulation can protect materials from the external environment while at the same time permitting selected materials to pass into and out of the microcapsules[24]. Microcapsules are known to protect live cells, enzymes, DNA, and other biological material from immune rejection and other extreme environments and have a number of biomedical and clinical applications. Many groups have shown that artificial cell microcapsules can be used for oral administration of live genetically engineered bacterial cells that can be effective for

therapy[26, 27]. The microcapsules remain intact as they pass through the GIT and are excreted with the stool in about 24h. The membranes of the microcapsules are permeable to smaller molecules, enabling the cells inside to metabolize nutrients, eliminate waste, act on substrate, and deliver biological products. During passage through the intestinal tract, bacterial cells can be retained inside the intact microcapsules and therefore do not enter into the circulation. This latter property limits the major safety concerns associated with the use of live bacterial cells for various clinical uses[24, 28]. Also, microencapsulation can be used to protect cells and enzymes in artificial systems such as bioreactors, fermenters, and medical devices. Active components of medical devices can be microencapsulated to protect the biologic material from other device components and the production process, increase the stability and shelf life of the biologic, bring the substrate and bioactive into proximity, and make handling delicate biologic material more simple.

The physiologic and therapeutic applications of NO appear to be endless and are the subject of an intense research effort to identify the most useful therapeutic applications and methods of delivery. One technical challenge is that NO has an extremely short half-life and must be produced at the site of physiologic action.

For oral applications, NiR-active probiotic bacteria present an interesting therapeutic option, as they produce NO from ingested inorganic nitrates which may have antithrombogenic, antihypertensive, antimicrobial, and mucosal protective effects. Produced NO protects the mucosa by maintaining blood flow, reduces intestinal epithelial permeability, inhibits platelet and leucocyte aggregation, down regulates mast cell reactivity, and modulates oxidative stress[29-33]. In addition, NO may alleviate the

symptoms of inflammatory bowel disease (IBD) due to its antimicrobial activity against infectious bacteria such as *E. coli*, *Salmonella*, or *Shigella*.

Topically, cell and enzyme systems that reduce nitrate to nitrite and subsequently acidify nitrite to NO, or systems that simply acidify nitrite and generate NO, are an appealing therapeutic option for antimicrobial, wound healing, and dermatologic disorder applications. On the surface of skin, exposure of NO gas (*g*NO) to wounds such as chronic non-healing ulcers can be beneficial in promoting healing and preparing the wound bed for treatment and recovery[34]. Application of exogenous gas has been shown to reduce microbial infection down regulating inflammation, manage exudates and secretion, up-regulate expression of endogenous collagenase to debride the wound, and regulate the formation of collagen[34]. Also, topical exposure of exogenous gas has been shown to reduce microbial infection, manage exudates and secretions by reducing inflammation, and regulate the formation of collagen[34].

1.2 Research Hypothesis

1 Bacterial metabolic activity can be exploited for the production of *g*NO and can be used for therapeutic purposes.

2 Bacteria can be used in the design of novel *g*NO-producing wound healing devices.

3 Novel *g*NO-producing wound healing devices can be used as topically applied antimicrobial dressings.

4 Novel *g*NO-producing wound healing devices can be used as dressings for ischemic and infected wounds.

5 Bacteria can be selected for a specific enzymatic activity that can be used to produce *g*NO when delivered orally for the prevention and treatment of metabolic disease.

1.3. Research Objectives

In the present project a treatment modality using probiotic and enzymatic production of gaseous nitric oxide (*g*NO) for wound healing, antimicrobial, cosmetic, and dermatologic uses is provided. In some examples, microencapsulation or immobilization is used to protect the biologic material, immobilize the active component, or improve handling and storage. In another aspect, the present project presents a treatment modality for metabolic disease and metabolic syndrome using nitrate reductase (NiR) active probiotic bacteria. The research objectives are:

1 To design equipment and develop an assay to measure *g*NO by free bacteria and devices containing bacteria or enzyme by chemiluminescence.

2 To screen probiotic bacteria for lactic acid producing characteristics *in-vitro* and determine the minimal substrate required for acid production.

3 To design several probiotic adhesive patch formulations/devices and test *g*NO production *in-vitro*.

4 To evaluate the antimicrobial activity of probiotic patches *in-vitro*.

5 To evaluate wound healing efficacy and safety of probiotic patches for ischemic and infected wounds *in-vivo* in a New Zealand White Rabbit model.

6 To design several enzymatic adhesive patch formulations/devices and test *g*NO production *in-vitro*.

7 To screen probiotic bacteria for nitrate reductase (NiR) activity *in-vitro.*

8 To optimize an oral formulation for the production of *g*NO using different food substrate and nitrate sources under GI conditions.

CHAPTER 2: LITERATURE REVIEW

2.1. Nitric oxide (NO) for wound healing dermatologic disorders, antimicrobial, and cosmetic applications

2.1.1. Nitric oxide for wound healing

Wound healing is a complicated process relying heavily on the integration of a multitude of control mechanisms, events, and factors. Inflammatory cells, keratinocytes, fibroblasts, and endothelial cells, as well as many enzymes and growth factors, must interact seamlessly for the normal healing process to occur[35]. These factors act together during the processes of clot formation, inflammation, re-epithelialization, angiogenesis, granulation, contraction, scar formation, and tissue remodelling to ensure adequate wound healing. Several pathological conditions, including diabetes and venous stasis, are associated with a number of changes at the molecular level which ultimately disrupt normal wound healing and can lead to the formation of chronic wounds[35].

One of these changes is the pathological change in the regulation of nitric oxide (NO) during the wound healing process[35]. During normal wound healing, the production of NO radical shows a very distinct time course with initially high concentrations which aid in inhibiting and clearing bacterial infection followed by lower levels of the free radical allowing for the normal wound healing processes to take place[35]. It is believed that the body's natural response to injury is with initially high NO concentrations for reducing the bacterial count, removing dead cells, and promoting healing. After a few days of this preparation of the wound bed, the body produces a new low NO level to promote further healing[34]. If a wound fails to heal, however, or becomes infected, the body maintains the

circulating NO at a high level and the wound is then caught in a vicious cycle preventing it from healing[34].

Mounting evidence points towards a deficiency in endogenously produced nitric oxide as a causative factor in non-healing diabetic ulcers and supplementation of endogenous NO as a potential therapy[36-38]. It has been shown that the deleterious effects of diabetes mellitus on wound healing are reflected in decreased wound NO synthesis and that diminished collagen deposition, mechanical strength, and NO synthesis can be partially restored by insulin treatment[39]. The same group had previously demonstrated that NO is produced in wounds and that its synthesis is critical to wound collagen accumulation[40]. A different group demonstrated that, while elevated matrix metalloproteinase (MMPs) activities in wound fluids impair endogenously produced or exogenously applied growth factors from acting as mitogenic agents, NO may circumvent this difficulty by acting as a mitogenic agent much the same way as a protein growth factor (EGF or KGF)[41]. In fact, it is believed that NO may be indistinguishable from protein-like growth factors, with respect to modulating mitogenic response of keratinocytes to external stimuli, and may act to improve wound healing by promoting proliferation of cells in a wound[42]. To this end, studies using polymeric NONOates and NO-donating agents in rats have shown promising results in wound repair[43]. Thus, it is believed that NO deficiency in chronic non-healing diabetic ulcers can be restored, improving collagen deposition, keratinocyte proliferation, and angiogenesis[44], circumventing elevated MMP levels or low tissue inhibitors of MMPs (TIMPs) levels, aiding in wound closure (Figure 2.1).

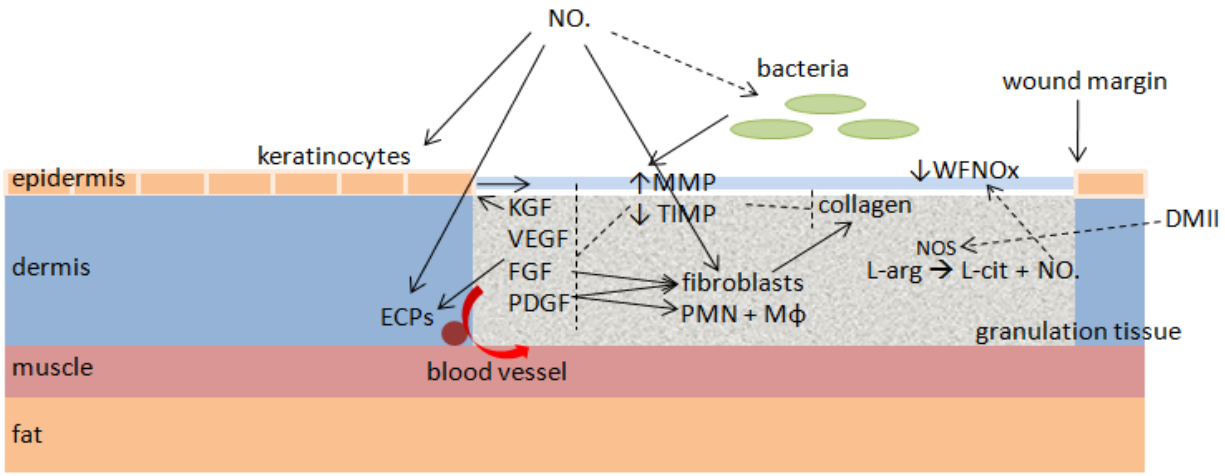

Figure 2.1.: Shows a possible mechanism of action for topically applied NO in DMII wounds.

2.1.2. Nitric oxide for infected wounds

Infected wounds pose a specific and significant problem to wound care specialists treating a chronic wound, non-healing ulcer, or healthy post-surgical wound for that matter. Typically, these wounds have been cared for by nurses, internists, plastic surgeons, and infectious disease specialists who use daily wet-to-dry dressing changes for debridement and topical or systemic antibiotics for treatment of the infection. Systemic and topical antibiotics, as well as other topical anti-microbial agents such as colloidial silver polymyxins or dye compounds, however, have become increasingly less effective against common pathogens.

Another problem in treating infected chronic wounds with systemic antibiotics is that such wounds often accompany reduced local and regional circulation. Patients often

acquire non-healing wounds due to chronic and massive atherosclerosis, venous stasis, or type II diabetes, which affects the peripheral and micro-circulation and often resulting from sedentary and poor eating habits. Patients with venous stasis ulcers have venous thrombosis, reduced circulation and poor regional blood flow; and patients with diabetic foot ulcers suffer from poor microcirculation due to deposition of glucose and reduced circulation. These patients become bed ridden, immobilized, and emaciated while trying to stay off the wounds on their lower extremities, only worsening their problem of sedentary living. Clinicians frequently appeal to surgeons to bypass arteries or provide surgical coverage of wounds; however, the patients frequently have multiple co-morbidities, are not well nourished, and are poor surgical candidates. This leaves the patient and clinician with the only remaining option of treating the chronic wound with daily dressing changes, a time consuming, costly, and relatively ineffective practice. Current practice is to treat chronic wounds with daily wet-to-dry dressing changes, keeping them clean and protected until the wound heals over. However, with a lack of compliance, poor circulation, poor nutrition, non-sterile conditions, and simply the time it takes to heal wounds in this way they often stay open for years and even decades. Systemic antibiotics can exacerbate this problem, due to constriction of the capillaries and small blood vessels, causing a further reduction in blood flow to the wound and reduced delivery of the antimicrobial agent. Topical agents are often more effective at concentrating the antimicrobial agent at the wound site; however, they are often less effective at eliminating infection for other reasons which include reduced circulation once again. Thus, traditional therapies often leave an infected wound untreated and a patient's limb or life in danger.

A worldwide increase in drug resistant strains of bacteria since the introduction of antimicrobial agents has documented this well accepted trend. Both Gorwitz and Anstead et al have recently reviewed *Methicillin-resistant Staphylococcus aureus* (MRSA) infections in skin and soft tissue, describing its emergence as a common cause of infection in children and adults in both community and hospital settings[45, 46]. Linares 2001 has recently reviewed the emergence of vancomycin intermediate resistant *Staphylococcus aureus* (VISA) and glycopeptide-intermediate *S. aureus* (GISA), for which few drugs and strategies to fight infection exist. Further, Nordmann et al. recently reviewed the new resistance problems that have emerged among hospital and community-acquired pathogens including *Enterococcus faecium* and *Pseudomonas aeruginosa*[47]. *P. aeruginosa* infection is particularly problematic, as patients are often immune suppressed or are severely disabled and artificially ventilated. Thus, as the common antimicrobial agents begin to fail, alternative treatments which do not rely on conventional antibiotics are needed.

It has recently been shown that topical exposure of NO gas (*g*NO) to wounds such as chronic, infected, non-healing ulcers can be beneficial in promoting healing and preparing the wound bed for treatment and recovery[34]. The application of exogenous gas has been shown to reduce microbial infection, manage exudates and secretions by reducing inflammation, up regulate expression of endogenous collagenase to locally debride the wound, and regulate the formation of collagen[34]. Furthermore, regimens have been proposed for the treatment of chronic wounds with *g*NO which specify high and low treatment periods to first reduce the microbial burden and inflammation and increase collagenase expression to debride necrotic tissue, and then restore the balance of NO and

induce collagen expression aiding in the wound closure respectively[48]. In fact, case studies have shown the efficacy of such a treatment by the exogenous application of *g*NO that was able to close a two year non-responsive, non-healing, venous stasis ulcer[48]. The NO delivery device, however, utilized many bulky and costly components including air pump systems, *g*NO source cylinders, internal pressure sensors, mechanical pressure regulators, and plastic foot boot with inflatable cuff to cover the patient's lower extremity[48]. Another drawback with the delivery of *g*NO is that NO rapidly oxidizes in the presence of oxygen (O_2) to form NO_2, which is highly toxic, even at low levels. A device for the delivery of NO must be anoxic, preventing NO from oxidizing to toxic NO_2 and preventing the reduction of NO which is required for the desired therapeutic effect[48]. Thus, since NO will react with O_2 to convert to NO_2, it is desirable to have minimal contact between the *g*NO and the outside environment.

2.1.3 Antimicrobial activity of nitric oxide (NO)

The antimicrobial effect of NO has been suggested by diverse observations[49]. First, NO production by inducible NO synthases has been stimulated by proinflammatory cytokines such as IFNɣ, TNF-α, IL-1, and IL-2 as well as by a number of microbial products like lipopolysaccharide (LPS) or lipoichoic acid[50]. Infections in humans and experimental animals triggered systemic NO production as evidenced by elevated nitrates in urine and plasma. Second, elevated expression of NO in animal models improved the abilities of host to fight infectious agents and inhibited microbial proliferation, overall improving the host response[51, 52]. Third, *in-vitro* studies demonstrated that inhibition of

NO synthases resulted in impaired cytokine-mediated activation of phagocytic cells and reduction of bactericidal and bacteriostatic activity[53]. And fourth, direct administration of NO-donor compounds *in-vitro*, induced microbial stasis and death. Importantly, NO-dependent antimicrobial[54] activity has been demonstrated in viruses, bacteria, fungi, and parasites[55, 56].

One of the plausible mechanisms of antimicrobial activity of NO involves the interaction of this free radical (and a reactive nitrogen intermediate) with reactive oxygen intermediates, such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) to form a variety of antimicrobial molecular species. In addition to NO itself, these reactive antimicrobial derivatives include peroxynitrite ($OONO^-$), S-nitrosothiols (RSNO), nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), and dinitrogen tetroxide (N_2O_4). It has been shown that these reactive intermediates target DNA, causing deamination, and oxidative damage including abasic sites, strand breaks, and other DNA alterations[57]. Reactive nitrogen intermediates can also react with proteins through reactive thiols, heme groups, iron-sulfur clusters, phenolic or aromatic amino acid residues, or amines[58]. Peroxinitrite and NO_2 can oxidize proteins at different sites. Additionally, NO can release iron from metalloenzymes and produce iron depletion. NO-mediated inhibition of metabolic enzymes may constitute an important mechanism of NO-induced cytostasis. Moreover, nitrosylation of free thiol groups may result in inactivation of metabolic enzymes[59].

Several examples of the antimicrobial effects of NO have been described in the literature. Antiviral activity of NO has been described by Kawanishi[60], in *in-vitro* cell culture experiments, where NO donors inhibited Epstein-Barr virus late protein synthesis, amplification of DNA preventing viral replication as a result of peroxynitrite formation.

In addition, NO and superoxide produced by macrophages lead to a peroxynitrite-related anti-parasitic effect in a murine model of leishmaniasis[61] and the use of a topical NO donor glyceryl trinitrate was successfully used to treat cutaneous leishmaniasis[62]. Moreover, recent observations indicate that murine macrophages exert antifungal activity against candida through peroxynitrite synthesis[63].The antibacterial effect of NO was shown through a variety of mechanisms such as S-nitrosothiol-mediated inhibition of spore outgrowth in Bacillus cereus[64] and several protein targets of nitrogen reactive species have been found in *Salmonella typhimurium*[65].

2.1.4. Nitric oxide for dermatologic disorders

Many dermatologic disorders are also amenable to topical NO therapy. Often diseases of the skin and underlying tissues are multi-factorial and can be treated topically or by elimination of an insulting agent. In many cases the mechanism of disease or its pathophysiology is associated with the complex interactions between epidermis, dermis, associated stem cells, extracellular matrix, nervous and vascular structures, complex cell signaling, and cell mediators of inflammation. In other cases the disease is directly related to an insulting agent that can be removed, eliminated, or neutralized by bioactive compounds.

Nitric oxide has been implicated in immunomodulation and T-lymphocyte responsiveness and has been shown to modulate functional maturation of T lymphocytes and can enhance their activation[66-68]. In mammalian cell assays, NO has been shown to

preferentially inhibit T-helper 1 (Th-1) clonal proliferation to antigen. The mature phenotype, in combination with specific concentrations of NO, has been shown to influence the modulatory effect of NO on human T cells. NO has also been implicated in regulation of monokine production and implicated as a factor contributing to the modulation of the immune response to different kinds of infections[68-70].

In addition, NO has been shown to act as either a proinflammatory or anti-inflammatory agent depending on concentration. Endogenous synthesis of NO is often correlated with production of proinflammatory cytokines. This effect can be simulated by short term topical treatment with an NO releasing agent which has been shown to have proinflammatory effects such as localized loss of Langerhans cells and apoptosis in keratinocytes in healthy skin[71]. Blockade of endogenous synthesis of NO reduces the proinflammatory effects of NO. On the other hand, NO has been shown to reduce recruitment of pro-inflammatory cells by down regulation of Endothelial Cell Adhesion Molecules such as ICAM 1[72]. NO synthesis through Nitric oxide synthase 2 (NOS2) is partially self-regulated by the NO induced inactivation of the transcription factor NF-κB[73].

Nitric oxide can also provide protection against apoptosis through protection against oxidative stress. NO can act directly to scavenge reactive oxygen species (ROS) thereby reducing ROS mediated cell damage such as lipid peroxidation and resultant apoptosis. NO also contributes to reducing apoptosis due to oxidative stress by inducing thioredoxin expression. NO has been demonstrated to protect cells from TNFα induced apoptosis in a cGMP dependent manner[74]. There is also evidence to suggest that

induction of Bcl-2 expression and suppression of caspase activation is another mechanism by which NO can protect cells from apoptosis[75].

Dysregulation of NOS2 expression is often correlated with impairment of barrier function in dermatitis. It is postulated that this NO inhibits terminal differentiation events in keratinocytes that result in the formation of the stratum corneum[76]. NO has been shown to inhibit the transcription of some terminal differentiation proteins essential to cornification and to inactivate others. Experimental addition of exogenous NO does not amplify this effect[77].

Several groups have developed NO producing patches or plastic containment devices holding *g*NO from complicated and expensive releasing devices; however, there remains a need for practical devices and compositions to produce NO for the treatment of dermatological disorders.

2.1.5. Nitric oxide (NO) for cosmesis

While there is a long history of topically applied chemicals and botanicals, some attached to specific health benefits, there has only recently been a significant research effort to define the biologically active compounds and to elucidate the mechanisms of action[78, 79]. The dichotomy is that there are many organically derived materials that contain some bioactive ingredients and that may have some biologic effect; however, most commercially available products either contain too little bioactive or simply purport science that is not true. A common difficulty is that the active compound is often only

naturally available at lower concentrations than its minimal therapeutic concentration[80, 81]. Also, some bioactives, such as anti-oxidants, are required at therapeutic levels for long durations to achieve a maximal therapeutic effect. Thus, while organically derived skin care products may be active, or chemically synthesized bioactives may be added, they may not be achieving a therapeutic effect because of low concentration or limited duration of action.

Oxidative damage is a time dependent process akin to rust formation on iron in the presence of oxygen. Biologically relevant free radicals are referred to as reactive oxygen species (ROS) because the most biologically significant molecules are oxygen-centered. Plants and lower organisms have evolved the biochemical machinery to make antioxidants for dealing with ROS and which prevent against their formation. Such antioxidants include vitamin E and vitamin C which are used to protect the outer layer lipophilic and hydrophilic constituents. Unfortunately, humans have lost the ability to make vitamin C, the predominant antioxidant in skin, due to a specific gene mutation. Vitamin C and other antioxidants help to protect the outer layer of cells, including biomembranes and DNA, against ROS formed endogenously by inflammatory reactions or exogenously by environmental oxidative stress (UV, ozone, etc). Such antioxidants can be divided into enzymatic and non-enzymatic antioxidants and those which are hydrophilic and those which are lipophilic. Nitric oxide is the most naturally occurring reducing agent which is biologically available and thus can be used to prevent the action of ROS. The pathophysiology of ROS includes damage to biomembranes, DNA, enzymes and to the extracellular matrix proteins. These biological components of skin are integral to the normal form and function of skin.

Nitric oxide is an integral component in the homeostasis of skin and cosmesis. Normally, NO is synthesized in small amounts by mammalian cells from L-arginine by endothelial nitric oxide synthase (NOS) for cell signaling. Nitric oxide is generated in much larger quantities by skin and inflammatory cells during inflammatory reactions by the inducible NOS. Also, microorganisms found on skin and in dermis have nitrate reductase (NiR) activity and produce *g*NO from nitrate in sweat and saliva[82, 83]. Nitrate present in the blood, and later in sweat, is acted on by the NiR possessed by these microorganisms[84, 85]. Superficial antimicrobial agents (chlorhexadine and other topical antibiotics) cannot kill these organisms and cannot diminish the *g*NO produced by skin. If you treat locally with a nitric oxide synthase (NOS) inhibitor (L-NMMA), you do not decrease the amount of NO produced and released by the skin[86]. However, if you treat with systemic wide-spectrum antibiotics, for an adequate duration, the skin loses it's NO producing capability[87]. Interestingly, the quantity of NO produced by the hands is much greater than that produced by the arm - this makes sense as the hands have a much greater need for the antimicrobial effects of NO[88]. Thus, there is a "map" of NO producing capability of the skin which is a product of the density of sweat glands and the presence/absence of bacteria with nitrate reductase (NiR) activity.

The appearance of skin can be enhanced by improved extracellular matrix deposition (ECM), increased moisture content, control of keratinocyte division and migration, improved blood flow, reduced inflammation, reduced pathogenic load, and increased anti-oxidative capacity. Nitric oxide can affect many of these factors and may have particular relevance by increasing regional blood flow and encouraging improved orderly collagen deposition.

2.2. Nitrate reductase (NiR) active probiotics for prevention and treatment of metabolic disease and metabolic syndrome (MetS)

2.2.1. Definition of metabolic syndrome (MetS)

MetS is a common condition that has many names: dysmetabolic syndrome, syndrome X, insulin resistance syndrome, obesity syndrome, and Reaven's syndrome. The syndrome identifies clinical symptoms and biochemical findings, including abdominal obesity, insulin resistance[89], hyperglycaemia, hyperlipidemia, and hypertension, that lead to an increased risk of cardiovascular disease (CVD)[90]. The Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (ATP III) highlights the importance of treating patients with MetS to prevent CVD and defines it as having at least three of the following[91]:

I. Central obesity: waist circumference ≥ 102 cm or 40 inches (male), ≥ 88 cm or 36 inches(female)
II. Dyslipidemia: TG ≥ 1.7 mmol/L (150 mg/dl)
III. Dyslipidemia: HDL-C < 40 mg/dL (male), < 50 mg/dL (female)
IV. Blood pressure ≥ 130/85 mmHg
V. Fasting plasma glucose ≥ 6.1 mmol/L (110 mg/dl)

In the US population, the unadjusted and age-adjusted prevalence of MetS are 21.8% and 23.7% respectively, with the highest prevalence occurring in Spanish Americans (31.9%) and older individuals (60-69 years old) (43.5%)[92]. The age-adjusted prevalence of MetS is similar for men (24.0%) and women (23.4%)[93]. Using 2000 census data, about 47 million US residents have MetS, demonstrating the serious implications the disease has on the health care sector[94, 95].

2.2.2. Etiology of metabolic syndrome

The exact etiology and pathophysiology of MetS is yet to be fully elucidated; however, the development of MetS strongly linked to body fat. Excess abdominal fat leads to excess free fatty acids in the portal vein, increasing fat accumulation in the liver and muscle cells causing insulin resistance and hyperinsulinemia[96]. Glucose metabolism is impaired, and dyslipidemias and hypertension develop. In addition, serum uric acid levels are typically elevated[97] and a prothrombotic state leading to the increased levels of fibrinogen and plasminogen activator inhibitor I and other factors leading to an inflammatory state causing MetS. Other risk factors include non-alcoholic steatohepatitis, chronic kidney disease, polycystic ovary syndrome in women and low plasma testosterone in men[98].

2.2.3. Current clinical management

Current clinical management of MetS includes advising patients on healthy lifestyle choices, including improved diet and exercise, and treating the signs and symptoms with a combination of prescription medications[99]. No single drug formulation exists that can prevent or treat MetS. Given limitations of presently available preventative techniques and therapies, an opportunity exists for the development of a safe, effective, and economical formulation for the treatment of MetS. To be efficacious, a formulation must address the broad spectrum of clinical symptoms and biochemical findings while assuring compliance to therapy.

2.2.4. Probiotics for metabolic syndrome?

There is strong evidence to suggest that probiotics have role in fat metabolism[100]. Oral administration of probiotics such as *L. gasseri, L. acidophilus, L. fermentum, L. crispatus, L. brevis,* and *B. subtilis,* have been suggested as possible adjunctive therapy to lower abdominal fat deposits. In fact, several reports have shown that probiotics can affect lipid metabolism, by reducing abdominal fat, and by reducing serum cholesterol[101]. Unfortunately, the therapeutic potential of probiotics for treating MetS has been hampered by inherent limitations of traditional probiotic therapies, including poor strain selection, little optimization of fermentation, and poor delivery due to harsh gastric transit.

2.2.5. Nitrate reductase (NiR) active probiotics for metabolic syndrome

Probiotic bacteria can generate high amounts of NO from nitrites due to the acidification of the environment as a result of fermentation and production of NO in the GIT from nitrites and nitrates has been observed in the presence of probiotics such as *Lactobacilli* and *Bifidobateria*[102, 103]. In fact, dietary nitrates and nitrites have been shown to lower blood pressure and present gastro protective effects in a rat model and the ingestion of fruits and vegetables rich in nitrates and nitrites may benefit health by the hypotensive effects[104, 105]. Further, a study in humans showed that provision of a nitrate-rich drink significantly reduced blood pressure and had vasoprotective and antiplatelet properties when compared to the individuals that were treated with water controls[106].

Recently, the National Heart, Lung and Blood Institute (a division of NIH) established a dietary approach to stop hypertension (DASH) suggesting 5-to-10 daily servings of raw vegetables and fruits. According to the DASH diet, the nitrate intake could vary between 174 and 1222 mg per day considering 4-to-5 daily servings of vegetables and fruit, depending on the food choices. Although sometimes considered a contaminant, nitrate is naturally present in our diet, mainly in leafy vegetables and in some roots. The nitrate content in vegetables may vary depending on the location of the fields where they are grown and the harvesting time of the year. Here is a list of some vegetable produce with the highest levels of nitrate: beet root (185mg/100g), beet root juice (170mg/100 mL), beet root powder (4600 mg/100g), spinach powder (5000 mg/100g), spinach (150mg/100g), kale (100-200 mg/100 g), mustard greens (116 mg/100 g), lettuce (100-200mg/ 100g).

Thus, we believe that the oral delivery of NiR-active probiotics could be utilized to facilitate the conversion of dietary nitrates throughout the GIT producing a hypotensive

and antithrombogenic effect in humans and thus ameliorating two common findings in patient with MetS and decreasing patient's risk of CVD.

PREFACE FOR CHAPTERS 3 TO 6

Presented in the following four chapters are the research and studies performed in order to investigate the stated research hypothesis and achieve the stated research objectives. Each chapter discusses various aspects of the thesis research project.

To evaluate the feasibility of the novel approach of delivering therapeutically relevant quantities of gaseous nitric oxide (*g*NO) using topically applied probiotic patches, *Lactobacillus fermentum* NCIMB 7230 was immobilized, microencapsulated, or added free to substrate and NO donors and *g*NO was measured. The preparation of such *g*NO-producing probiotic patches is described in **Chapters 4 and Chapter 5**.

To accurately measure *g*NO produced by the probiotic patches, novel equipment was designed and an assay for measuring gaseous nitric oxide (*g*NO) was developed. The experimental apparatus and assay for measuring *g*NO in probiotic patches are outlined in **Chapter 4**.

To evaluate the antimicrobial activity of probiotic patches *in-vitro,* the *g*NO-producing probiotic adhesive patches were applied to cultures of common bacterial and fungal pathogens: *E. coli, S. aureus, P. aeruginosa, MRSA, T. mentagrophytes, and T. rubrum*, and *A. Baumannii*. The antibiotic, bacteriostatic, and anti-fungal efficacy of *g*NO-producing probiotic patches are outlined in **Chapter 4**.

To evaluate wound healing efficacy and safety of the *g*NO-producing probiotic patches *in-vivo,* the novel probiotic wound healing patch, using lactic acid producing bacteria (LAB) in an adhesive gas permeable membrane, was used to treat ischemic and infected full-thickness dermal wounds in a New Zealand White Rabbit model for

ischemic wound healing. The efficacy and safety of *g*NO-producing probiotic patches in treating ischemic and infected full-thickness dermal wounds are outlined in **Chapter 5**.

To screen probiotic bacteria for nitrate reductase (NiR) activity *in-vitro* using different substrate and nitrate sources, several probiotic organisms were screened for *g*NO production and microencapsulated or free *g*NO producers were chosen for further study. As probiotics can be ingested free or in combination with a food product, a diary product, or juices, or with encapsulated nitrates that allow for slow release of the NO donor in the lower gastrointestinal tract, *g*NO-producing capacity was determined in simulated GI conditions and with various food matrices. The results of screening probiotic bacteria for the ability to produce *g*NO *in-vitro*, under simulated GI conditions, and in the presence of various food matrices are outlined in **Chapter 6**.

The results obtained in my research have been presented in the following papers:

Research articles:

1. Jones ML, Jorge Gabriel Ganopolsky, Alain Labbé, Wahl C, and Prakash S. Antimicrobial properties of nitric oxide and its application in antimicrobial formulations and medical devices. *Appl Microbiol Biotechnol. 2010 Sep;88(2):401-7. Epub 2010 Aug 3*.
2. Jones ML, Ganopolsky JG, Labbé A, Prakash S. A novel nitric oxide producing probiotic patch and its antimicrobial efficacy: preparation and in vitro analysis. *Appl Microbiol Biotechnol. 2010 Jun; 87(2):509-16. Epub 2010 Mar 19.*
3. Jones ML, Ganopolsky JG, Alain Labbé, Gilardino M, Wahl C, Martoni C, and Prakash S. Novel nitric oxide producing probiotic wound healing patch: preparation and *in-vivo* analysis in a New Zealand white rabbit model of ischemic and infected wounds. *J Biomed Biotechnol*. (Under consideration).
4. Jones ML, Ganopolsky JG, and Prakash S. Nitric oxide producing nitrate reductase (NiR) active probiotic bacteria for oral therapy. (Not yet submitted).

Patents:

1. Jones ML and Prakash S. *Nitric Oxide Device and Method for Wound Healing, Treatment of Dermatologic Disorders and Microbial Infections*. Priority: U.S. 61/075,040 filed June 24, 2008 and U.S. 61/097,978 filed September 18, 2008, and

U.S. 61/166,430 filed April 3, 2009. International: PCT/CA2009/000858 filed June 23, 2009; WO/2009/155689.

2. Jones ML and Prakash S. *Nitric oxide compositions and devices and methods for cosmesis.* Priority: U.S. 61/153,696 filed February 19, 2009. International: PCT/2009/000859 filed June 23, 2009; WO/2009/155690.

3. Jones ML, Bhathena-Changlani J, and Prakash S. *Prevention and therapy of metabolic syndrome using probiotics*. Priority: U.S. 61/349,270 filed May 28, 2010.

In accordance with McGill University regulations, three of the above manuscripts (the major publications 1 to 3) are reported in their original form in full as individual chapters (Chapters 3 to 5).

CHAPTER 3: ANTIMICROBIAL PROPERTIES OF NITRIC OXIDE AND ITS APPLICATION IN ANTIMICROBIAL FORMULATIONS AND MEDICAL DEVICES

*Mitchell Lawrence Jones, Jorge Ganopolsky, Alain Labbé, Christopher Wahl, and Satya Prakash**

Preface: This chapter reviews the pathophysiologic rationale for the use of therapeutic topical nitric oxide gas (*g*NO) and provides a mechanism of action for the use of *g*NO in antimicrobial applications. This chapter also examines several devices and methods for *g*NO delivery, evaluates and describes limitations of current designs, and provides a probiotic based *g*NO producing system as an example of a design with advantages. A thorough review of the literature and design successes and failures was required for the effective design of a *g*NO-producing device.

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3.1. Abstract

We review the antimicrobial properties of nitric oxide (NO) and explore its application as an antimicrobial agent in different formulations and medical devices. We describe the eukaryotic biosynthesis of NO and its physiologic functions as a cell messenger, and as an antimicrobial agent of the cell-mediated immune system. The NO produced by bacteria on the skin surface and in the gastrointestinal tract by reduction of nitrite, acts as a mediator of the innate immune system. We analyze the antimicrobial activity of NO, and the eukaryotic protective mechanisms against NO for the purpose of delineating the therapeutic NO dosage range required for an efficacious and safe antimicrobial activity. We also examine the role of NO produced by virulent bacteria in lessening the efficacy of traditional antimicrobials. In addition, we discuss the efficacy of NO in infected wound healing and describe different NO producing devices by category, analyzing therapeutic levels and duration of NO production, as well as commercial considerations. Finally, we provide current and future prospects for the design and use of NO producing devices.

3.2. Introduction

Since the discovery that endothelium derived relaxing factor (EDRF) is in fact nitric oxide (NO), it has become evident that NO is a widely distributed and multifunctional cellular messenger[107], that it has a physiologically relevant endogenous

metabolism (nitrate-nitrite-nitric oxide pathway)[108], and it is utilized by the human innate and cellular immune systems as an antimicrobial agent[109, 110].

Normally, NO is produced by the enzyme nitric oxide synthase (NOS) in the presence of oxygen from the amino acid L-arginine[111]. NO is a transitory free radical responsible for the regulation of blood pressure, the control of platelet aggregation[112], protection against vascular injury caused by tissue deposition of immune complexes[113], and is used as a broad spectrum antimicrobial agent by both the innate and cell mediated immune systems[114, 115]. Also, nitrates and nitrites that are consumed in the diet, and are produced by bacteria in the gastrointestinal tract (GIT), are now believed to make up bio-inactive pools of NO, stored in blood, which have NO-like bioactivity and help mammals maintain homeostasis of a nitrogen cycle capable of producing NO[116].

Here we review the antimicrobial properties of nitric oxide (NO) and explore its application as an antimicrobial agent in various formulations and medical devices.

3.3. Eukaryotic Biosynthesis of Nitric Oxide

Nitric oxide (NO) is a free radical that plays a metabolic regulatory a role in eukaryotes and as an autocrine, paracrine, and endocrine messenger in a variety of tissues[117]. In particular, NO initiates a signal transduction pathway in the endothelium, regulating vascular tone and blood pressure, modulating of homeostasis and proliferation of vascular smooth muscle cells[118]. In leukocytes, NO signalling attenuates inflammatory responses through regulation of cytokine expression. In the GIT, NO functions as a protective agent against early inflammatory insults and a protector of a normal, intact

mucosal barrier[119, 120]. NO is synthesized *in-vivo* by a family of NO synthases (NOS) that catalyze the oxidation of L-arginine to L-citrulline. Whereas endothelial NOS (eNOS) and neuronal NOS (nNOS) are responsible for constitutive NO synthesis in endothelial and neural associated cells, there is an inducible form (iNOS) found in epithelial, endothelial and inflammatory cells, whose expression is up regulated by cytokines, microbes or bacterial products.

3.4. Microbial Biosynthesis of Nitric Oxide

Bacterial and eukaryotic nitric oxide synthases (NOS) are structurally and mechanistically related and catalyze the oxidation of L-arginine to L-citrulline to produce NO. Although bacterial NOS (bNOS) lack an essential reductase domain, bacteria use eukaryotic cellular reductases to generate NO *in-vivo*[121]. Gusarov, et al. recently found that low levels of NO, produced by bNOS with the help of eukaryotic reductases, may protect bacteria against oxidative stress applied by systemic antibiotics and increase the resistance of bacteria to broad spectrum of antibiotics.

While synthesis of bacterial NO is an important factor on the antimicrobial landscape, other approaches to NO production may prove more relevant protecting humans against infection. The reduction of nitrate to nitrite, and its subsequent dismutation to NO appears to be an important protective process on skin and within the gastrointestinal tract (GIT)[15, 122-124]. Weller et al. showed that NO is generated on the surface of human skin and proposed that nitrate in sweat is reduced to nitrite by bacterial nitrate reductase in anaerobic niches, with the resulting nitrite converted to NO[125]. They

showed that NO synthesis is not inhibited by arginine analogs, and NO production is increased by topical acidification of the skin surface. In addition, patients on long-term antibiotics show significantly reduced NO production on skin, and concluded that NO generation on skin is dependent on bacterial nitrate reduction to nitrite and subsequent reduction by acidification. They proposed a physiologic role in the inhibition of infection by improving cutaneous T-cell function, keratinocyte differentiation, and skin blood flow[126].

Nitric oxide (NO) can also be generated in the GIT of mammals from inorganic nitrate and nitrite by the action of commensal bacteria[15]. Under anaerobic conditions, bacteria presenting nitrate reductase (NiR) activity can generate nitrite anions from nitrates present either in saliva or ingested. The produced nitrite results in NO once it reaches the stomach due to the acidic environment. Consequently, NO enters the blood stream through the mesenteric circulation and gets reconverted to nitrate, which can then be eliminated in urine, or get recycled to the saliva where it is concentrated and becomes available for further NO generation[127]. Lactic acid bacteria (LAB) can generate high amounts of NO from nitrites due to the acidification of the environment as a result of fermentation[128]. Production of NO in the GIT from nitrites and nitrates was observed in the presence of probiotics such as *Lactobacilli* and *Bifidobateria*[129]. Finally, ammonia-oxidizing bacteria such as nitrosomonas produce NO from ammonia and hydroxylamine[130].

3.5. Eukaryotic Protective Mechanisms

An important eukaryotic protective measure against NO is metallothionein (MT) production. Metallothionein expression has been shown to be up regulated by eukaryotes in response to oxidative stress among other factors. Metallothionein is a protein thiol induced in cells exposed to cytokines and bacterial products capable of forming iron-dinitrosyl thiolates *in-vitro*. Particularly, nucleolus-associated MT may be able to reduce NO mediated genotoxicity[131]. The overproduction of this cell protein product is one protective mechanism that eukaryotes use against elevated levels of NO.

3.6. Antimicrobial Applications of Nitric Oxide

The antimicrobial effect of NO has been suggested by diverse observations. First, NO production by inducible NOS has been stimulated by proinflammatory cytokines such as IFNɣ, TNF-α, IL-1, and IL-2 as well as by a number of microbial products like lipopolysaccharide (LPS) or lipoichoic acid[132]. Second, elevated expression of NO in animal models improved the abilities of host to fight infectious agents and inhibited microbial proliferation, overall improving the host response[133-135]. Third, *in-vitro* studies demonstrated that inhibition of NO synthases resulted in impaired cytokine-mediated activation of phagocytic cells and reduction of bactericidal and bacteriostatic activity[136]. Finally, direct administration of NO-donor compounds *in-vitro*, induced microbial stasis and death. Importantly, NO-dependent antimicrobial activity has been demonstrated in viruses, bacteria, fungi, and parasites[137, 138].

One of the plausible mechanisms of antimicrobial activity of NO involves the interaction of this free radical (and a reactive nitrogen intermediate) with reactive oxygen

intermediates, such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) to form a variety of antimicrobial molecular species (Figure 3.10.1). In addition to NO itself, these reactive antimicrobial derivatives include peroxynitrite ($OONO^-$), S-nitrosothiols (RSNO), nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), and dinitrogen tetroxide (N_2O_4). It has been shown that these reactive intermediates target DNA, causing deamination, and oxidative damage including abasic sites, strand breaks, and other DNA alterations[139]. Reactive nitrogen intermediates can also react with proteins through reactive thiols, heme groups, iron-sulphur clusters, phenol or aromatic amino acid residues, or amines[140]. Peroxynitrite and NO_2 can oxidize proteins at different sites. Additionally, NO can release iron from metalloenzymes and produce iron depletion. NO-mediated inhibition of metabolic enzymes may constitute an important mechanism of NO-induced cytostasis. Moreover, nitrosylation of free thiol groups may result in inactivation of metabolic enzymes[141]. As well, the antibacterial effect of NO was shown through a variety of mechanisms such as S-nitrosothiol-mediated inhibition of spore outgrowth in *Bacillus cereus*[142] and several protein targets of nitrogen reactive species have been found in *Salmonella typhimurium*[143].

In addition to the antibacterial activity of NO, several examples of the antiviral, antifungal, and anti-parasitic effects of NO have been described in the literature. Kawanishi et al. demonstrated the antiviral activity of NO when they showed that NO donors inhibited Epstein-Barr virus late protein synthesis and amplification of DNA preventing viral replication as a result of peroxynitrite formation[60]. Activity against parasites was established with a murine model of leishmaniasis; NO and superoxide produced by macrophages lead to a peroxynitrite-related anti-parasitic effect[144], and the

use of a topical NO donor glyceryl trinitrate was successfully used to treat cutaneous leishmaniasis[145]. Finally, the antifungal effects are evidenced by recent observations that indicate that murine macrophages exert antifungal activity against Candida through peroxynitrite synthesis[146].

A common source of infections is wounds. Wound healing is a complicated process relying heavily on the integration of a multitude of control mechanisms, events, and factors. Inflammatory cells, keratinocytes, fibroblasts, and endothelial cells, as well as many enzymes and growth factors, must interact seamlessly for the normal healing process to occur[35]. Several pathological conditions, including diabetes, venous stasis, and infection are associated with cellular and molecular changes that ultimately disrupt normal wound healing[35]. One of these changes is the pathological change in the regulation of nitric oxide (NO) during the wound healing process[35]. During normal healing, the production of NO radical shows a very distinct time course with initially high concentrations which aid in inhibiting and clearing bacterial infection followed by lower levels of the free radical allowing for the normal wound healing processes to take place. If a wound fails to heal or becomes infected, however, the body maintains high levels of circulating NO and the wound enters a vicious cycle preventing it from healing[34].

Infected wounds pose a specific and significant problem as systemic and topical antibiotics, as well as other topical anti-microbial agents such as colloidal silver polymyxins or dye compounds, have become increasingly less effective against common pathogens. A worldwide increase in drug resistant strains of bacteria since the introduction of antimicrobial agents has documented this well accepted trend[45, 147, 148].

Thus, as the common antimicrobial agents begin to fail, alternative treatments which do not rely on conventional antibiotics are needed.

Another difficulty in treating infected chronic wounds with systemic antibiotics is that such wounds often accompany reduced local and regional circulation. Patients with venous stasis ulcers, diabetic foot ulcers and infection can suffer from poor microcirculation and systemic antibiotics exacerbate this problem due to constriction of the small blood vessels, causing a further reduction in blood flow to the wound and reduced delivery of the antimicrobial agent.

It has recently been shown that topical exposure of NO to infected wounds can be beneficial in promoting healing and preparing the wound bed for treatment and recovery[34]. The application of exogenous gas has been shown to reduce microbial infection, manage exudates and secretions by reducing inflammation, and regulate the formation of collagen[34]. Furthermore, regimens have been proposed for the treatment of chronic wounds with NO g which specify high and low treatment periods to first reduce the microbial burden and inflammation, and increase collagenase expression to debride necrotic tissue, and then restore the balance of NO and induce collagen expression aiding in the wound closure respectively[34].

3.7. Design of NO Producing Devices

Devices for production and delivery of topical NO have been constructed in several arrangements. A considerable challenge in the delivery of NO is that NO rapidly oxidizes in the presence of oxygen (O_2) to form NO_2, which is highly toxic, even at low

levels. A device for the delivery of NO must be anoxic, preventing NO from oxidizing to toxic NO_2 and preventing the reduction of NO which is required for the desired therapeutic effect[34]. Thus, since NO will react with O_2 to convert to NO_2, it is desirable to have minimal contact between the *g*NO and the outside environment.

Initial designs utilized observations regarding the acidification of nitrite in sweat on skin and reproduced the *g*NO generating mechanism by acidifying nitrite salts with agents such as ascorbic acid[149-152]. Other attempts have been made to increase endogenous production of NO by stimulating eukaryotic synthesis of NO from arginine[153]. Also, a number of groups have tried adsorbing the free gas under pressure to various polymeric compounds, or trapping NO under pressure in silica metal chambers until the time of delivery[142, 154-157]. Another group used a combination of chemical generation of *g*NO from nitrite using electrons from glucose with an adsorption method so that in situ produced NO is incorporated to a chitosan-tetramethylorthosillicate matrix which is lyophilised to form nanoparticles. In this case, the NO is released when the matrix becomes in contact with an aqueous phase[158]. Still other groups have tried to control the delivery of free gas using a tank of compressed NO, controller, regulator, delivering the gas through a tube to an adhesive patch[159, 160]. Finally, devices using enzymatic and cellular systems have been developed[161]. A review of these devices is provided in Table 3.10.1 which outlines the categories of NO producing devices, provides information regarding the capability of a device to maintain NO levels within the therapeutic window, provides information on therapeutic duration, and discusses commercial considerations. Table 3.10.2 presents the chemical mechanisms of

production for categories of NO producing devices and presents examples of NO generating mechanisms for each.

3.8. Trends and Future Prospects

It is becoming increasingly clear that NO plays an important role in human specific and non-specific immunity and that it is a particularly good broad spectrum antimicrobial agent. Evidence for endogenous NO production on skin, within the gut, and by the cellular immune system to protect and fight disease is growing. Furthermore, the importance of systemic nitrate, nitrite, and nitrosylated compounds in blood is becoming better understood and the implications in fighting infectious disease more apparent. Stimulation of endogenous NO or application of exogenous NO to infected human tissue appears to be an effective method for treating microbial infections; however, considerable hurdles with design of a commercially viable device exist. Current design trends aim to achieve therapeutic antimicrobial levels below what is considered toxic to Eukaryotic cells. Devices that release high levels initially and weaning levels over time appear less commercially viable as antimicrobial therapies, particularly if the device is to be applied daily or more frequently. Devices that sustain consistent therapeutic release over time, utilize inexpensive mass producible ingredients, and that make use of substances that are generally regarded as safe appear ideal, especially if extended shelf life and stability can be established. Such devices may prove commercially viable in treating a wide variety of bacterial, fungal, parasitic, and viral infections and in healing infected wounds. In stark

contrast to more exotic and expensive antimicrobials, NO may prove an ideal and commercially viable antimicrobial agent for the first and developing worlds alike.

3.9. Acknowledgements

We acknowledge financial support from the Industrial Research Assistance Program of the National Research Council of Canada (IRAP-NRC) and from Micropharma Limited.

3.10. Tables and Figures

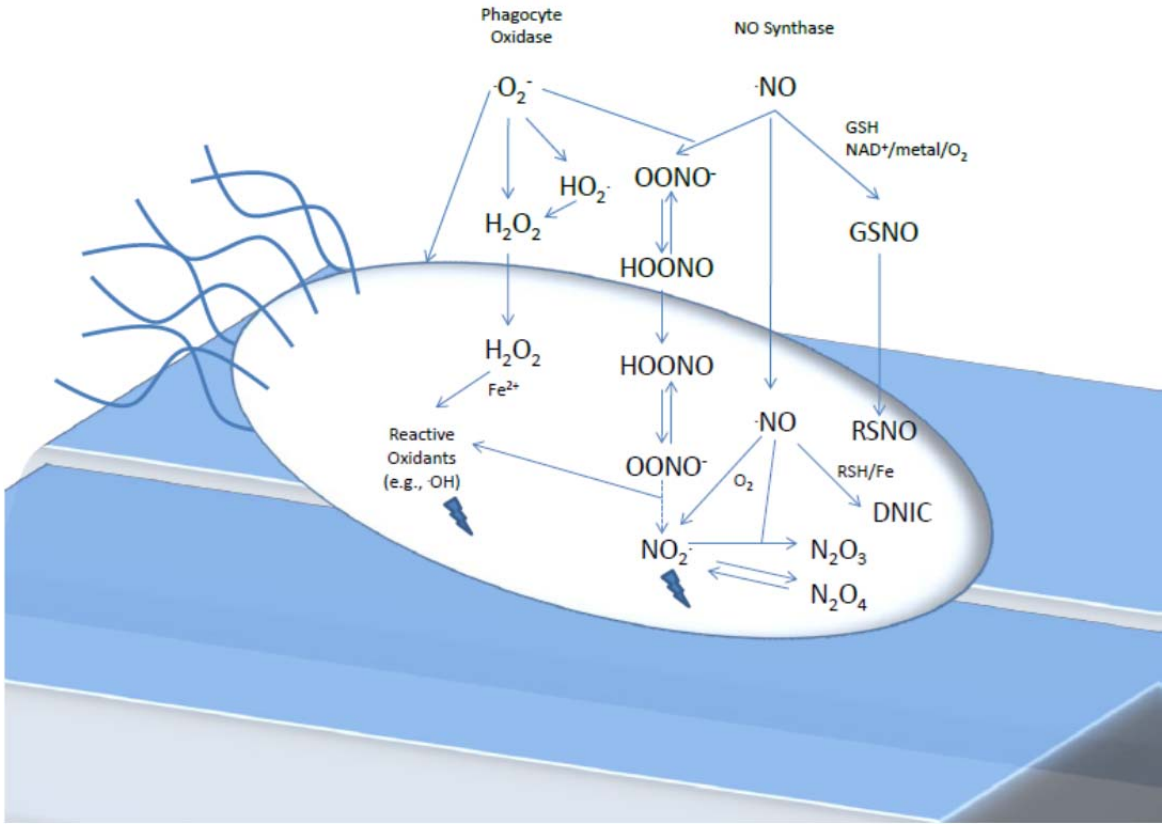

Figure 3.10.1. Potential interactions between phagocyte-derived reactive oxygen and NO-derived species during antimicrobial activity. The schematic shows possible reactions between products originating from phagocyte oxidase and NO synthase. *Fe*, iron; *GSH*, glutathione; *GSNO*, *S*-nitrosoglutathione; *H2O2*, hydrogen peroxide; *HOONO*, peroxynitrous acid; *NAD*, nicotinamide adenine dinucleotide; *NO*•, nitric oxide; *NO2* •, nitrogen dioxide; *N2O3*, dinitrogen trioxide; *N2O4*, dinitrogen tetroxide; *O2*, molecular oxygen; •*OH*, hydroxyl; *OONO2*, peroxynitrite; *RSNO*, *S*-nitrosothiol. These oxygen and nitrogen reactive species cause bacterial death by modifying DNA, inactivating metabolic

enzymes, or damaging structural proteins among other mechanisms. The figure was modified from Fang[162].

Type of device	Therapeutic Window	Therapeutic Duration	Commercial Considerations	Ref.
Endogenous Production	Production limited by endogenous nitric oxide synthase (NOS) cellular machinery and availability of arginine	Production of endogenous levels limited to synthesis by eukaryotic NOS	**Cost:** dependent on cost of endogenous NO production stimulating molecule **Ease of use:** cream application	163
Free Gas	Complete control of *g*NO levels within desired therapeutic window	Maintains levels in therapeutic window for desired therapeutic duration; however, limited by time patient can remain non-ambulatory	**Cost:** expensive device: tank, regulator, computer control system **Ease of use:** complicated set up and use of device, patient non-ambulatory during therapy, tethered to device	164, 165
Adsorption/Release	Production of therapeutic levels dependent on conditions (exudates, temperature, etc.)	High levels of *g*NO early with relatively short half life	**Cost:** moderate **Ease of use:** patch application	142, 166-169
Chemical	Poor control of therapeutic levels with short production dictated by chemical reaction	High levels of *g*NO early with relatively short half life	**Cost:** inexpensive materials **Ease of use:** patch application	170-174
Cell and Enzyme	Excellent control of *g*NO levels within a therapeutic window dictated by enzymatic reaction	Maintains therapeutic levels of *g*NO for 24-72h	**Cost:** inexpensive materials **Ease of use:** patch application	175, 176

Table 3.10.1: Categories of NO producing devices, Capability of a device to maintain NO levels within the therapeutic window, and information on therapeutic duration and commercial considerations.

Type of Device	Mechanism	Description of NO Production
Endogenous Production	$\text{L-Arg} + \text{NADPH} + H^+ + O_2 \xrightarrow{\text{NOS}} \text{NOHLA} + \text{NADP+} + H_2O$ $\text{NOHLA} + ½\ \text{NADPH} + ½\ H^+ + O_2 \rightarrow$ $\text{L-citruline} + ½\ \text{NADP+} + \text{NO}(g) + H_2O$	NO is synthesized *in-vivo* by a family of NO synthases (NOS) that catalyze the oxidation of L-arginine to L-citrulline.
Free Gas	$\text{NO}(p) \rightarrow \text{NO}(g)$ (atm)	NO is stored under pressure and released over time at controlled levels.
Adsorption/Release	$\text{Zeolite xNO} \rightarrow \text{xNO}(g)$	Transition metal-exchanged zeolites adsorb and store NO (up to 1 mmol of NO/g of zeolite). Stored NO is released on contact with an aq. environment. The release of the NO can be tuned by altering the chemical composition, amount of water, temperature and pH.
Chemical Production	$3\ NO_2^- + 3\ H^+ \overset{\text{keq}}{\leftrightarrow} 2\ \text{NO}(g) + H_2O + NO_3^-$	The acidification of nitrite salts produces nitric oxide gas at a rate dependent on availability of nitrite, protons, water, temperature and pH.
Chemical Release	$\text{Chitosan-based NONOate} + H_2O \rightarrow 2\ \text{NO}(g)$	Chitosan-based diazenium diolates (NONOates) are synthesized by reacting NO with modified chitosan polymers and release NO in aq. media. The rate at which NO is released is dependent on amount of water, temperature and pH.
Chemical Release	$2\ \text{RSNO} \rightarrow 2\ \text{NO}\ (g) + \text{RSSR}$	Interpolymer complexes with nitrosothiosols derived from glutathione are decomposed in the presence of light, metal or heat. The rate of NO release is dependent on the wound temperature.
Cell and Enzyme	$\text{Glucose} \overset{\textit{L. fermentum}}{\rightarrow} \text{Lactic Acid}$ $3\ NO_2^- + 3\ H^+ \overset{\text{keq}}{\leftrightarrow} 2\ \text{NO}\ (g) + H_2O + NO_3^-$	Probiotic bacteria and enzymatic systems can be used to produce organic acids which will dismutate nitrite salts releasing NO over time depending on temperature and pH.

Table 3.10.2: Chemical mechanisms of production for categories of NO producing devices and examples of each method.

Original Research Article 2

CHAPTER 4: A NOVEL NITRIC OXIDE PRODUCING PROBIOTIC PATCH AND ITS ANTIMICROBIAL EFFICACY: PREPARATION AND *IN-VITRO* ANALYSIS

*Mitchell Lawrence Jones, Jorge Ganopolsky, Alain Labbé, and Satya Prakash**

Preface: This chapter investigates the hypotheses that probiotic *g*NO-producing patches can be designed to deliver bacteriostatic, bactericidal, and fungicidal levels of *g*NO to a wide spectrum of virulent and drug resistant organisms. Throughout this process, probiotic patches, utilizing the metabolic activity of immobilized lactic acid bacteria (LAB), glucose, and nitrite salts, were designed and used for the production of antimicrobial levels of *g*NO over therapeutically relevant durations. Results indicated that *g*NO producing probiotic patches were highly antimicrobial against common wound pathogens which allowed for evaluation of the *g*NO-producing probiotic patches in an ischemic and infected *in-vivo* wound model which evaluated the efficacy of *g*NO in normal, infected, ischemic, and ischemic-infected wounds. The *in-vivo* antifungal efficacy of *g*NO-producing probiotic patches was not further evaluated.

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Contribution of Authors: I would like to acknowledge laboratory assistance of Jorge Gabriel Ganopolsky and Alain Labbé, as well as editing of my co-authors Jorge Gabriel Ganopolsky, and Alain Labbé.

4.1. Abstract

Microbial and fungal infections are a significant consideration in the etiology of all wounds. Numerous antimicrobial and antifungal formulations have been developed with varying degrees of efficacy and stability. Here we report a nitric oxide producing probiotic adhesive patch device and investigate its antimicrobial and antifungal efficacy *in-vitro*. This probiotic patch utilizes the metabolic activity of immobilized lactic acid bacteria (LAB), glucose, and nitrite salts for the production of gaseous nitric oxide (*g*NO), which is used as an antimicrobial agent against bacterial and fungal pathogens. Results show that application of *g*NO-producing probiotic patches to cultures of *E. coli, S. aureus, P. aeruginosa, MRSA, T. mentagrophytes, and T. rubrum* resulted in complete cell death at between 4 and 8 hours, and application to cultures of *A. baumannii,* resulted in fewer than 10 colonies detected per ml at 6 hours. We believe these results demonstrate that a *g*NO producing probiotic patch device containing bacteria, glucose, and nitrite salts can produce sufficient levels of *g*NO over a therapeutically relevant duration to kill common bacterial and fungal wound pathogens in humans.

4.2. Introduction

The antimicrobial efficacy of nitric oxide gas (*g*NO) has been suggested by several research groups and is evidenced by a number of diverse observations[177]. Firstly, pro-inflammatory cytokines up-regulated upon microbial infection, such as IFNɣ, TNF-α, IL-1, and IL-2, and microbial products such as lipopolysaccharide (LPS) and lipoichoic

acid, have been shown to increase NO production by inducing the nitric oxide synthase (iNOS)[178]. Also, infections in both animals and humans have been shown to trigger systemic NO production, as evidenced by elevated nitrates in blood and urine. Secondly, expression of nitric oxide has been shown to improve the host capacity to fight infectious agents and inhibit microbial proliferation improving immune response in several animal models[179, 180]. Thirdly, *in-vitro* studies have demonstrated that inhibition of NO synthases results in impaired cytokine-mediated activation of phagocytic cells, and reduction of bactericidal and bacteriosatic activity[181]. Finally, direct administration of NO donor compounds to pathogenic microbial culture *in-vitro* has been shown to induce stasis and bacterial death[182-184].

Broad-spectrum antimicrobial effects of nitric oxide have been described for viruses, parasites, fungus, and bacteria[185]. The antiviral action of NO was described by Kawanishi et al., using cell culture experiments, in which NO donors lead to peroxynitrite formation and inhibited Epstein-Barr virus late protein synthesis and amplification of viral DNA preventing viral replication[60]. Nitric oxide and superoxide radicals produced by macrophages have been shown to lead to a peroxynitrite-related anti-parasitic effect in a murine model of leishmaniasis and the topical NO donor glyceryl trinitrate was successfully used to treat cutaneous leishmaniasis[186, 187]. Also, recent observations indicate that murine macrophages exert antifungal activity against Candida through peroxynitrite synthesis[188]. Finally, the antibacterial effect of NO has been described as acting through S-nitrosothiol-mediated inhibition of spore outgrowth in *Bacillus cereus*[189] and several protein targets of nitrogen reactive species in *Salmonella typhimurium*[190].

Suggested mechanisms involve the interaction of the free radical, and reactive nitrogen intermediate with reactive oxygen intermediates, such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-), to form a variety of antimicrobial molecular species[191]. These antimicrobial derivatives include peroxynitrite ($OONO^-$), S-nitrosothiols (RSNO), nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), and dinitrogen tetroxide (N_2O_4). It has been shown that these reactive intermediates target DNA, causing deamination, and oxidative damage including abasic sites, strand breaks, and other DNA alterations[192]. These intermediates can also react with proteins through reactive thiols, heme groups, iron-sulfur clusters, phenolic or aromatic amino acid residues, or amines[193]. Additionally, peroxinitrite and NO_2 can oxidize proteins at different sites and NO can release iron from metalloenzymes and cause iron depletion. NO-mediated inhibition of metabolic enzymes may constitute an important mechanism of NO-induced cytostasis. Moreover, nitrosylation of free thiol groups may result in inactivation of metabolic enzymes[194].

A number of research groups have investigated use of *g*NO to kill bacteria and fungus *in-vitro*; however, they have realized mixed results heavily dependent on the level of NO and duration of sustained release[195-197]. Hetrick et al. demonstrated that NO-releasing silica nanoparticles had greater antibacterial activity than the NO donor PROLI/NO against *P. aeruginosa* and showed that the nanoparticles had antimicrobial activity against bacteria in biofilms of *E. coli, S. aureus, S. epidermidis,* and *C. albicans*. The nanoparticles, however, were not capable of sustained release and had a *g*NO releasing half-life of only 6 and 18 minutes[198, 199]. Ghaffari et al. delivered free medical grade *g*NO at 200 ppmV over 24 hours to reduce bacterial growth of *S. aureus*, MRSA, *E. coli*, Group B *Streptococcus*, *P. aeruginosa*, and *Candida albicans*; however, the

delivery system is cumbersome and expensive and requires that the patient be tethered to the device and non-ambulatory for the duration of therapy[200]. Weller et al. investigated the antimicrobial activity and sensitivity of *C. albicans*, *T. rubrum*, *T. mentagraphytes*, *S. aureus*, and *P. acnes* to acidified (HCl) nitrite (KNO_2) and found that growth was inhibited, but that it was dependent on acidity of the media and required very high levels of nitrite[201]. Importantly, both Hetrick and Ghaffari report no cytotoxic effects of *g*NO on human dermal fibroblasts grown in tissue culture. Although these reports are excellent evidence for the antimicrobial efficacy and safety of *g*NO, an efficacious and economical antimicrobial dressing has not yet been developed. Thus, there is a need for a simple and cost effective method for generating clinically effective dosages of *g*NO, in an easily applied dressing, so that the potential of this novel antimicrobial molecule can be fully realized.

For the first time here, we report a novel probiotic patch utilizing lactic acid producing bacteria in adhesive gas permeable patches for the continuous production of *g*NO through the dismutation of nitrite salts to nitric oxide gas (*g*NO) and investigate their antibacterial and antifungal potentials *in-vitro*.

4.3. Materials and Methods

Nitric Oxide producing probiotic patch design and active ingredients

To prepare a probiotic patch, a pocket was created (approximately 8 x 10 cm) by heat sealing three sides of a 10 cm x 12 cm rectangular gas permeable membrane (Tegaderm™, 3M, MN) with a polyethylene/nylon heat sealable plastic film (Seal-a-

meal®, MA). The resulting pocket was filled with 10ml of physiologic solution (0.85% NaCl) containing 10% glucose, 30mM $NaNO_2$ and 1 gram of lyophilized alginate-immobilized log phase *Lactobacillus fermentum* NCIMB 7230. The fourth side of the pocket was then heat sealed to create a waterproof pocket. A layer of aluminized tape was applied to the outermost layer of plastic film to make the patch occlusive. Vehicle control patches were identical to the active patches as described; however, they did not contain the nitric oxide donor nitrite salt ($NaNO_2$). Details of the chemistry and *g*NO-producing probiotic patch design are described in Figures 4.7.1 and 4.7.2 respectively.

Bacteria and culture conditions

The bacterial strain used for production of probiotic patches was *Lactobacillus fermentum* NCIMB 7230. It was grown in MRS broth (Difco) at 37°C in an Innova 4300 chest incubator (New Brunswick Scientific) and harvested at 16-18 hours.

Nitric oxide producing probiotic patch antimicrobial efficacy experiments were performed with commonly known virulent bacterial strains. Specifically, *Methicillin Resistant Staphylococcus aureus* ATCC 43300 was grown in tryptic soy broth and plated in tryptic soy agar, *Staphylococcus aureus* ATCC 10832 was grown in tryptic soy broth and plated in tryptic soy agar, *Pseudomonas aeruginosa* ATCC 10145 was grown in nutrient broth and plated in nutrient agar, *Acinetobacter baumannii* ATCC 19606 was grown in nutrient broth and plated in nutrient broth agar, and *Escherichia coli* BL21 was grown in LB and plated in LB agar. All cultures were grown at 37°C and incubated overnight.

Fungi and culture conditions

The antifungal efficacy of the probiotic patch was investigated using *Trichophyton rubrum* ATCC 10218 and *Trichophyton mentagrophytes* ATCC 4807 grown in Sabouraud broth (Quelab, Montreal) and plated in Sabouraud agar (Quelab, Montreal). Once the colonies became confluent (usually after 7-10 days) 25 ml of sterile physiologic solution was added to the plates and the fungal colonies were scraped with a sterile scalpel. The colonies in the physiologic solution were then transferred to a 50 ml tube and vortexed to break cells apart. A 1:4 dilution of the suspension was prepared in saline and filtered through a layer of gauze to eliminate large particles. A total of 3 ml of the filtered suspension was transferred to each assay chamber to proceed with the bactericidal assay. All cultures were grown at RT.

Methods of immobilization of Lactobacillus fermentum

A 2% solution of low viscosity alginate (Sigma) was prepared and autoclaved for 15 minutes. *Lactobacillus fermentum* NCIMB 7230 was grown overnight, centrifuged for 15 minutes at 4°C at 4000 x g, the supernatant was poured off and the culture resuspended in physiologic solution (8.5g NaCl/L). The concentrated cell suspension was added to the sterile alginate to achieve a final cell concentration of 8% cells by mass. The alginate/microorganism mixture was immobilized, using a 400 µm nozzle, into a filtered solution of $CaCl_2$ (0.1 M) with an Inotech Encapsulator IER-20 (Inotech Biosystems International Inc., MD). The immobilized *Lactobacillus fermentum* NCIMB 7230 alginate beads were washed in sterilized physiological solution (8.5g NaCl/L),

lyophilized using a bench-top lyophilizer (Virtis, NY), and stored in a desiccator prior to production of *g*NO producing patches.

Bacteriosatic assay

Petri dishes filled with approximately 40 ml of growth medium-agar, to minimize head gas space, and were plated with between 30 and 100 CFU of bacteria. A *g*NO-producing patch or a vehicle control patch was placed on the dish lid. Dishes were then sealed with Parafilm®, and incubated overnight at 37°C. Bacterial cell colonies were counted the following day.

Bactericidal and fungicidal assay

To test the bactericidal and fungicidal effect of *g*NO-producing probiotic patches, assay chambers were designed using a high density polyethylene (Lee Valley, Canada) consisting of a 6 ml cylindrical cavity with liquid and gas sampling ports. The chambers were filled with 3 ml of bacterial suspension in saline (approximately 10^5 CFU/ml) or 3 ml of the fungal suspension prepared as mentioned above, and were sealed with a control or active *g*NO-producing patch. Liquid samples were obtained every 2 hours from the liquid port and serial dilutions were plated on the appropriate growth medium/agar plates. Bacterial colonies were counted after overnight incubation at 37°C. Fungal colonies were counted after 72 hours incubation at RT.

Nitric oxide measurements

The gNO gas was measured by a chemiluminescence nitric oxide analyzer (Sievers®, GE, USA). For this, a known volume of gas was sampled every hour with a 100 µL glass syringe (Hamilton, USA) from the gas port of the assay chamber and *g*NO concentration (ppmV) and standard curve was prepared and then samples were analyzed.

4.4. Results

Bacteriosatic efficacy of the gNO-producing probiotic patch

To investigate *bacteriostatic* efficacy of the novel *g*NO-producing probiotic patches, inhibition of growth of several common pathogenic bacteria was investigated by bacteriostatic plating assay. Results show excellent bacteriostatic efficacy of the probiotic patch towards *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Results show that the *g*NO-producing patches inhibited growth of colonies as compared to vehicle control patches for each of the organisms challenged (Figure 4.7.3).

Bactericidal efficacy of the probiotic patch

To show the capability of *g*NO-producing probiotic patches in killing several common pathogenic bacteria, bactericidal assays were performed in specially designed chambers (Figure 4.7.3). Bactericidal assays were performed for *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa, Acinetobacter baumannii, and Methicillin-Resistant Staphylococcus aureus* (MRSA).

Result shows that *g*NO-producing patches were able to generate greater than 250 ppmV *g*NO (Figure 4.7.4, left) and were found to have excellent bactericidal efficacy.

Application of *g*NO-producing probiotic patches to chambers growing *Escherichia coli* in a chamber resulted in complete cell death after 6 hours compared to maintained viability of pathogenic bacteria in chambers treated with vehicle control patches over the full 8 hour treatment period (Figure 4.7.4, right). It was shown that the *g*NO-producing patches maintained a nitric oxide concentration above 200 ppmV over the 8 hour duration of the experiment while the control patches produced no *g*NO (Figure 4.7.4, left).

Application of *g*NO-producing probiotic patches to chambers growing *Staphylococcus aureus* resulted in complete cell death after 5 hours of treatment compared to maintained viability of pathogenic bacteria in chambers treated with vehicle control patches (Figure 4.7.5, right). It was shown that the *g*NO-producing patches maintained a nitric oxide concentration above 200 ppmV over the 5 hour duration of the experiment while the control patches produced no *g*NO (Figure 4.7.5, left).

Application of *g*NO-producing probiotic patches to chambers growing *Pseudomonas aeruginosa* resulted in complete cell death after 4 hours of treatment compared to maintained viability of pathogenic bacteria in chambers treated with vehicle control patches (Figure 4.7.6, right). It was shown that the *g*NO-producing patches maintained a nitric oxide concentration above 200 ppmV over the 5 hour duration of the experiment while the control patches produced no *g*NO (Figure 4.7.6, left).

Application of *g*NO-producing probiotic patches to chambers growing *Acinetobacter baumannii* resulted in almost complete cell death (10 colonies were detected per ml) after 6 hours of treatment compared to maintained viability of pathogenic bacteria in chambers treated with vehicle control patches (Figure 4.7.7, right). It was shown the *g*NO-producing patches maintained a nitric oxide concentration above

approximately 150 ppmV for the 6 hour duration of the experiment while the control patches produced no *g*NO (Figure 4.7.7, left). Despite a less than optimal level of *g*NO production (<200 ppmV) the bactericidal effect was observed.

Application of *g*NO-producing probiotic patches to chambers growing *MRSA* resulted in complete cell death after 6 hours of treatment compared to maintained viability of pathogenic bacteria in chambers treated with vehicle control patches (Figure 4.7.8, right). It was shown that the *g*NO-producing patches maintained nitric oxide concentrations above approximately 100 ppmV for the 6 hour duration of the experiment while the control patches produced no *g*NO (Figure 4.7.8, left). Again, despite a less than optimal level of *g*NO production (<200 ppmV), the bactericidal effect of *g*NO was observed.

Fungicidal efficacy of the probiotic patch

The fungicidal efficacy of the *g*NO-producing probiotic patches in killing several common pathogenic fungi was determined by fungicidal assay in specially designed chambers. Two common pathogenic fungal strains were investigated: *Trichophyton mentagrophytes,* and *Trichophyton rubrum*. It was shown that application of *g*NO-producing patches to chambers growing *Trichophyton mentagrophytes* resulted in complete cell death after 7 hours of treatment compared to maintained viability of pathogenic fungi in chambers treated with vehicle control patches (Figure 4.7.9, right). It was shown that the *g*NO-producing patches maintained *g*NO concentrations above the desired 200 ppmV level for the 7 hour duration of the experiment while the control patches produced no *g*NO (Figure 4.7.9, left).

Application of *g*NO-producing patches to chambers growing *Trichophyton rubrum* resulted in complete cell death after 8 hours of treatment compared to maintained viability of pathogenic fungi in chambers treated with vehicle control patches (Figure 4.7.10, right). It was shown the *g*NO-producing patches maintained nitric oxide concentrations above the desired 200 ppmV level for the 8 hour duration of the experiment while the control patches produced no *g*NO (Figure 4.7.10, left).

4.5. Discussion

Infections, especially bacterial biofilms, are an important part of the etiology of all wounds whether chronic or acute, and whether surgical, traumatic, or caused by underlying pathology. Nitric oxide, in addition to having a regulatory function in blood vessels (eNOS) and acting as a cellular messenger in the nervous system (nNOS), acts as an effector molecule used by both the innate and cell mediated immune systems to prevent and eliminate infections (iNOS)[202, 203]. Here we demonstrate the antimicrobial efficacy of a *g*NO-producing probiotic patch device, dependent on the metabolism of LAB for the production of protons which dismutate nitrite salts to produce *g*NO, towards several common bacterial and fungal pathogens.

Vehicle control patches containing all active components except for the nitric oxide donor nitrite salt ($NaNO_2$) were used because they did not produce measurable levels of NO and were not effective in killing bacteria in the chambers. Thus, it was decided that vehicle control patches would contain the live immobilized bacteria and not the nitrite salts, as it was believed that the bacteria may alter the gaseous environment in

the test chambers resulting in a more anaerobic environment. In fact, we have shown in previous experiments that a reaction mixture containing nitric oxide donor salts ($NaNO_2$) and glucose/saline does not produce *g*NO (data not shown).

Here we demonstrate, for the first time, a novel *g*NO-producing probiotic patch, comprised of immobilized bacteria and active ingredient, that can generate effective levels of *g*NO over therapeutically relevant periods of time and show its antimicrobial efficacy against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa, Acinetobacter baumannii,* and a *Methicillin-Resistant Staphlococcus aureus* (MRSA). This device also showed efficacy in bacteriostatic assays for *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Additionally, we show that this *g*NO-producing probiotic patch has antifungal activity as demonstrated by fungicidal assays against *Trichophyton mentagrophytes,* and *Trichophyton rubrum*.

These results confirm the capacity of *g*NO to eradicate a wide spectrum of bacterial strains and for the first time shows the use of low levels of *g*NO to kill fungal strains in liquid media. Also, these results show the novel use of probiotic bacteria in metabolizing glucose to lactic acid, generating protons, and dismutating nitrite salts for the production of *g*NO. The results demonstrate the sustained release of *g*NO at a level that is antimicrobial to common pathogens and for a duration that is therapeutically relevant and useful in hospitals and in the community. Further *in-vivo* studies are required to demonstrate therapeutic efficacy of these patches for antimicrobial applications including wound healing.

4.6. Acknowledgements

We acknowledge financial support from the Industrial Research Assistance Program of the National Research Council of Canada (IRAP-NRC) and from Micropharma Limited.

4.7 FIGURES:

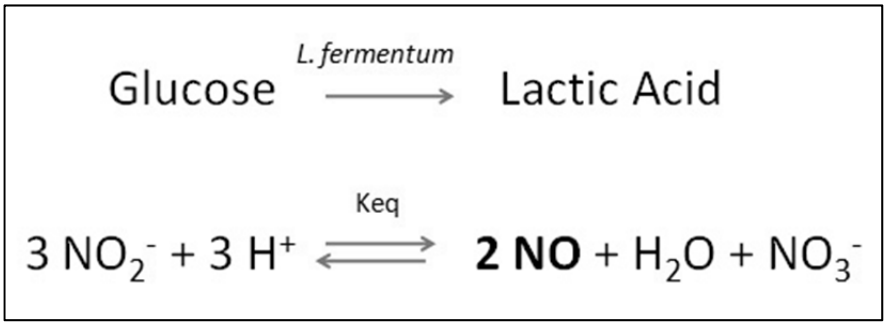


Figure 4.7.1: Production of nitric oxide gas (*g*NO) from nitrite and protons released from lactic acid generated by fermentation of glucose by immobilized *Lactobacillus fermentum* NCIMB 7230.

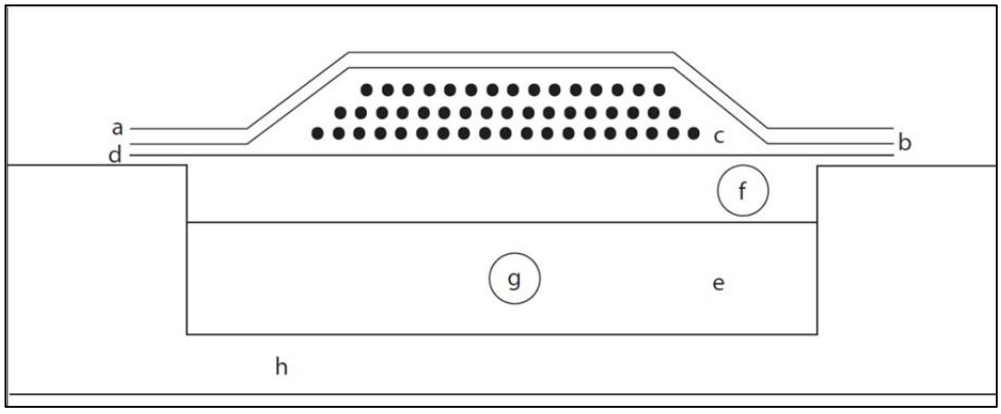


Figure 4.7.2: The cross section of the probiotic patch and chamber assembly. The patch components are shown and include, from the outermost to innermost layers, aluminized tape (a), polyethylene/nylon heat sealable plastic film (b), immobilized *Lactobacillus fermentum* NCIMB 7230 and 10 ml of 10% glucose, 30 mM $NaNO_2$, and 0.85% NaCl (c), and a layer of adhesive non-occlusive Tegaderm® (d). The reaction chamber contains a well for microbes and media (e), has gas sampling (f) and liquid sampling (g) ports and was constructed of high density polyethylene plastic (h).

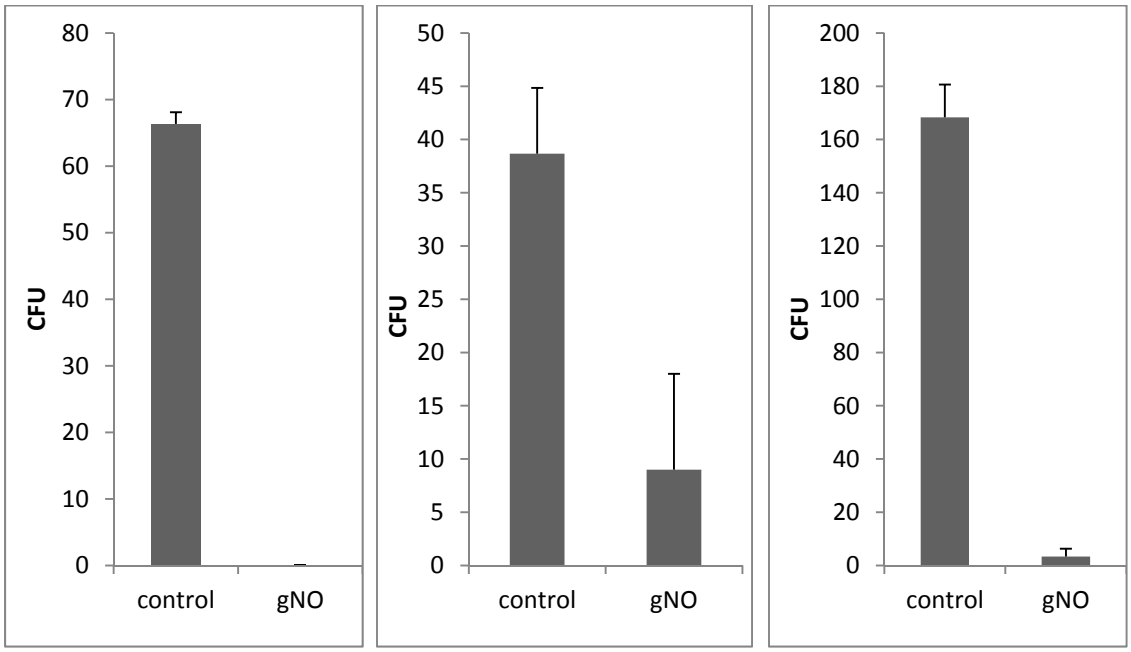


Figure 4.7.3: The bacteriostatic efficacy of *g*NO-producing patches on *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* is shown above. Treatment of *Escherichia coli* plates with *g*NO-producing probiotic patches inhibited the growth of colonies as compared to vehicle control patches (left). Treatment of *Staphylococcus*

aureus plates with *g*NO-producing patches reduced the growth of colonies as compared to vehicle control patches (middle). Treatment of *Pseudomonas aeruginosa* plates with *g*NO-producing patches reduced the growth of colonies as compared to vehicle control patches (right).

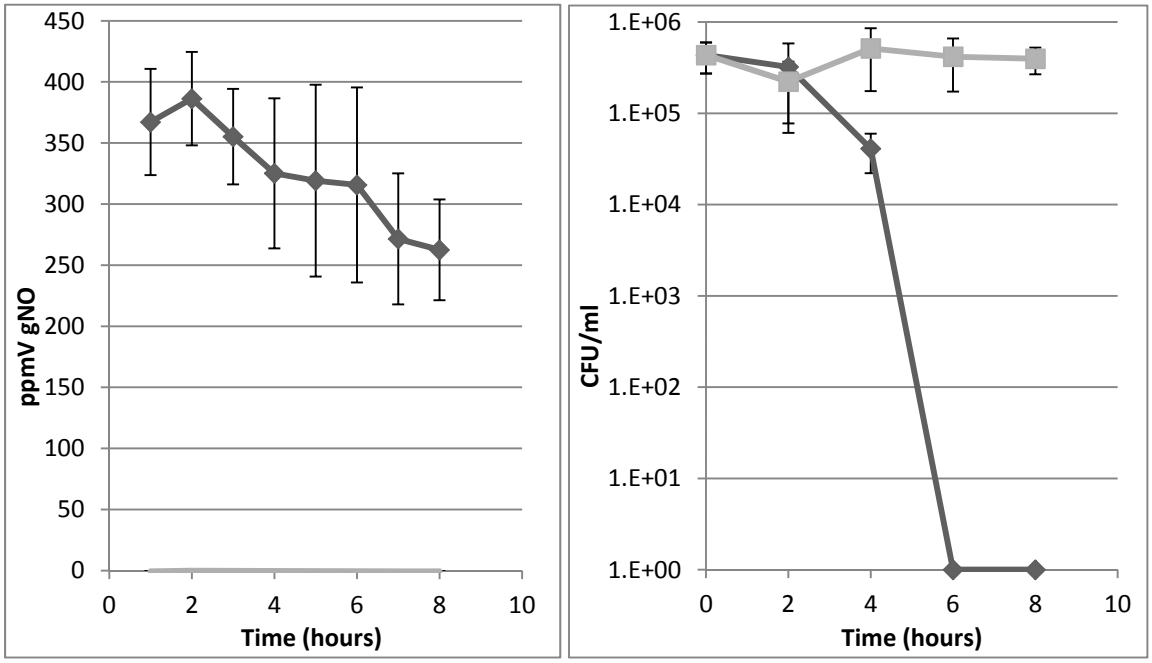


Figure 4.7.4: The bactericidal effect of *g*NO-producing patches on *Escherichia coli* (right). After 6 hours of *g*NO-producing patch application no *E. coli* colonies were detected (black); however, bacterial growth remained constant over the 8-hour treatment period when challenged with vehicle control (gray). Levels of *g*NO produced by active patches (black) or vehicle control patches (grey) were monitored hourly (left).

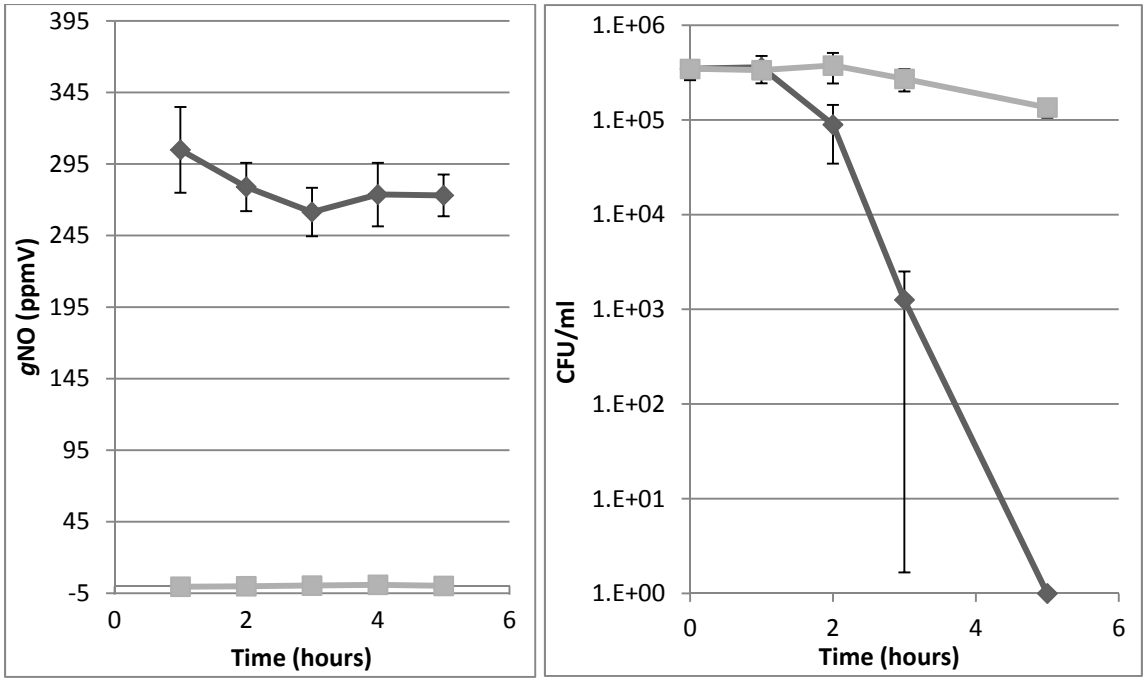


Figure 4.7.5: The bactericidal effect of *g*NO-producing probiotic patches on *Staphylococcus aureus* is shown (right). After 5 hours of *g*NO-producing patch application no colonies were detected (black); compared to control (grey). Levels of *g*NO produced by active patches (black) or vehicle control patches (grey) were monitored hourly (left).

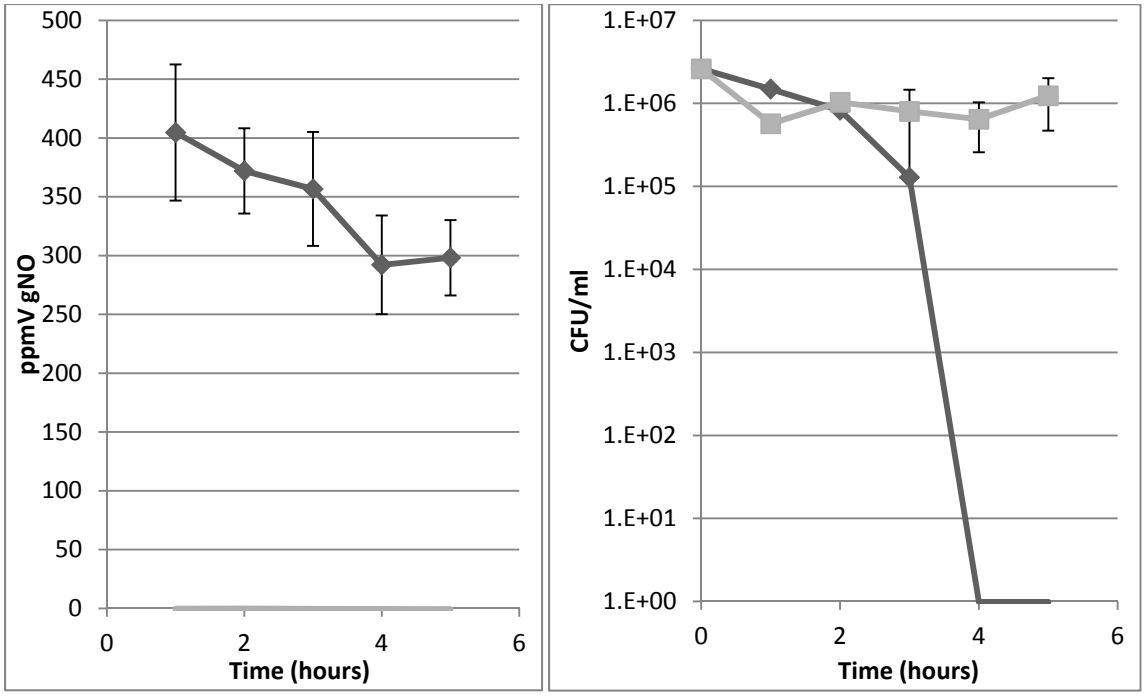


Figure 4.7.6: The bactericidal efficacy of *g*NO-producing probiotic patches on *Pseudomonas aeruginosa* is shown (right). After 4 hours of *g*NO-producing patch application no colonies were detected (black); however, bacterial growth remained constant over the 5-hour treatment period when challenged with vehicle control (gray). Levels of *g*NO produced by active patches (black) or vehicle controls (grey) were monitored over the test period (left).

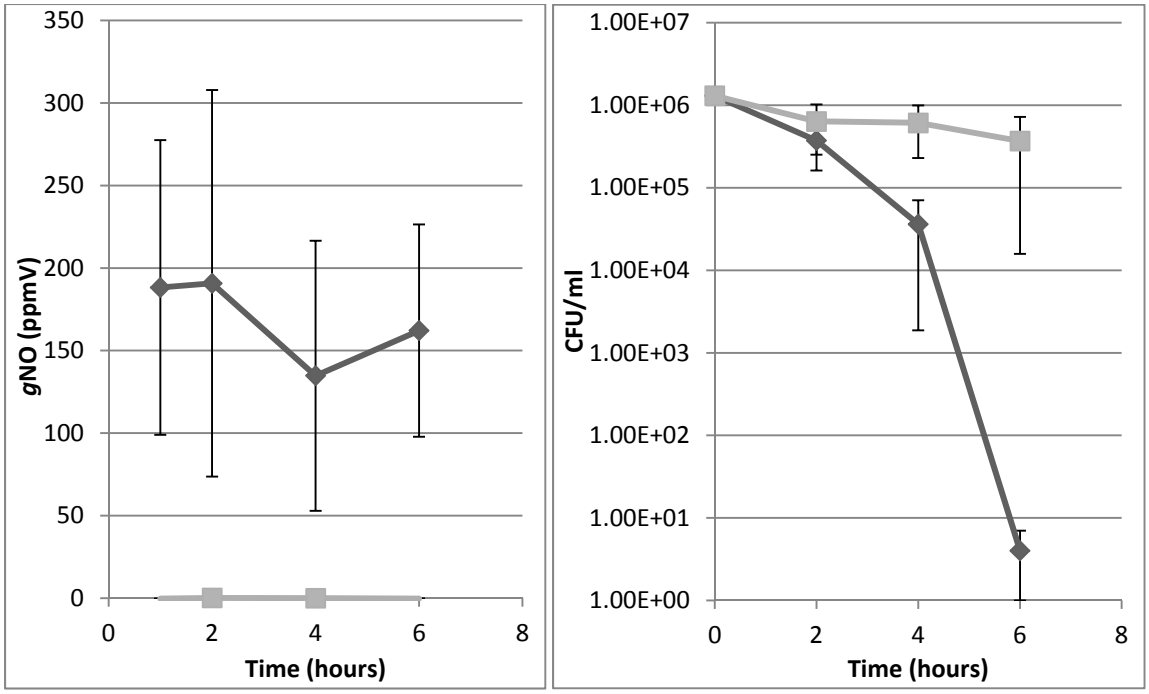


Figure 4.7.7: The bactericidal effect of *g*NO-producing probiotic patches on *Acinetobacter baumannii* is shown (right). After 6 hours of *g*NO-producing patch application fewer than 10 colonies were detected per ml (black); however, bacterial growth remained constant over a 6-hour treatment period when challenged with vehicle control (gray). Levels of *g*NO produced by active patches (black) or vehicle controls (grey) were monitored over the test period (left).

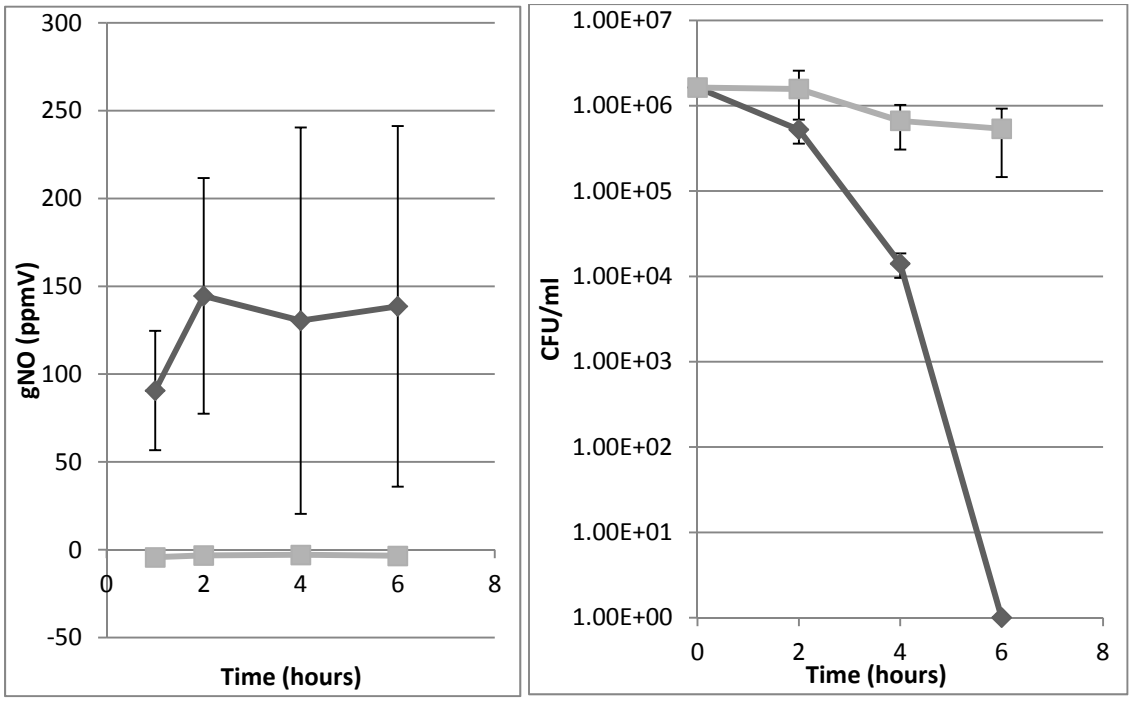


Figure 4.7.8: The bactericidal effect of *g*NO-producing probiotic patches on MRSA is shown (right). After 6 hours of *g*NO-producing patch application no colonies were detected (black); however, bacterial growth remained constant over the 6-hour treatment period when challenged with vehicle control (gray). Levels of *g*NO produced by active patches (black) or vehicle controls (grey) were monitored hourly (left).

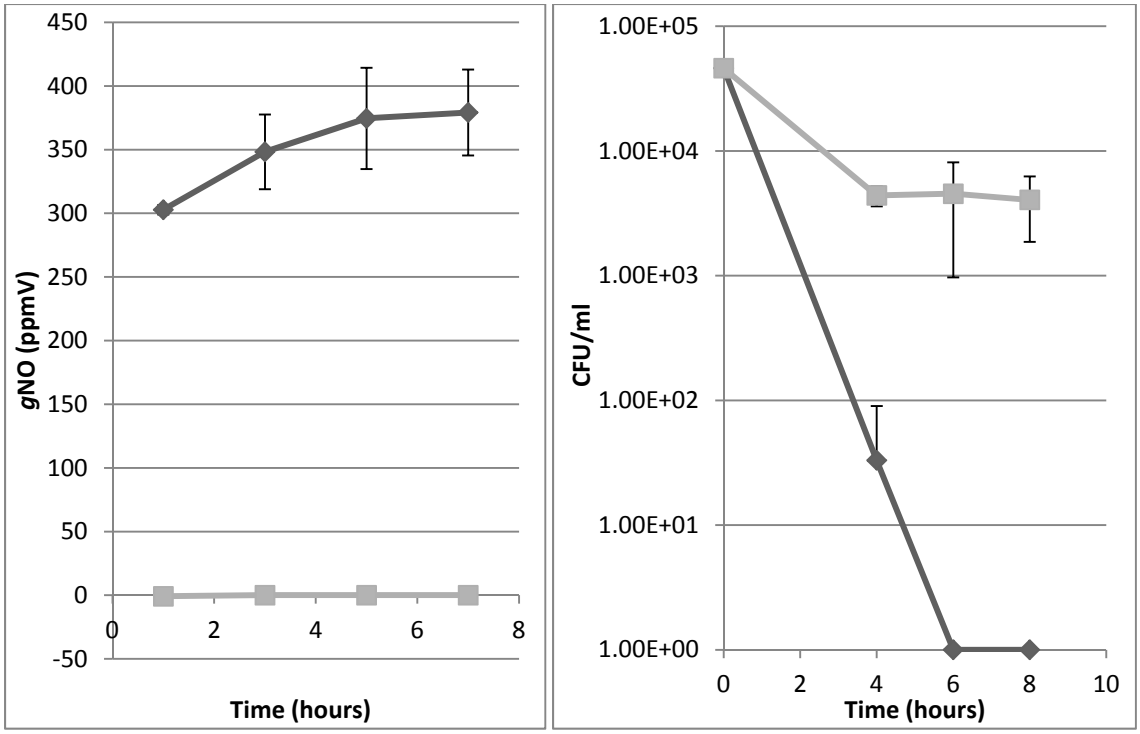


Figure 4.7.9: The fungicidal effect of *g*NO-producing probiotic patches on *Trichophyton mentagrophytes* is shown (right). After 6 hours of *g*NO-producing patch application no colonies were detected (black); however, fungal growth remained constant over an 8-hour treatment period when challenged with vehicle control (gray). Levels of *g*NO produced by active patches (black) or vehicle controls (grey) were monitored hourly (left).

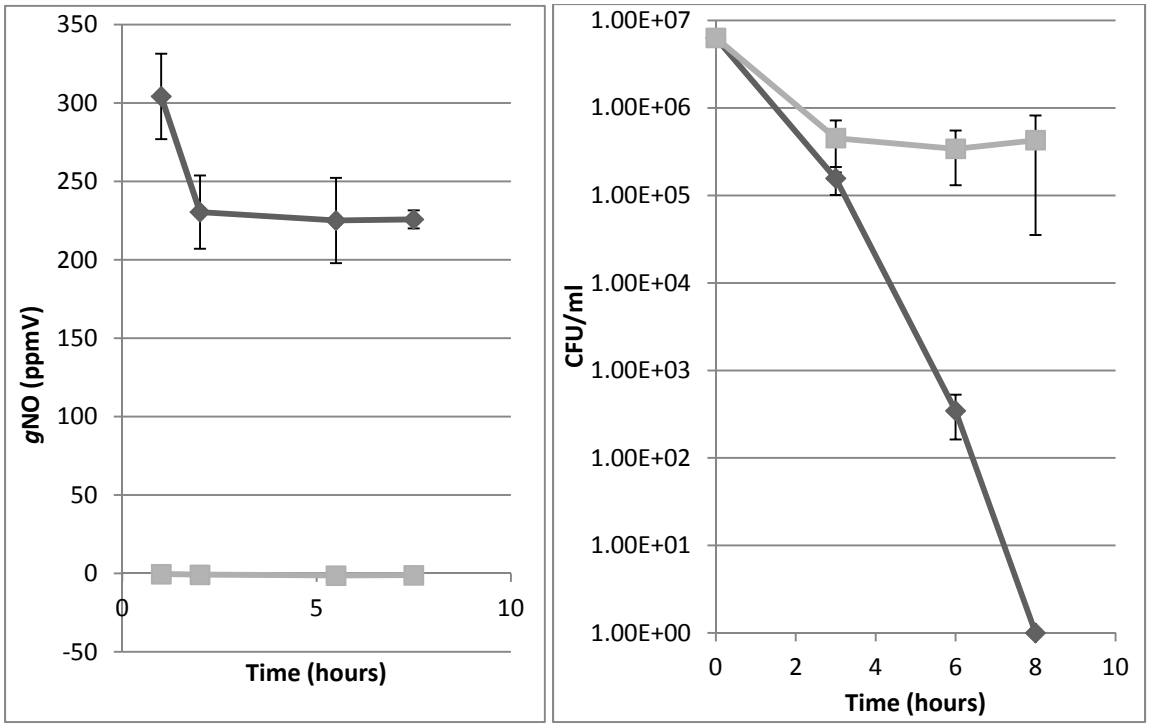


Figure 4.7.10: The fungicidal effect of *g*NO-producing probiotic patches on *Trichophyton rubrum* is shown (right). After 8 hours of *g*NO-producing patch application no colonies were detected (black); however, fungal growth remained constant over the 8-hour treatment period when challenged with vehicle control (gray). Levels of *g*NO produced by active patches (black) or vehicle controls (grey) were monitored over the test period (left).

Original Research Article 3

CHAPTER 5: NOVEL NITRIC OXIDE PRODUCING PROBIOTIC WOUND HEALING PATCH: PREPARATION AND *IN-VIVO* ANALYSIS IN A NEW ZEALAND WHITE RABBIT MODEL OF ISCHEMIC AND INFECTED WOUNDS

*Mitchell Jones, MD; Jorge G. Ganopolsky, PhD; Alain Labbé, PhD; Mirko Gilardino, MD; Christopher Wahl, MD[2]; Christopher Martoni, PhD[2]; and Satya Prakash, PhD **

Preface: This chapter investigates the efficacy and safety of *g*NO-producing probiotic patches for treating normal, ischemic, infected, and ischemic-infected full-thickness dermal wounds in a New Zealand White Rabbit model for ischemic wound healing. The antimicrobial activity of the *g*NO-producing probiotic patches was investigated in chapter 4. The likelihood of wound closure and decrease in wound area was assessed and the endpoint histopathological characteristics were characterized. Furthermore, a safety profile of the gNO-producing probiotic patch was evaluated.

Article under consideration for publication in ***Journal of Biomedicine and Biotechnology.***

* Corresponding Author

Contribution of Authors: I would like to acknowledge laboratory assistance of Jorge Gabriel Ganopolsky, and Alain Labbé, as well as surgical assistance of Mirko Gilardino. Finally, I would like to acknowledge editing of my co-authors Jorge Gabriel Ganopolsky, Alain Labbé, Mirko Gilardino, Christopher Wahl, and Christopher Martoni.

5.1. Abstract

The treatment of chronic wounds poses a significant challenge for clinicians and patients alike. Here we report design and preclinical efficacy of a novel nitric oxide gas (*g*NO) producing probiotic patch for use in wound healing. Specifically, a novel probiotic wound healing patch using lactic acid producing bacteria (LAB) in an adhesive gas permeable membrane has been designed and investigated for treating ischemic and infected full-thickness dermal wounds in a New Zealand White Rabbit model for ischemic wound healing. Kaplan-Meier survival curves showed increased wound closure with *g*NO-producing patch treated wounds over 21 days of therapy (Log-Rank P=0.0225, and Wilcoxon P=0.0113), Cox proportional hazard regression showed that *g*NO-producing patch treated wounds were 2.52 times more likely to close compared to control patches (Hazard P=0.0375, Score P=0.032, and Likelihood ratio P=0.0355), and histological analysis showed improved wound healing in *g*NO-producing patch treated animals. This study may provide an effective, safe, and less costly alternative for treating chronic wounds.

5.2. Introduction

The treatment of chronic wounds poses a significant challenge for clinicians and patients alike. Chronic wound healing is often slow or stagnant, causing prolonged patient morbidity. The prevalence of unfavourable wound environments, such as ischemia, venous stasis, or infection further hinders successful treatment of these difficult wounds. In addition, several pathological healing conditions, including diabetes and venous stasis, are associated with dysregulation of wound healing mediators which disrupts wound healing and facilitates the formation of chronic wounds[35, 204, 205].

Currently used methods such as the use of topical antibiotics, as well as other anti-microbial agents such as colloidal silver polymyxins and dye compounds have become increasingly ineffective against common pathogens. In fact, recent reviews support the well accepted fact that there is a worldwide increase in drug resistant strains of bacteria since the introduction of antimicrobial agents[45, 206-208]. Among these bacteria are methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin intermediate resistant *Staphylococcus aureus* (VISA), glycopeptide-intermediate *Staphylococcus aureus* (GISA), and community-acquired *Enterococcus faecium* and *Pseudomonas aeruginosa.* Thus, as the common antimicrobial agents begin to fail alternative treatments which do not rely on conventional antibiotics will be required.

Reduced local and regional circulation resulting from systemic antibiotic use is another problem when treating infected chronic wounds. Patients with venous stasis ulcers have venous thrombosis, reduced circulation and poor regional blood flow, and patients with diabetic foot ulcers suffer from poor microcirculation due to deposition of

glucose in microcirculatory endothelium. Systemic antibiotics can exacerbate this problem, due to constriction of the capillaries and small blood vessels, causing a further reduction in blood flow to the wound and reduced delivery of the antimicrobial agent. Topical applications are often more effective at concentrating the antimicrobial agent at the wound site; however, they are often less effective at eliminating infection for reasons which again include reduced circulation. Thus, traditional therapies frequently leave infected wounds untreated and a patient's limb and life in danger

Nitric oxide is one such biological mediator of wound healing as it acts as a signaling molecule in endothelial, neuronal, and immunological cells and is involved in many phases of wound healing including inflammation, angiogenesis, collagen deposition, re-epithelialization, and collagen rearrangement[35, 209]. During the inflammatory phase, nitric oxide acts as a chemo attractant for monocytes and neutrophils, and stimulates the production of multiple pro-inflammatory cytokines such as IL-1 and TGF-β[210]. Throughout re-epithelialization NO regulates other chemo attractants, such as RANTES, and is important for the proliferation of keratinocytes[211]. During the later stages of wound healing, nitric oxide acts in collagen deposition and in the activity of matrix metalloproteinase involved in the rearrangement and maturation of the newly deposited collagen at the wound site[212-215].

It has recently been shown that topical exposure of NO gas (*g*NO) to wounds such as chronic non-healing ulcers can be beneficial in promoting healing and preparing the wound bed for treatment and recovery[34]. Application of exogenous gas has been shown to reduce microbial infection down regulating inflammation, manage exudates and secretion, up-regulate expression of endogenous collagenase to debride the wound, and

regulate the formation of collagen[34]. Furthermore, treatment regimens have been proposed for chronic wounds which specify high and low *g*NO treatment periods to first reduce the microbial burden and consequent inflammation, increase collagenase expression in a process of debridement, and then restore the balance of NO which induces collagen expression and aids in wound closure[34]. The *g*NO delivery device, however, utilized cumbersome and expensive components and required that the patient be tethered and remain non-ambulatory during the duration of therapy[34]. Other methods for delivering *g*NO have been explored and include adsorbing nitric oxide to polymers[216], zeolite[217], or silica particles[218, 219] requiring vast amounts of purified *g*NO under pressure, or delivering exogenous nitrosothiols[220] which can be expensive and is limited by shelf life (GSNO is thermally and photolytically liable). These methods are each able to deliver some nitric oxide; however, the level is often not physiologically relevant or the length of *g*NO release is inadequate. For this reason we are proposing a probiotic bacteria based system for the production of nitric oxide so that the control of *g*NO production relies on the metabolism of bacteria that can be sustained for the longer durations required for treatment of chronic wounds both in a hospital and community setting.

Here we report design of a novel probiotic patch for treating ischemic and infected full-thickness dermal wounds and investigation of the preclinical efficacy in an experimental New Zealand White Rabbit animal model of chronic wound healing.

5.3. Materials and Methods

Nitric oxide producing probiotic patch design

The *g*NO producing dressings were designed using two Tegaderm™ adhesive patches, one in contact with the skin and the other acting as a top layer of the device (Figure 5.8.1). The active ingredients of the dressing, namely lyophilized alginate microbeads containing *Lactobacillus fermentum* NCIMB 7230, and a solution containing glucose (10%), NaCl (0.85%), and sodium nitrite (30mM), were placed in a heat sealed pocket made between the Tegaderm™ in contact with the skin and a layer of polyethylene (Figure 5.8.1). Control patches were built similarly, but were prepared with glucose (10%), NaCl (0.85%), and no sodium nitrite.

Animals

Male New Zealand white rabbits (1.5 to 2.5 kg) were obtained from Charles River Laboratories (Montreal). The animals were housed at the UQAM animal center in individual cages with food and water *ad libitum*. The animals were kept on a 12 hour light-dark cycle and were left to acclimatize for 4 weeks prior to surgery.

Surgical procedures

The animals were sedated with glycopyrolate (0.1mg/kg SC) and anaesthesia was started (ketamine (35 mg/kg IM) and xylazine (5mg/kg IM)). Once asleep, the anaesthesia was maintained with 4-5 % isofluorane and the animals were monitored for heart and breathe rates. The surgical procedures for the induction of ischemia and the

creation of full-thickness wounds were based on a modified method published by Chien et al.[221]. Briefly, the ventral side of each ear was shaved as well as the areas at the base of the left ear, where the incisions were made for the induction of ischemia. The surgical areas were wiped with ethanol and the rabbit's body temperature was maintained on a heated pad during.

Three vertical incisions were made about 1 cm from the base of the ear, closely located to the central, cranial and caudal bundles (composed of the artery, vein and nerves). The central artery was surgically divided from the nerve and veins. The central artery was ligated with 5-0 silk while the venous circulation was preserved. The entire cranial bundle was ligated with 5-0 silk. The caudal bundle was identified and separated from surrounding tissues, but left untouched. After disruption of the arteries, a subcutaneous tunnel was created between the 3 incisions. Using a sharp pair of scissors, the tissues under the skin were cut and removed to expose cartilage all around the base of the ear, working through the three incisions. Once the disruption was complete, any bleeding was controlled by applying pressure with a sterile gauze pad before closing the incisions using 4-0 nylon sutures.

Four full thickness wounds were created on the ventral side of the ears using a sterile sharp 6 mm biopsy punch. Very light pressure was applied to avoid breaking through the thin cartilage of the ear. The wounds were created on the upper portion of the rabbit's ears, to allow easy access and to maintain the wounds on a flat surface. Skin was removed from the cartilage and special care was taken to remove the perichondrial membrane and expose the cartilage. Following the creation of the wounds, the ears were covered with sterile gauze. Cotton was used to pack the ventral side of the ear. Gauze

bandages were used to wrap the ears and were immobilized with surgical tape. The wrapping was kept relatively tight to prevent the loss of the dressing but not so tight as to limit blood flow in the non-ischemic control ear. Elizabethan collars were also fitted to the rabbits to prevent damage caused by the rabbits scratching their wounds. An injection of antibiotics (enrofloxacin, Baytril®, 5mg/kg IM) was given intra-operatively, to reduce the risk of systemic infections. A Fentanyl patch (25 µg/hr) was applied for 3 days following surgery on a shaved area located 10 cm distally from the base of the skull, on the rabbits back.

Infection

Experimental rabbits were infected with *Staphylococcus aureus* to assess the effect of *g*NO-producing patches on infected wounds. An overnight culture of *S. aureus* was diluted to approximately $1x10^6$ CFU/ml in saline (0.85% NaCl) and applied with sterile cotton tipped applicators to the four wounds on each rabbit ear for 10 min. before the control or treatment patches were applied.

Wound treatment

Starting one day after surgery, the wounds were treated with control or *g*NO-producing patches designed to produce *g*NO levels of approximately 200ppmV for 24h (Figure 5.8.3). The patches were replaced daily after gently wiping the wounds using sterile gauze. The wounds were dressed as described at the end of the surgical procedure. The treatment was maintained for 20 or 21 days, irrespective of wound closure.

Wounds of rabbits 1 and 2 were not infected. Wounds on rabbit 3 and 4 were infected with *S. aureus*. Wounds of rabbits 1 and 3 were treated with vehicle control patches; wounds of rabbits 2 and 4 were treated with *g*NO-producing patches.

Data Collection

Wound healing was monitored daily and photographic records were kept for morphometric analysis. Starting on the day of surgery, photographs were taken of the wounds on each ear.

Euthanasia and Sample Collection

Rabbits were euthanized under general anaesthesia using ketamine (35 mg/kg IM) and xylazine (5mg/kg IM) followed by inhalation of isoflurane (5% in 1l/min O_2). Cardiac puncture was performed until complete exsanguination. Blood was collected in EDTA collection tubes for haematological analysis and in glass tubes for serum collection after clotting. Urine samples were also collected directly from the bladder after death of the animals. The ears were removed and the 4 wounds on each ear were cut out and placed in buffered formalin for histopathological analysis.

Histopathology

Histopathology was performed on all the wounds. Briefly, the tissue sections were left to fix for at least 24 hours in formalin. The samples were bisected, placed in cassettes and processed to paraffin. Sections were sectioned at approximately 5µm, mounted on glass slides and stained with haematoxylin and eosin (H&E) and Masson's trichrome

stains. Fixation, mounting, staining and analysis of the stained samples were performed by blinded pathologists using a semi-quantitative grading system. Inflammation was graded from 0 (absent or very few inflammatory cells) to 3 (abundant inflammatory cells). Maturity was graded based on the organisation and quantity of granulation tissue. A grade of 0 indicates the absence of granulation tissue while a score of 4 indicates a dense granulation tissue with bundles of mature dermal-type collagen.

Haematology

Haematological analysis was performed with an ADVIA 120 analyser. The following parameters were evaluated: red blood cell counts (RBC), haemoglobin (Hg), hematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count, white blood cells (WBC), WBC differential counts, cell morphology, reticulocyte count. Blood smears were also prepared to evaluate morphology.

Blood Chemistry

Serum samples were analyzed for sodium (Na^{+}), potassium (K^{+}), chloride (Cl^{-}), bicarbonate (HCO^{3-}), creatinine, urea, glucose, calcium (Ca^{2+}), phosphate (PO_4^{3-}), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lipase, and C-reactive protein (CRP) using a Hitachi 911 blood clinical chemistry analyzer.

Methemoglobin Measurements

Blood samples were haemolysed with distilled water and phosphate buffer pH 7.0. Percentage of blood methemoglobin in the samples were determined according to a modified Evelyn-Mallow method[222]. Briefly, OD_{632} of hemolysed blood was measured to determine the concentration of methemoglobin. Total haemoglobin was determined by OD_{632} after addition of potassium ferricyanide to haemolysed blood. The baseline correction values were determined by subtracting OD_{632} values of both solutions after the addition of sodium cyanide. The percentage of methemoglobin was calculated as the quotient of the corrected OD_{632} values.

Statistical Analysis

Statistical analysis of wound morphometric data was performed by repeated measures ANOVA with mixed models (SAS version 9.2).

Statistical analysis of complete wound closure was performed by Kaplan-Meier curves and Cox proportional hazard regression analysis. Kaplan-Meier curves (survival curves) express the likelihood of survival over time. Here the statistical method was used as a representation of the likelihood of wound closure over time. The data was plotted using time-to-closure of each wound separately, on a Kaplan-Meier graph and statistical analysis was performed using each of the variables present in the study: time-to-closure, treatment, ischemia and infection. The Cox proportional hazard model is a statistical method used to determine the multiplicative effect on a subject's hazard rate. The hazard ratio represents the increase (or decrease) in the likelihood of an event occurring (wound closure). Data analysis was performed using EPI info from the Center for Disease Control (CDC).

5.4. Results

Nitric oxide producing probiotic patch design

Novel probiotic patches were designed. For this, 0.5 grams immobilized *Lactobacillus fermentum* (NCIMB 7230) (20% cells w/v) in 2.5 ml glucose (10% w/v) and sodium nitrite (30mM) in saline (0.85% w/v) were used. Figure 5.8.1 depicts the *g*NO producing probiotic patch design and shows the active ingredients in the gas permeable patches. *g*NO production was evaluated in three representative patches over a 24 hour period and was measured by chemiluminescence (Sievers®, GE, USA) and is shown in Figure 5.8.3. There was no gNO detected in the control patches (not shown).

Photographic evaluation and morphometric analysis of wounds treated with nitric oxide producing probiotic patch

Photographs of infected and non-infected wounds, ischemic and non-ischemic wounds, treated with either *g*NO-producing probiotic patches or vehicle control patches taken at days 1, 13, and 20 are presented in Figure 5.8.4. Non-infected wounds generally appeared dry for the duration of the trial whereas infected wounds presented purulent exudates. Also, non-ischemic wounds closed between 13 and 15 days post-surgery, independent of infection, as previously reported[223]. The efficacy of *g*NO-producing probiotic patches versus vehicle control patches was determined by morphometric analysis of wound area under four experimental conditions: (i) ischemic non-infected wounds (Figure 5.8.5, upper left); (ii) ischemic infected wounds (Figure 5.8.5, upper

right); (iii) non-ischemic non-infected wounds (Figure 5.8.5, lower left); and (iv) non-ischemic infected wounds (Figure 5.8.5, lower right). Morphometric analysis was performed by measuring the wound areas from daily photographs of wounds treated with *g*NO-producing or vehicle control patches for each of the above conditions. Repeated measures analysis of the entire data set, across all experimental conditions (n=32), showed that treatment with *g*NO-producing probiotic patches resulted in a significant decrease in wound area over the duration of the study (P=0.0001), that the presence of ischemia significantly slowed wound closure (P=0.0009), but that the presence of infection did not significantly affect the healing process (P=0.2578). The statistics for ischemic wounds only (n=16) showed that treatment with *g*NO-producing patches resulted in a significant decrease in wound area over the duration of the study (P<0.0001). There was also a significant decrease in wound area for *g*NO-producing probiotic patch-treated ischemic non-infected wounds (P=0.0006) (Figure 5.8.5, upper left) and ischemic infected wounds (P=0.015) (Figure 5.8.5, upper right) compared to the respective vehicle control-treated wounds. Finally, treatment of non-ischemic non-infected wounds with *g*NO-producing patches did not significantly accelerate the rate of healing (P=0.3539) (Figure 5.8.5, lower left) and treatment of non-ischemic infected wounds did not significantly accelerate the rate of wound healing (P= 0.1988, through day 11) (Figure 5.8.5, lower right) when compared to treatment with vehicle control patches.

Histopathological analysis of wounds treated with nitric oxide producing probiotic patch

The histological results show a trend towards improved wound healing in tissues of the *g*NO-producing probiotic patch treated animals when compared to those treated

with vehicle control patches, although a larger sample size would be required to show significance (Table 5.7.1). In almost every case, there was improvement in the average score (defined in materials and methods) given to histological observations consistent with improved wound healing (Table 5.7.1). At the wound surface less depression was observed (mean grade of 0.14±1.68 versus -1.00±1.77) and less crusting and exudates was observed (mean grade of 0.5±1.07 versus 1.63±1.41). The epidermal layer showed improved mean coverage (87.5±31.5% versus 79.4±29.8%), less hyperplasia (mean of 2.29±0.76 versus 2.63±0.74 in multiples of epidermal thickness), and improved epidermal maturity (mean score of 3.13±0.64 versus 2.38±0.91). The granulation tissue and dermal layer showed increased thickness (1.13±0.64 versus 0.84±0.76 multiples of dermal thickness), less inflammation and infiltration of inflammatory cells (2.13±0.83 versus 2.38±0.74), and improved maturity (mean grade of 1.88±0.99 versus 1.13±0.83) (Table 5.7.1). Figure 5.8.6 shows representative Masson's trichrome stained sections for the evaluation of wound healing, inflammation, and maturity.

Safety of nitric oxide producing probiotic patch

A complete hematologic profile showed that markers for rabbits 1, 2 and 4 were all within normal ranges. Nitric oxide producing probiotic patch safety was further evaluated through a complete blood biochemical analysis: All values were within normal ranges except for elevated serum potassium for rabbits 3 (9.79 mmol/L) and 4 (11.44 mmol/L) (treated and control infected rabbits). Analysis of methemoglobin was performed on whole blood samples and showed normal levels of methemoglobin in both

*g*NO-producing probiotic patch treated rabbits (0.1± 0.1 %) and vehicle control patch treated rabbits (0.3± 0.2 %) (Table 5.7.2).

Kaplan-Meier curve and Cox proportional hazard regression

A Cox proportional hazard survival regression analysis was performed for all wounds (n=32) for the variables: treatment, ischemia, and infection, and for ischemic wounds (n=16) for the variables: treatment and infection (Table 5.7.3). The hazard ratio represents the increase or decrease in the likelihood of an event occurring (wound closure). Analysis of all wounds showed a 2.45 fold increase in the likelihood of wound closure for *g*NO-producing probiotic patch treated wounds versus vehicle control patch treated wounds (P=0.0533) and that ischemic wounds were 0.21 times less likely to close (P=0.0039). An analysis of ischemic wounds only (n=16, 8 treated, 8 non-treated) showed a 17.95 fold increase in the likelihood of wound closure for *g*NO-producing probiotic patch treated wounds versus control patch treated wounds (P=0.0302) and that infected ischemic wounds are 0.068 times less likely to close (P=0.0371).

The likelihood of wound closure over time was evaluated in wounds treated with *g*NO-producing probiotic patches versus wounds treated with vehicle control patches by plotting a Cox proportional hazard regression plot independent of co-variables (Figure 5.8.7). The analysis shows that *g*NO-producing probiotic patch treated wounds are 2.52 times more likely to close than vehicle control treated wounds (Hazard P=0.0375, Score P=0.032, and Likelihood ratio P=0.0355).

The likelihood of wound closure over time was evaluated in wounds treated with *g*NO-producing probiotic patches versus wounds treated with vehicle control patches by

plotting a Kaplan-Meier plot and calculating the Log-Rank and Wilcoxon statistics (Figure 5.8.8). The analysis shows that wounds treated with *g*NO-producing probiotic patches closed before wounds treated with vehicle control patches (Log-Rank P=0.0225, and Wilcoxon P=0.0113, 16 wounds per group) and that several of the control-treated wounds remained open.

The likelihood of wound closure over time was evaluated for ischemic and non-ischemic wounds (Figure 5.8.9). The data shows that ischemia significantly delays wound closure in this animal model (Log-Rank P=0.005, and Wilcoxon P=0.0056, 16 wounds per group). Finally, analyzing only the ischemic wounds (n=8, 4 wounds per group) the same analysis shows a significant improvement in wound closing for the NO-treated groups as compared to the vehicle controls despite the number of wounds (Log-Rank P=0.04, and Wilcoxon P=0.046).

5.5. Discussion

This study demonstrates for the first time, the efficacious and safe use of a *g*NO-producing probiotic patch for ischemic and infected wound healing. Specifically, this study describes the design of a probiotic patch, its fabrication, *in-vitro* testing, and investigates the potential of a *g*NO-producing probiotic patch on healing of ischemic and/or infected full-thickness wounds in New Zealand white rabbits. For these experiments, animals were operated to induce ischemia, four full-thickness wounds were created on each ear, and two sets of wounds were infected with *S. aureus* while two sets of wounds remained non-infected. Half of the wounds were treated with *g*NO-producing

patches while the other half were treated with vehicle control patches. Wounds were monitored, dressings changed, and photographs taken daily for the study duration.

The efficacy of the probiotic gNO producing patch on wound healing was measured by morphometric and time to closure analyses. Results show that wound healing was significantly accelerated in ischemic wounds treated with *g*NO-producing probiotic patches. This accelerated closure was shown to be significant for both non-infected and infected ischemic wounds. Morphometric analysis did not reveal a significant effect of *g*NO on wound healing in non-ischemic wounds, both infected and non-infected. A non-significant improvement, however, was seen in treated ischemic and non-ischemic infected wounds when compared to vehicle control-treated wounds, whereas no such trend was evident in non-infected wounds. Thus, while morphometric analysis shows that ischemia plays a role in the pathophysiology of delayed wound healing, and that *g*NO-producing probiotic patches can be used to accelerate wound closure in infected or non-infected ischemic wounds, only a promising trend exists for treating non-ischemic infected wounds with *g*NO. However, based on the wide spectrum antimicrobial activity of nitric oxide[224], the proven antimicrobial and antifungal activity of this *g*NO-producing probiotic patch[225], and the known pathologic action of bacterial biofilms in slowing wound closure, a real difference may be apparent in a larger population of wounds.

While improved wound healing (decreased wound area) over time was easily quantifiable, and successfully revealed by morphometric analysis, the definitive endpoint for efficacy in chronic wound healing is complete wound closure[226]. Thus, Log-Rank and Wilcoxon statistics for Kaplan-Meier survival probability plots and Cox proportional

hazard regression analyses were performed to determine the increased or decreased likelihood of complete closure. Analysis of all wounds showed that treatment with *g*NO-producing probiotic patches significantly improved the likelihood of wound closure and that the presence of ischemia decreased the likelihood of closure. Also, analysis showed that ischemic wounds that were treated with *g*NO-producing probiotic patches were significantly more likely to close than vehicle control patch-treated ischemic wounds. Finally, infected ischemic wounds were found less likely to close than non-infected ischemic wounds. These results corroborate those seen in analysis of morphometric data and strengthens the hypothesis that the pathophysiology of non-healing ischemic wounds is closely tied to a dysregulation of endogenous nitric oxide production and that the exogenous application of *g*NO may have a therapeutic effect[31, 209, 227, 228].

Histological analyses was used to, assess the degree of healing, presence of inflammation, level of tissue reorganization, and overall maturity of the wound and supports the above results showing improved wound healing and complete closure with *g*NO-producing probiotic patches. The results show improved scores for surface, epidermal and dermal observations and shows a tendency towards improved wound healing in the *g*NO-producing probiotic patch treated wounds. These histological results provide growing support for the hypothesis that improved wound healing and maturity due to the topical application of *g*NO may be due to increased collagen deposition, increased stimulation of endothelial progenitor cells, stimulation of neovascularization, endothelial cell relaxation, and improved keratinocyte migration[35, 229].

Determination of the safety and toxicity profile of the *g*NO-producing probiotic patches was performed by blood analysis. *g*NO-producing probiotic patch treated and

vehicle control treated samples were all found to be within normal ranges except for potassium values in serum samples for rabbits 3 and 4 (infected rabbits), which were found to be hyperkalemic and thought to be the result of either a compensated metabolic acidosis, due to infection and/or poor perfusion, or haemolysis of blood samples prior to centrifugation and serum collection. A complete hematologic profile was preformed, and showed treated and non-treated animals to be comparable and no abnormal values were found to be associated with *g*NO-producing probiotic patch treatment. One non-*g*NO treated animal was found to be leucopenic which may have been the result of stress from the surgical procedure and daily manipulation or may be a statistical outlier. The results of blood analysis for safety and toxicity markers corroborate reports in the literature that the topical application and even inhalation of *g*NO is non-toxic and safe[230].

Finally, one possible consequence of *g*NO absorption is an increase in the blood level of methemoglobin which is a haemoglobin molecule in which the iron is oxidized from Fe^{2+} to Fe^{3+} [231]. Analysis of levels of methemoglobin, however, showed normal levels in both the *g*NO-producing probiotic patch treated rabbits and vehicle control patch treated rabbits, indicating that the *g*NO induced formation of methemoglobin was undetectable or that clearance was adequate.

While several groups are working on *g*NO releasing wound healing devices, the barriers to commercial use (sustained *g*NO release, shelf life, ease of use, and cost) have not yet been overcome[34, 232-236]. This study shows that *g*NO-producing probiotic patches can be designed and used for topical applications, including treating ischemic and infected full-thickness dermal wounds that approximate chronic non-healing ulcers, and that *g*NO-producing probiotic patches may provide an elegant solution to the obstacles to

commercial development. This study also provides further evidence that the dysregulation of NO may be intimately associated with the pathophysiology of chronic ischemic wounds and that exogenous application can accelerate wound healing. Human clinical efficacy is yet to be evaluated.

5.6. Acknowledgements

We gratefully acknowledge financial support from the Industrial Research Assistance Program (IRAP) of the National Research Council (NRC) of Canada and research grant from Micropharma Limited.

5.7 TABLES:

[1]**Table 5.7.1:** Histological analysis.

		Control	Treatment	Improvement
Wound Surface	Wound Width (microscopic fields at 4x magnification)	1.00±0.27	1.08±0.19	N/C
	Raised (+) /Depressed (-) (-3 to 3)	-1.00±1.77	0.14±1.68	Improved
	Central Protrusion (0 to 3)	0.13±0.35	0.57±0.98	
	Crusting/Exudates (0 to 3)	1.63±1.41	0.5±1.07	Improved
Epidermis	Coverage (estimated %)	79.4±29.8	87.5±31.5	Improved
	Hyperplasia (multiple of normal epidermal thickness)	2.63±0.74	2.29±0.76	Improved
	Maturity (1 to 4)	2.38±0.91	3.13±0.64	Improved
Granulation Tissue/Dermis	Thickness (multiple of normal dermal thickness)	0.84±0.76	1.13±0.64	Improved
	Inflammation/ Infiltration (0 to 3)	2.38±0.74	2.13±0.83	Improved
	Maturity (0 to 4)	1.13±0.83	1.88±0.99	Improved

[1] **Table 5.7.1**. Shows histological analysis of ischemic wounds on ears of rabbits treated with *g*NO-producing probiotic patches or those treated with vehicle control patches. The results show a trend towards improved wound healing as evidenced by less surface depression, less crusting and exudates, less inflammation/infiltration, greater epithelial coverage, reduced hyperplasia, greater dermal thickness, and improved overall epidermal and dermal maturity. The results are averages of *g*NO-producing patch treated (n=16) and vehicle control patch treated wounds (n=16).

[2]**Table 5.7.2:** Safety and toxicological data.

			Rabbit 1	Rabbit 3	Rabbit 2	Rabbit 4	Finding
Body Weight			-0.1kg	-0.3kg	-0.1kg	-0 kg	No difference
Blood Morphology			Normal	Normal	Normal	Normal	No difference
Hematology	WBC	($x10^9$/L)	6.16	**1.54**	7.81	4.66	
	RBC	($x10^{12}$/L)	5.91	6.10	6.07	5.98	
	HGB	(g/L)	122	121	119	122	
	HCT	(L/L)	0.35	0.34	0.35	0.36	
	MCV	(fL)	59.9	55.2	57.7	60.3	
	MCH	(pg)	20.6	19.8	19.7	20.5	
	MCHC	(g/L)	344	359	341	339	
	PLT	($x10^9$/L)	315	444	247	583	
	Neut	($x10^9$/L)	1.85	0.38	1.52	1.32	
	Lymp	($x10^9$/L)	3.69	1.07	5.75	2.98	
	Mono	($x10^9$/L)	0.06	0.01	0.07	0.05	
	Eos	($x10^9$/L)	0.11	0.03	0.15	0.09	
	Luc	($x10^9$/L)	0.01	0.00	0.01	0.00	
	Baso	($x10^9$/L)	0.43	0.05	0.31	0.22	
	Retic	($x10^{12/}$L)	0.211	0.086	0.163	0.137	
Blood	Na^+	mmol/l	146.8	146.3	148	148	

[2] **Table 5.7.2.** Shows safety and toxicological data of the *g*NO-producing probiotic patch treated and vehicle control patch treated animals. No significant differences were observed between *g*NO-producing probiotic patch treated and vehicle control patch treated animals in body weight, blood morphology, hematology, blood chemistry, or methemoglobin levels. Bolded values are those outside of the normal range.

Chemistry							
Electrolytes	K^+	mmol/l	5.79	**9.79**	5.55	**11.44**	
	Cl^-	mmol/l	101.5	102.9	107.5	109.2	
	HCO_3^-	mmol/l	34.13	30.86	27.52	22.76	
Kidney	Creat	µmol/l	126.84	162.61	144.78	127.26	
	Urea	mmol/l	6.99	6.17	7.15	6.57	
Endocrine	Glucose	mmol/l	13.73	11.22	12.56	14.74	
	Ca^{2+}	mmol/l	3.29	3	3.26	3.16	
	PO^{4-}	mmol/l	2.11	2.06	2.74	1.97	
Liver	ALT	U/l	50.83	31.01	28.71	32.12	
	AST	U/l	32.89	33.3	54.95	19.32	
Pancreas	Lipase	U/l	190.59	158.6	148.37	194.28	
Inflammatory	CRP	nmmol/l	0.14	1.88	-0.38	1.34	
Methemoglobin			0.3% ±0.2%		0.1%±0.1%		Normal levels

[3]**Table 5.7.3:** Cox proportional hazard survival regression analysis for all wounds.

	Variable	Hazard Ratio	P-Value
All Wounds	Treatment	**2.4488**	**0.0533**
	Ischemia	**0.2111**	**0.0039**
	Infection	0.6825	0.4192
Ischemic	Treatment	**17.9542**	**0.0302**
	Infection	**0.0676**	**0.0371**

[3] **Table 5.7.3.** shows Cox proportional hazard survival regression analysis for all wounds (n=32) and for ischemic wounds (n=16) under the conditions: treatment, ischemia and infection. Hazard ratios represent an increase (or decrease) in the likelihood of an event occurring (wound closure). The data was analyzed using EpiInfo software (CDC, USA).

5.8 FIGURES:

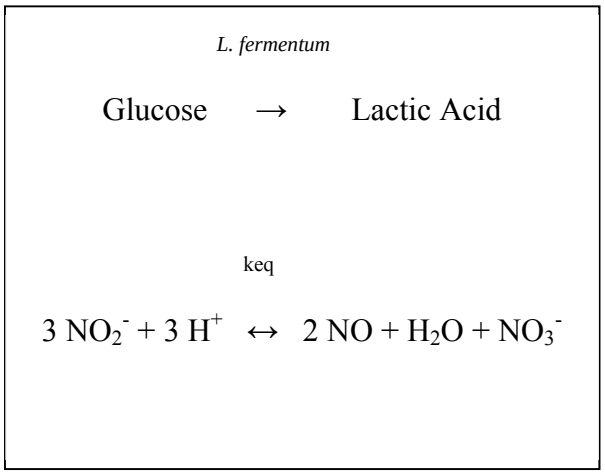


Figure 5.8.1. Chemistry for novel nitric oxide gas (*g*NO) producing probiotic patch in which *g*NO is generated from nitrite and protons produced by *Lactobacillus fermentum* NCIMB 7230 bacteria from glucose.

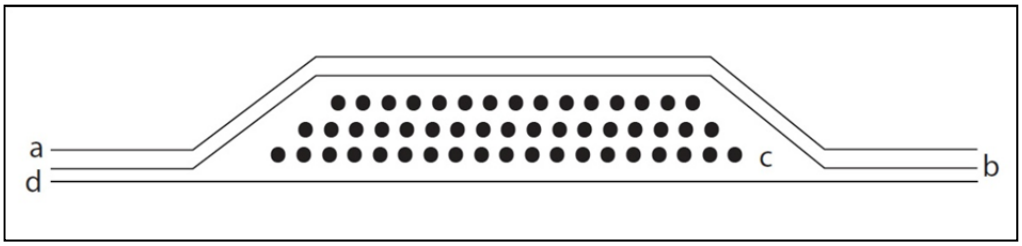


Figure 5.8.2. Cross section of novel *g*NO-producing probiotic patch design. From the outermost to innermost layers, the patch is composed of: (a) adhesive non-occlusive Tegaderm™, (b) polyethylene/nylon laminated film, (c) immobilized bacteria *Lactobacillus fermentum* NCIMB 7230 and of 10% glucose, 30 mM $NaNO_2$, and 0.85% NaCl, and (d) a layer of adhesive non-occlusive Tegaderm™.

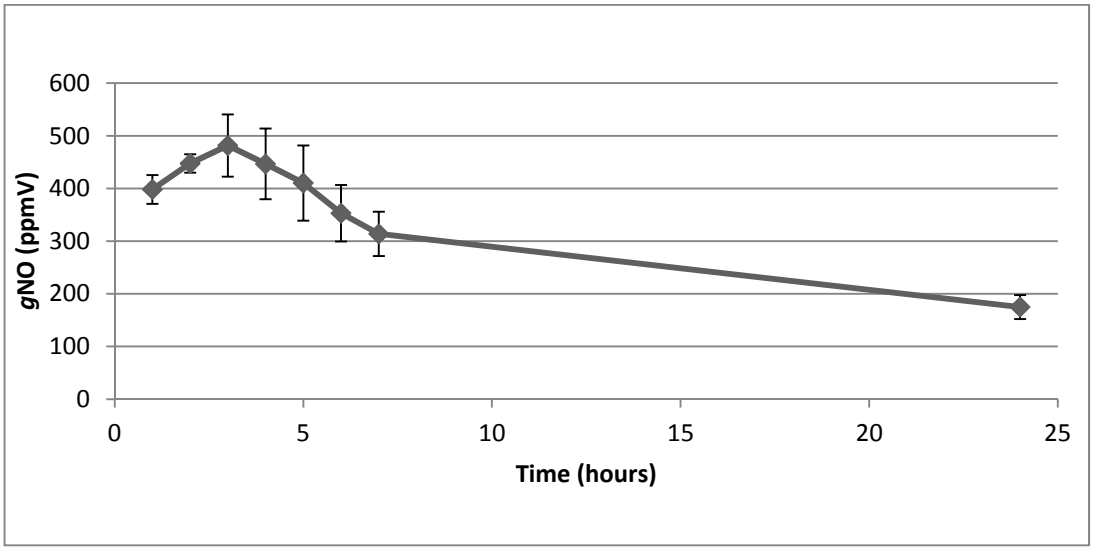


Figure 5.8.3. Nitric oxide gas (*g*NO) production by *g*NO-producing probiotic patch containing 2.6 ml of glucose (10% w/v), sodium nitrite (30 mM), and 0.5 g lyophilized alginate microbeads containing 20% w/v immobilized *Lactobacillus fermentum* NCIMB 7230 by weight. Nitric oxide gas was detected using a chemilumiescent nitric oxide analyzer (Sievers®, GE, USA) in a custom designed high density polyethylene chamber.

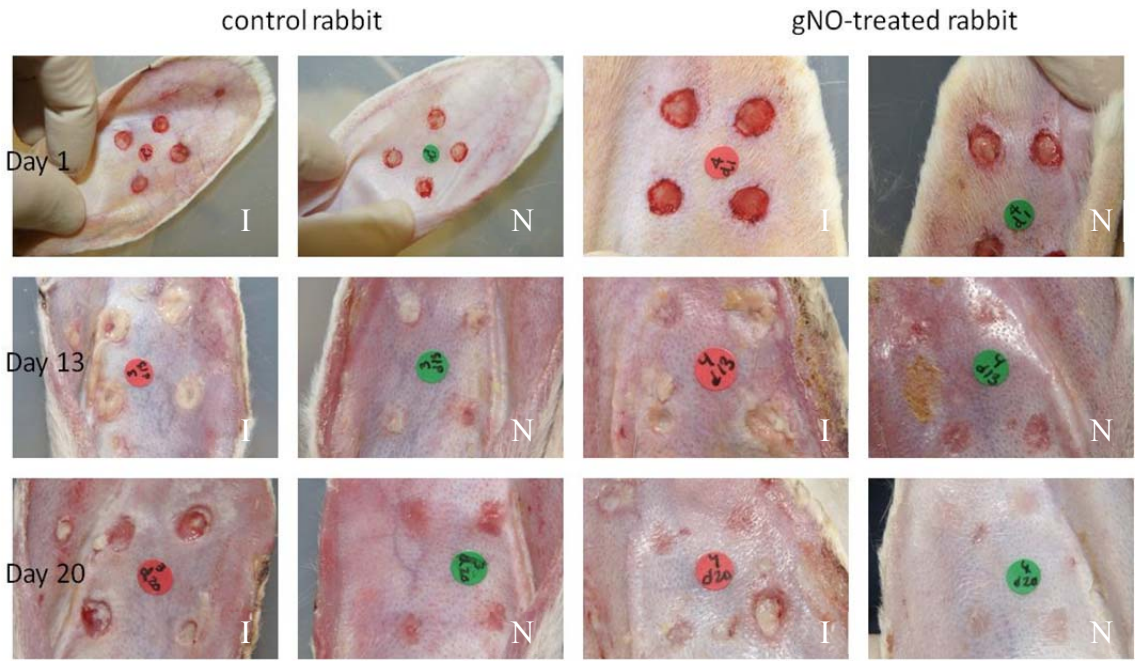


Figure 5.8.4. Photographs of infected full-thickness dermal wounds on ears that are either ischemic "I" or non-ischemic "N" and treated with *g*NO-producing probiotic patches or treated with vehicle control patches at days 1, 13, and 20 post-surgery. Wound healing was monitored daily and photographic records were kept for computer aided morphometric analysis.

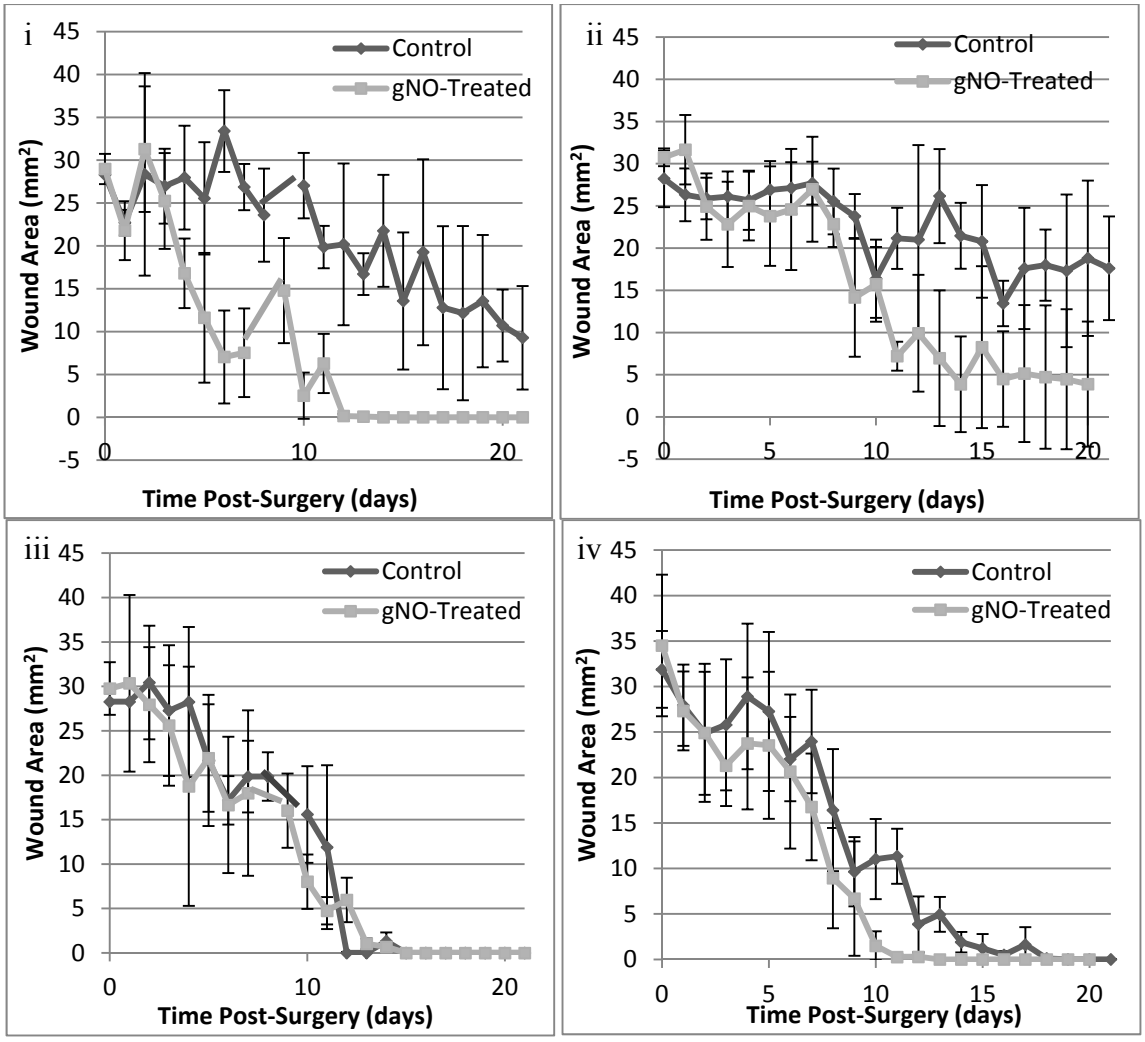
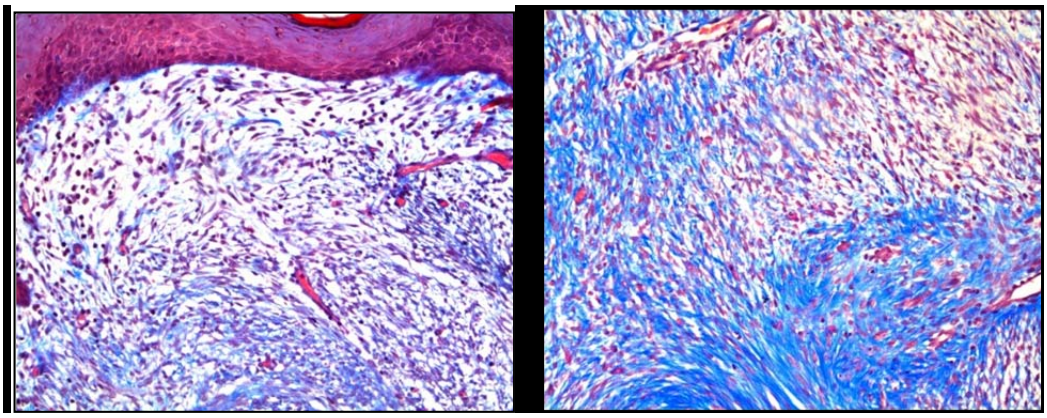
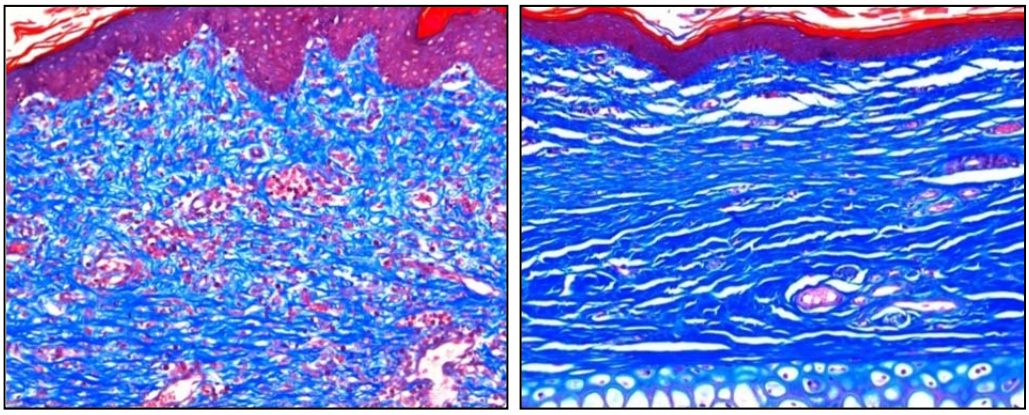


Figure 5.8.5. Effect of treatment with a *g*NO-producing probiotic patch compared to a vehicle control patch under four experimental conditions as seen by daily morphometric analysis: (i) ischemic, non-infected wounds treated with active *g*NO-producing probiotic patches versus vehicle control patches (ii) ischemic, infected wounds treated with active *g*NO-producing probiotic patches versus vehicle control patches; (iii) non-ischemic, non-infected wounds treated with active *g*NO-producing probiotic patches versus vehicle

control patches); (iv) non-ischemic, infected wounds treated with active *g*NO-producing probiotic patches versus vehicle control is shown Error bars represent one standard deviation from the mean.



A B



C D

Figure 5.8.6. Representative Masson's Trichrome stained sections for the evaluation of wound healing, inflammation, and maturity by probiotic patch. A: section showing with no granulation tissue and almost absent staining for collagen (Grade 1). B: shows loose granulation tissue with low collagen (Grade 2). C: dense granulation tissue with a high

concentration of disorganized collagen (Grade 3). D: dense granulation tissue with bundles of dermal-type collagen (Grade 4).

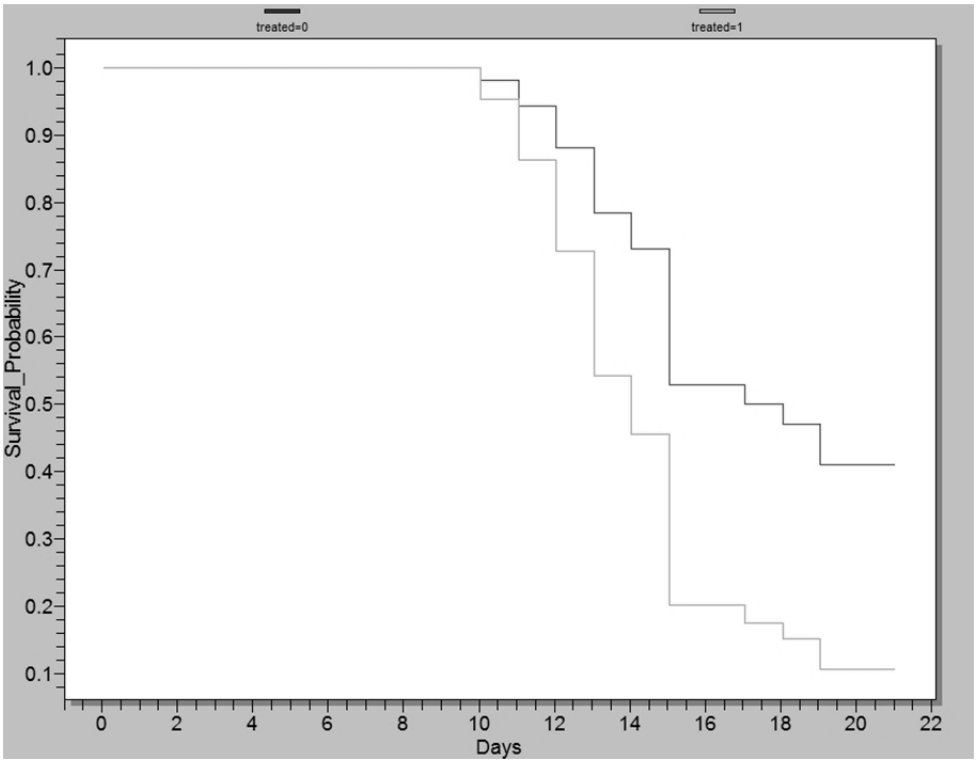


Figure 5.8.7. Cox proportional hazard regression plot investigating likelihood (survival probability) of wound closure over time in *g*NO-producing patch treated wounds versus wounds treated with vehicle control patches independent of co-variables. The data shows that *g*NO-producing probiotic patch treated wounds are 2.52 times more likely to close compared to wounds treated with vehicle control patches (Hazard P=0.0375, Score P=0.032, and Likelihood ratio P=0.0355) and that several non-treated wounds remained open. The light line represents *g*NO-producing probiotic patch treated wounds (16 wounds) and the dark gray line represents wounds treated with vehicle control patches (16 wounds). The data was graphed using EpiInfo software (CDC, USA).

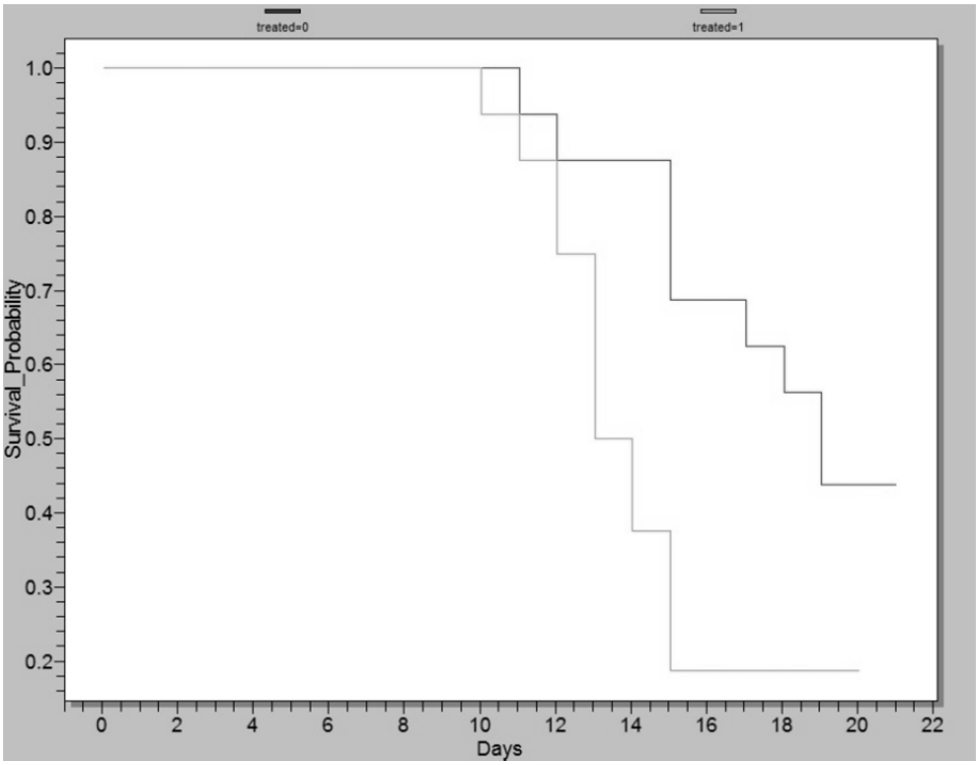


Figure 5.8.8. Kaplan-Meier plot showing the likelihood (survival probability) of wound closure in wounds treated with *g*NO-producing probiotic patches versus wounds treated with vehicle control patches. The data shows that *g*NO-producing probiotic patch treated wounds closed before wounds treated with vehicle control patches (Log-Rank P=0.0225, and Wilcoxon P=0.0113) and that several non-treated wounds remained open. The light gray line is *g*NO-producing probiotic patch treated wounds (16 wounds) and the dark gray line represents vehicle control treated wounds (16 wounds). The data was graphed using EpiInfo software (CDC, USA).

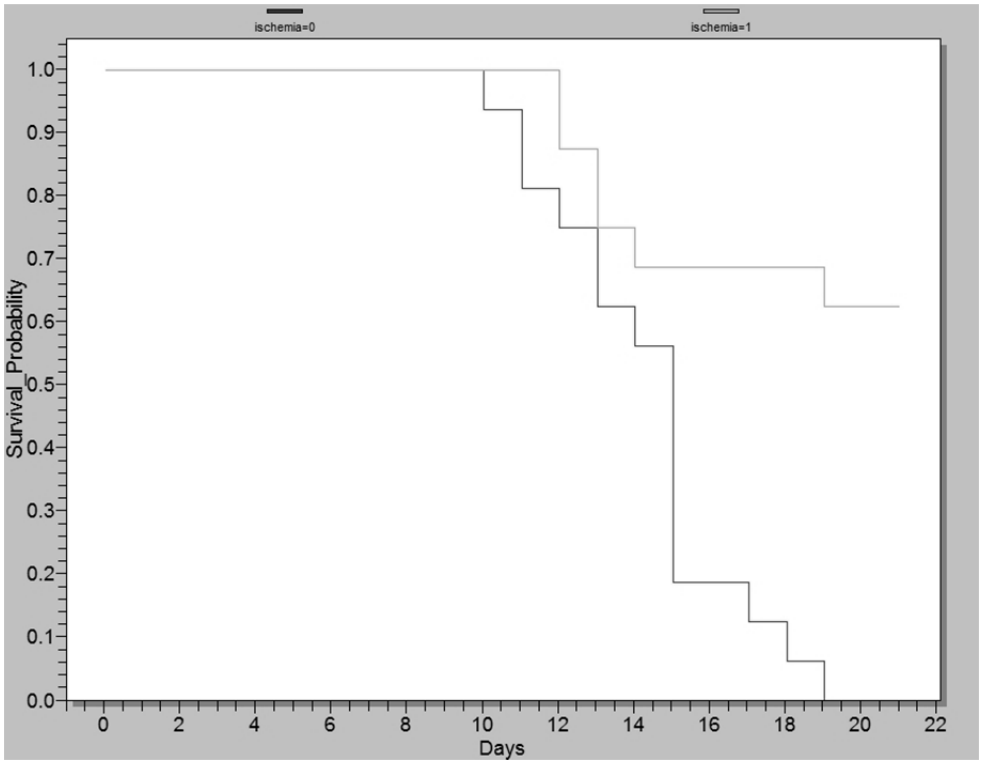


Figure 5.8.9. Kaplan-Meier plot showing the likelihood (survival probability) of wound closure over time for ischemic wounds versus non-ischemic wounds. The data shows that ischemia delays wound closure (Log-Rank P=0.005, Wilcoxon P=0.0056) and that some ischemic wounds remain open at 21 days. The light grey line represents ischemic wounds (16 wounds), whereas the dark grey line represent non-ischemic (16 wounds). The data was graphed using EpiInfo software (CDC, USA).

Original Research Article 4

CHAPTER 6: NITRIC OXIDE PRODUCING NITRATE REDUCTASE (NIR) ACTIVE PROBIOTIC BACTERIA FOR ORAL THERAPY

*Mitchell Jones, MD; Jorge G. Ganopolsky, PhD; and Satya Prakash, PhD **

Preface: Chapters 3-5 investigate the use of probiotic bacteria for the design of *g*NO-producing devices for topical application. During this line of research it was hypothesized that probiotic bacteria could be selected for their ability to produce *g*NO when delivered orally for potential therapeutic applications such as: hypertension, inflammatory bowel disease, gastric ulcers, diabetes and thrombosis. To investigate this research hypothesis, this chapter examines the selection of probiotic bacteria for the ability to reduce dietary nitrate *in-vitro,* using different substrate and nitrate sources, microencapsulated or delivered free, under simulated GI conditions, and in the presence of various food matrices.

Manuscript not yet submitted. (In preparation)

* Corresponding Author

Contribution of Authors: I would like to acknowledge laboratory assistance and editing of my co-author Jorge Gabriel Ganopolsky.

6.1. Abstract

Here we report novel orally administered nitric oxide-producing probiotics, that produce gaseous nitric oxide (*g*NO) using inorganic nitrate or organic nitrate as substrate, which could be used to treat, ameliorate, or prevent hypertension (HTN), inflammatory bowel disease (IBD), gastric ulcers, diabetes mellitus type II (DMII) and reduce the risk of thrombosis. Several probiotic organisms were screened for *g*NO production and microencapsulated or free *g*NO producers were chosen for further study. As probiotics can be ingested free or in combination with a food product, a diary product, or juices, or with encapsulated nitrates that allow for slow release of the NO donor in the lower gastrointestinal tract, *g*NO-producing capacity was determined in simulated GI conditions and with various food matrices.

6.2. Introduction

MetS is a common condition that identifies clinical symptoms and biochemical findings, including abdominal obesity, insulin resistance[237], hyperglycaemia, hyperlipidemia, and hypertension, that lead to an increased risk of cardiovascular disease (CVD)[238]. The Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (ATP III) highlights the importance of treating patients with MetS to prevent CVD and defines it as having at least three of the following[239]: waist circumference ≥ 102 cm or 40 inches (male), ≥ 88 cm or 36 inches(female); TG ≥ 1.7 mmol/L (150 mg/dl); HDL-C <

40 mg/dL (male), < 50 mg/dL (female); blood pressure ≥ 130/85 mmHg; and fasting plasma glucose ≥ 6.1 mmol/L (110 mg/dl). In the US population, the unadjusted and age-adjusted prevalence of MetS are 21.8% and 23.7% respectively, with the highest prevalence occurring in Spanish Americans (31.9%) and older individuals (60-69 years old) (43.5%)[240]. The age-adjusted prevalence of MetS is similar for men (24.0%) and women (23.4%)[241]. Using 2000 census data, about 47 million US residents have MetS, demonstrating the serious implications the disease has on the health care sector[242, 243].

The exact etiology and pathophysiology of MetS is yet to be fully elucidated; however, the development of MetS strongly linked to body fat. Excess abdominal fat leads to excess free fatty acids in the portal vein, increasing fat accumulation in the liver and muscle cells causing insulin resistance and hyperinsulinemia[244]. Glucose metabolism is impaired, and dyslipidemias and hypertension develop. In addition, serum uric acid levels are typically elevated[97] and a prothrombotic state leading to the increased levels of fibrinogen and plasminogen activator inhibitor I and other factors leading to an inflammatory state causing MetS. Other risk factors include non-alcoholic steatohepatitis, chronic kidney disease, polycystic ovary syndrome in women and low plasma testosterone in men[245].

Current clinical management of MetS includes advising patients on healthy lifestyle choices, including improved diet and exercise, and treating the signs and symptoms with a combination of prescription medications[246]. No single drug formulation exists that can prevent or treat MetS. Given limitations of presently available preventative techniques and therapies, an opportunity exists for the development of a safe, effective, and economical formulation for the treatment of MetS. To be efficacious,

a formulation must address the broad spectrum of clinical symptoms and biochemical findings while assuring compliance to therapy.

Nitric oxide (NO) is a free radical that plays a role as an autocrine/paracrine and endocrine messenger in a variety of tissues, regulating cellular processes[247]. In particular, NO initiates a signal transduction pathway in the endothelium, regulating vascular tone and blood pressure, modulating of hemostasis and proliferation of vascular smooth muscle cells[248]. NO signalling in leukocytes attenuates inflammatory responses through regulation of cytokine expression. In the gastrointestinal tract, NO not only functions as a protective agent against early inflammatory insults but also as a protector of a normal, intact mucosal barrier[249, 250]. NO is synthesized in vivo by a family of NO synthases (NOS) from arginine as a donor. Whereas endothelial NOS (eNOS) and neuronal NOS (nNOS) are responsible for constitutive NO synthesis in endothelial cells, and neurally associated cells, there is an inducible form (iNOS) found in epithelial, endothelial and inflammatory cells, whose expression is up regulated by cytokines, microbes or bacterial products.

There is vast evidence of the physiologic role of NO in vascular diseases such as hypertension[251], portal hypertension[252], in platelet activation during thrombotic events[253], in atherosclerosis[254], as well as in inflammatory bowel diseases[255] including ulcerative colitis, Crohn's disease, collagenous colitis, lymphocytic colitis, ischemic colitis, diversion colitis, Behçet's syndrome, and infective colitis. In vascular diseases, NO functions as a vasodilator, and retards atherogenesis by inhibiting inflammatory cell recruitment and platelet aggregation[253]. In intestinal inflammation, administration of NO protects the mucosa by maintaining blood flow, reducing intestinal epithelial

permeability, inhibiting platelets and leucocyte adhesion and aggregation, down regulating mast cell reactivity, and modulating oxidative stress[31, 256-259]. In addition, NO may alleviate inflammatory bowel disease due to its antimicrobial activity against infectious bacteria such as *E. coli*, *Salmonella*, or *Shigella*. One of the plausible mechanisms of antimicrobial activity of NO involves the interaction of this free radical (and a reactive nitrogen intermediate) with reactive oxygen intermediates, such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) to form a variety of antimicrobial molecular species. In addition to NO itself, these reactive antimicrobial derivatives include, S-nitrosothiols (RSNO), nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), and dinitrogen tetroxide (N_2O_4)[260].

NO could be also generated in the gastrointestinal system of mammals from inorganic nitrate and nitrite by the action of commensal bacteria[15]. Under anaerobic conditions, oral bacteria presenting nitrate reductase activity can generate nitrite anions from nitrates present either in saliva or ingested. The produced nitrite will become NO once it reaches the stomach due to the acidic environment. Consequently, NO will enter the blood stream through the mesenteric circulation and get reconverted to nitrate, which could be then eliminated by urine, or get recycled to the saliva where it is concentrated and becomes available for further NO generation[261]. Lactic acid bacteria can generate high amounts of NO from nitrites due to the acidification of the environment as a result of fermentation[262]. Production of NO in the gastrointestinal tract from nitrites and nitrates was observed in the presence of probiotics such as lactobacilli and bifidobateria[263]. Finally, ammonia-oxidizing bacteria such as nitrosomonas produce NO from ammonia and hydroxylamine.

The ingestion of fruits and vegetables rich in nitrates and nitrites, such as leafy greens or beet root may benefit health by the blood pressure lowering effects of the Dietary Approaches to Stop Hypertension (DASH) diet[264, 265]. In fact, oral administration of nitrates increases mucosal blood flow and mucosal defense in a rat model of gastric injury[266]. In addition, dietary nitrates and nitrites lower blood pressure and present gastro protective effects in a rat model of gastric injury[267]. These results were abolished by spraying the rats with an antibacterial mouth wash, indicating that oral bacteria are indispensable for the beneficial effects of NO derived from dietary nitrates. Moreover, a study in humans showed that provision of a nitrate-rich drink such as beet root juice significantly reduced blood pressure and had vasoprotective and antiplatelet properties when compared to the individuals that were treated with water controls[268].

As mentioned above, nitrate conversion to nitrite is achieved in the mouth by the action of resident bacteria. The oral delivery of probiotics could be utilized as a method to colonize the gastrointestinal tract, and thus facilitate and improve the conversion of dietary nitrates not only in the mouth but in the whole digestive system. The alginate/poly-L-lysine microencapsulation of probiotics is a technology used to immobilize and protect bacteria from harsh conditions while allowing the free transport of nutrients and waste products with the milieu. Such technology is suitable to deliver probiotics through the acidic stomach environment, preserving viabilities and allowing further colonization of the lower gastrointestinal tract. The administration of nitrate-reducing free or microencapsulated probiotics, in combination with a nitrate rich diet, presents a potential opportunity to generate physiologically relevant amounts of NO in the upper and lower gastrointestinal tract. This invention can thus provide health benefits

to individuals suffering or with a propensity of developing cardiovascular or inflammatory bowel diseases.

6.3. Materials and Methods

Bacterial seeding and growth:

The surfaces of frozen glycerol bacterial stocks were scratched with a sterile wooden stick to streak MRS agar plates. After an overnight incubation at 37˚C under anaerobic conditions, a single colony was picked with a metallic loop under sterile conditions and transferred into a tube containing 10 mls of MRS. The cultures were incubated overnight at 37˚C for experimental use.

Preparation of APA microcapsules:

Selected bacteria were incubated in 900 ml Beckmann bottles and grown overnight at 37 C in MRS. The following day, the bacterial cultures were centrifuged and the supernatants were discarded. The pellets were weighed and resuspended in saline solution (8.5 g/l sodium chloride) and sodium alginate solution (2 % W/V in saline) to obtain a final bacterial load of 8% W/V in 1.75% sodium alginate. The resulting solution was dispensed through a 400 μM nozzle using a microencapsulator to allow for electromagnetic separation and dispersion of micro droplets that were collected in a calcium chloride solution (0.1M). The produced microbeads were washed for 15 minutes in the calcium chloride solution, and then transferred into a meshed bag and washed in a saline solution. The microencapsulation finished by consecutive 15 minute-incubations of

the beads under continuous stirring, first in a poly-l-lysine solution (0.1%), then in saline, then in a sodium alginate solution (0.1%), and finally in saline. The microcapsules were then stored in 10 % MRS at 4˚C.

Growth media:

Nitrate reductase medium contains glucose (10%), casein hydrolasate (1%), KNO_3 10 mM, $NaNO_3$ (20 mM), $MgSO_4$ (1mM), NH_4Cl (30 mM), Na_2MoO_4 (0.5 mM), and Ferric Citrate (30 μM). Food component medium contains starch (3 g/l), pectin (2 g/l), mucin (4 g/l), arabinogalactan (1 g/l), xylan (1 g/l), yeast extract (3 g/l), peptone (1 g/l), glucose (0.4 g/l), and cysteine (0.5 g/l).

Gastrointestinal simulated conditions:

To simulate stomach conditions, the pH of the bacterial growth medium was adjusted to pH 2.0 with 1 M HCl and the bacterial cultures were allow to incubate for 120 min at pH 2.0 at 37 C. To simulate intestinal conditions, the pH of acidified food/bacterial suspension was readjusted 6.5 with 0.5 M NaOH. After the pH of 6.5 was reached, the suspension was supplemented with a ¼ volume of pancreatic juice (bovine bile (6.0 g/l), pancreatin (0.9 g/l), sodium bicarbonate (12.6 g/l)).

NO determinations:

For experiments with free bacteria, 2 ml-HPLC vials were filled with 900 μl of MRS/ sodium nitrate (30 mM), or nitrate reductase medium, or food component medium containing 30 mM sodium nitrate. Overnight grown bacterial cultures (100 μl) were

transferred to the HPLC tubes which were tightly closed with septum caps. For experiments with encapsulated bacteria, 4 ml-HPLC vials were filled with 1.4 ml of MRS/sodium nitrate (30 mM), or nitrate reductase medium, or MRS supplied with a $1cm^2$ nitro-glycerine patch (GTN), or food component medium supplied 30 mM sodium nitrate or fruit juices. Wet APA microencapsulated bacteria (0.6 g), were transferred to the tubes which were then tightly closed with septum caps. Cultures of both free and encapsulated bacteria were incubated at 37˚C during the whole experiment.

A known volume of the head gas was sampled periodically from the septum of assay tube with a Hamilton syringe and *g*NO content was determined by chemiluminescence with an NO analyzer (Sievers).

6.4. Results

6.4.1. Production of *g*NO by free lactic acid bacteria

The production of *g*NO by lactic acid bacteria (LAB) in the presence of nitrate was analyzed at different time points. Several strains of *Lactobacillus fermentum* and *Lactobacillus reuteri* have been identified as positives for *g*NO production under these conditions 2-to-3 hours after inoculation and up to 24 hours as shown in figure 6.6.1. The production of *g*NO by the positive strains was verified in the presence of nitrate in nitrate reductase medium as shown in figure 6.6.2. To analyze whether the selected LAB strains are able to produce significant amounts of *g*NO in the presence of other NO donors, *g*NO generation was analyzed in MRS containing glyceryl trinitrate (GTN). All the identified

strains produced very significant amounts of *g*NO with GTN as a donor from 2 hours after inoculation as seen in figure 6.6.3. The generation of *g*NO by the positive strains in the presence of nitrate was also tested in a food simulate containing a variety of components in the proportions found on a typical diet. All of the positive LAB produced significant quantities of *g*NO as early as 2 hours after inoculation as seen in figure 6.6.4.

6.4.2. Production of *g*NO by encapsulated bacteria

The production of *g*NO from sodium nitrate was evaluated in the presence of selected LAB strains that have been microencapsulated in alginate/poly-l-lysine/alginate (APA) as described in the materials and methods. Significant levels of the *g*NO were produced when the microcapsules were tested in MRS (figure 6.6.5) or in food component simulate medium (figure 6.6.6).

6.4.3. Production of *g*NO from a nitrate source by encapsulated bacteria under gastrointestinal conditions

The production of *g*NO by selected encapsulated LAB was evaluated in MRS/nitrate, nitrate reductase medium, or beet root juice under different gastrointestinal conditions. To simulate stomach conditions, the pH of the suspension was adjusted to 2.0 under anaerobic conditions, after 2 hours of anaerobic growth of the capsules. For intestinal conditions, after 2 hours at stomach environment, the pH of the suspension as readjusted to 6.5, and a solution containing bile salts and pancreatin under anaerobic

conditions. In MRS containing sodium nitrate (30 mM) significant *g*NO production was observed for *Lactobacillus fermentum* 23 after 4 hours and up to 24 hours with no pH adjustments (figure 6.6.7A). Under stomach conditions, very significant production of *g*NO was observed for every strain except for *Lactobacillus fermentum* 38 (figure 6.6.7B). Under simulated, gastrointestinal conditions (2 hours pH 2, then pH 6.5 + pancreatin and bile salts), the NO production dropped 5 hours after inoculation but increased significantly by 24 hours for strains *Lactobacillus fermentum* 22, *Lactobacillus fermentum* 23, and *Lactobacillus fermentum* 38 (figure 6.6.7C). In nitrate reductase medium (sodium nitrate 30 mM) under simulated stomach conditions, *Lactobacillus fermentum* 23 and *Lactobacillus reuteri* 64 generated significant amounts of NO 2 hours after inoculation and the *g*NO production reached a maximum 4 hours after inoculation (figure 6.6.8A). NO production decreased initially after exiting stomach conditions due to the loss of acidity, but kept increasing for at least 24 hours after inoculation for all the strains. Under these conditions, NO production was maximal for *Lactobacillus reuteri* 64 (figure 6.6.8B). Finally, microencapsulated LAB were tested for NO production in beet root juice under different simulated gastrointestinal conditions. Under no pH adjustments, NO production was very significant for *Lactobacillus fermentum* 23 and *Lactobacillus reuteri* 64 with a maximum at 6 hours after inoculation (figure 6.6.9A). Under simulated stomach conditions, the generation of *g*NO was very significant for *Lactobacillus reuteri* 64 with a maximum at 6 hours after inoculation and lasted for at least 24 hours under these harsh pH conditions (figure 6.6.9B). Under simulated gastrointestinal conditions, NO production was very significant for *Lactobacillus reuteri* 64 for up to 5 hours after inoculation.

6.5. Discussion

Several strains of *Lactobacillus* were identified as capable of producing gNO for several hours after inoculation in nitrate reductase media and for up to 24 hours. The production of *g*NO did not occur in the absence of nitrate; thus, it was hypothesized that the strains produced gNO due to an intrinsic ability to reduce nitrate to nitrite and through the further dismutation of nitrite to *g*NO. This capacity is likely due to the enzymatic activity of a nitrate reductase enzyme (NiR). Strains capable of producing *g*NO in the presence of nitrate were also found to produce significant amounts of *g*NO in the presence of another nitric oxide donor glyceryl trinitrate (GTN), indicating that the ability to reduce other nitric oxide donors was possible as well. Despite the presence of a more complex and more physiologically relevant food simulate media, it was found that all positive LAB produced significant quantities of *g*NO indicating that the probiotic bacteria may be used orally to produce *g*NO in the complex milieu of the human GI.

As the reduction of nitrate to nitrite requires anoxic conditions, and probiotic bacteria are sensitive to upper GI conditions, the production of *g*NO from sodium nitrate was evaluated for alginate/poly-l-lysine/alginate (APA) microencapsulated strains. All microencapsulated strains proved capable of producing significant quantities of *g*NO in MRS media. Thus, APA microencapsulation may present an ideal solution for the protection of the strain and enzymatic machinery from storage conditions and the harsh upper GI conditions while maintaining the capacity to produce *g*NO from salivary and dietary inorganic nitrate.

Finally, several microencapsulated strains were shown to produce *g*NO in simulated gastric and intestinal conditions. Under gastric conditions, significant production of *g*NO was observed for every strain except for *L. fermentum* 38. Once placed in simulated intestinal conditions, *L. fermentum* 22, *L. fermentum* 23, and *L. fermentum* 38 produced *g*NO well for 24h. In nitrate reductase medium and under simulated gastric conditions, *L. fermentum* 23 and *L. reuteri* 64 generated significant amounts of *g*NO; however, *g*NO production was decreased after exiting stomach conditions, most likely due to the loss of acidity, but despite this loss in activity the strains produced *g*NO for at least 24 hours after inoculation. Under these conditions, NO production was maximal for *L. reuteri* 64. Finally, in the presence of a natural source of inorganic nitrates, beet root juice, and under simulated gastric conditions, the generation of *g*NO was significant for *L. reuteri* 64 making it an excellent choice for the generation of *g*NO from dietary sources of nitrate in the conditions of the upper GI.

The results presented indicated that novel NiR-active probiotic bacteria can be selected for nitrate reductase (NiR) activity *in-vitro,* using different substrate and nitrate sources, and NiR activity can be maintained when microencapsulated or delivered free. Novel NiR-active probiotic bacteria can be selected for the ability to produce *g*NO *in-vitro*, under simulated GI conditions, and in the presence of various food matrices confirming the lab scale feasibility of the approach *in-vitro* and providing a starting point for *in-vivo* studies aimed at proving the efficacy of this potential therapeutic for treating hypertension, inflammatory bowel disease, gastric ulcers, diabetes and thrombosis.

6.6 Figures

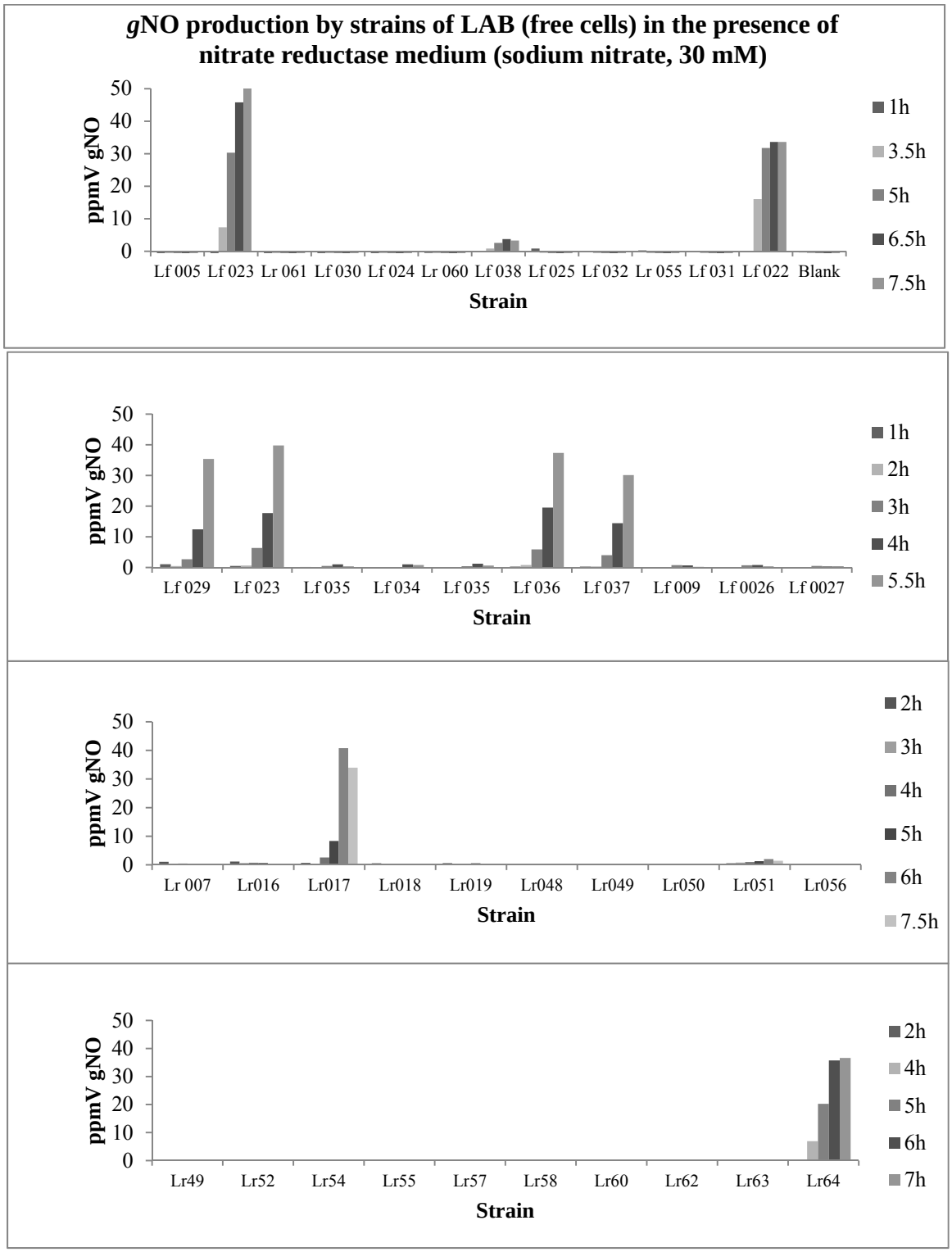

gNO production by strains of LAB (free cells) in the presence of nitrate reductase medium (sodium nitrate, 30 mM)
ppmV gNO
50
40
30
20
10
0
Lf 005 Lf 023 Lr 061 Lf 030 Lf 024 Lr 060 Lf 038 Lf 025 Lf 032 Lr 055 Lf 031 Lf 022 Blank
Strain
1h
3.5h
5h
6.5h
7.5h
ppmV gNO
50
40
30
20
10
0
Lf 029 Lf 023 Lf 035 Lf 034 Lf 035 Lf 036 Lf 037 Lf 009 Lf 0026 Lf 0027
Strain
1h
2h
3h
4h
5.5h
ppmV gNO
50
40
30
20
10
0
Lr 007 Lr016 Lr017 Lr018 Lr019 Lr048 Lr049 Lr050 Lr051 Lr056
Strain
2h
3h
4h
5h
6h
7.5h
ppmV gNO
50
40
30
20
10
0
Lr49 Lr52 Lr54 Lr55 Lr57 Lr58 Lr60 Lr62 Lr63 Lr64
Strain
2h
4h
5h
6h
7h

Figure 6.6.1. The production of *g*NO by different strains of lactic acid bacteria (LAB) in the presence of nitrate reductase medium as measured at different time points. Some strains generate significant amounts of NO after 2-to-3 hours. Lf = *Lactobacillus fermentum* and Lr = *Lactobacillus reuteri*.

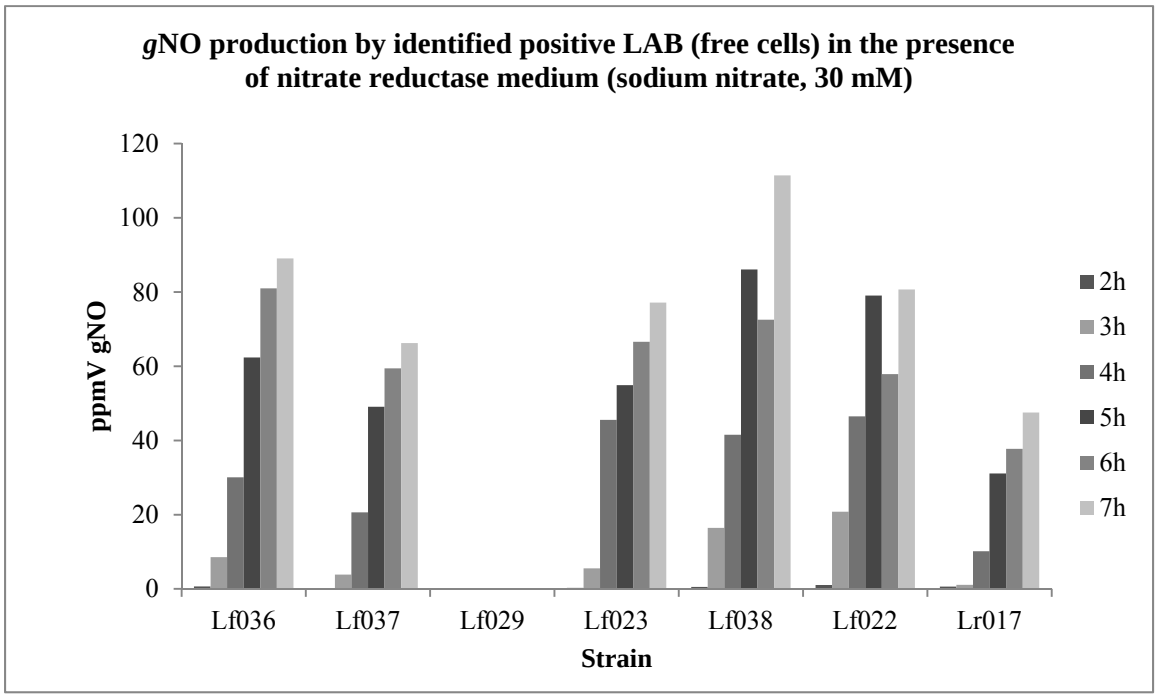


Figure 6.6.2. Production of gaseous nitric oxide (*g*NO) in the presence of nitrate reductase medium was evaluated on previously identified positive strains. Lf = *Lactobacillus fermentum* and Lr = *Lactobacillus reuteri*.

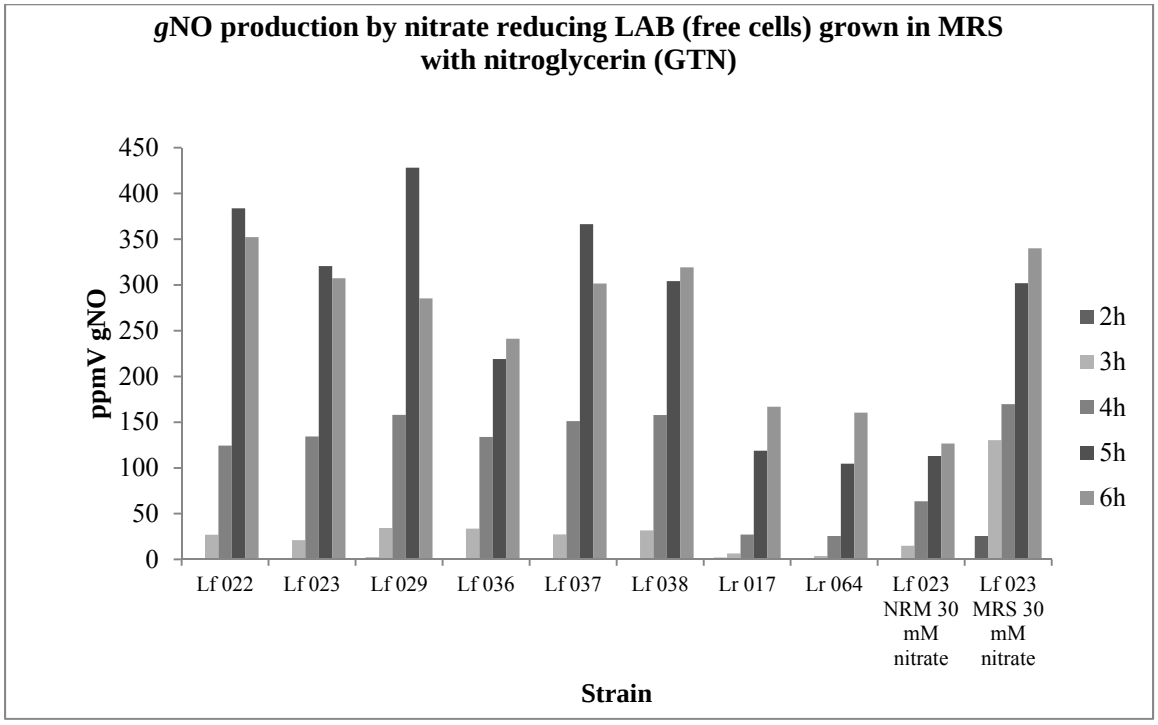


Figure 6.6.3. LAB strains previously identified as positive for *g*NO production in the presence of nitrate were tested for *g*NO production in MRS supplied with a 1cm^2 nitro-glycerine patch. These strains produced very significant levels of *g*NO under these conditions. Lf = *Lactobacillus fermentum* and Lr = *Lactobacillus reuteri*.

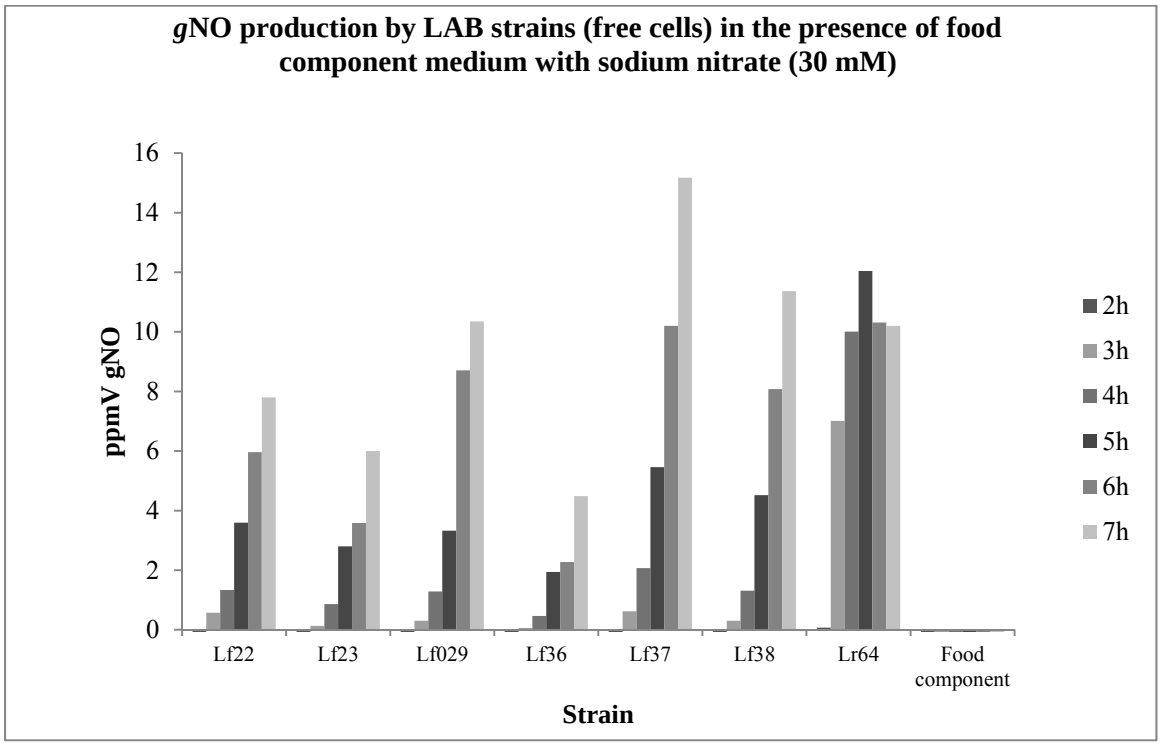


Figure 6.6.4. Previously identified positive LAB strains (free cells) were tested for *g*NO production in the presence of a food simulate medium supplied with 30 mM sodium nitrate. All the strains generated significant amounts of *g*NO over time starting 2-to-3 hours after inoculation. Lf = *Lactobacillus fermentum* and Lr = *Lactobacillus reuteri*.

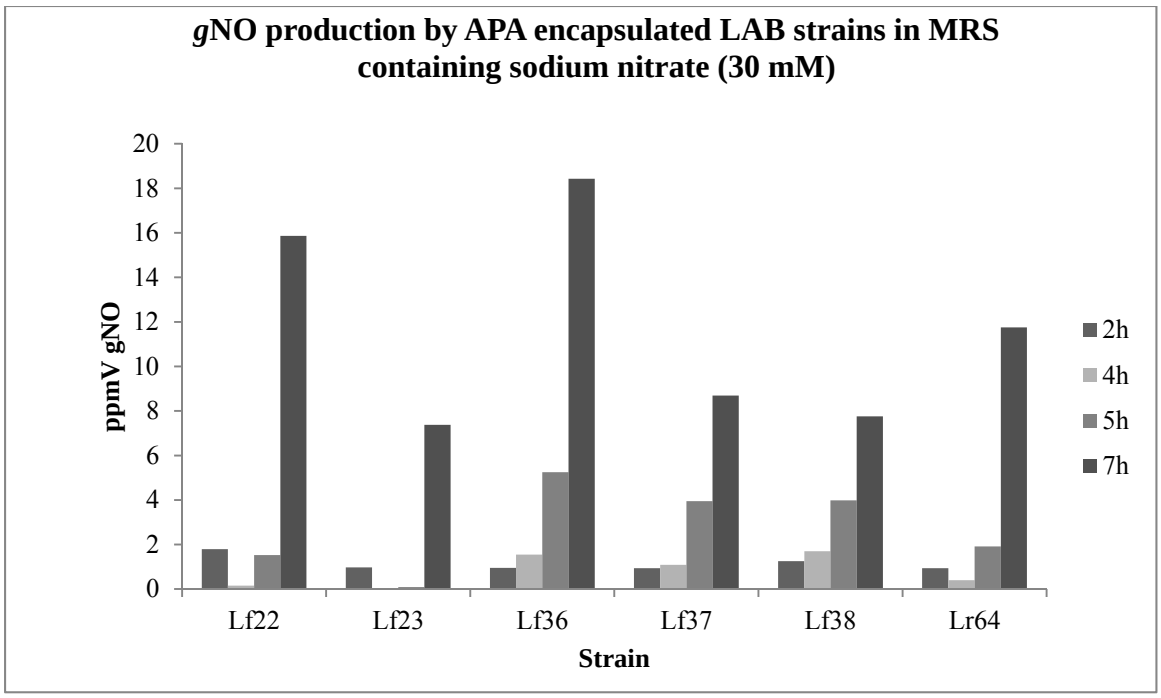


Figure 6.6.5. LAB strains previously identified as positive for *g*NO production from nitrate were microencapsulated in APA microcapsules. Generation of *g*NO by microencapsulated LAB strains in MRS containing sodium nitrate (30 mM) was measured over time. Significant *g*NO was produced 2 hours after inoculation. Lf = *Lactobacillus fermentum* and Lr = *Lactobacillus reuteri*.

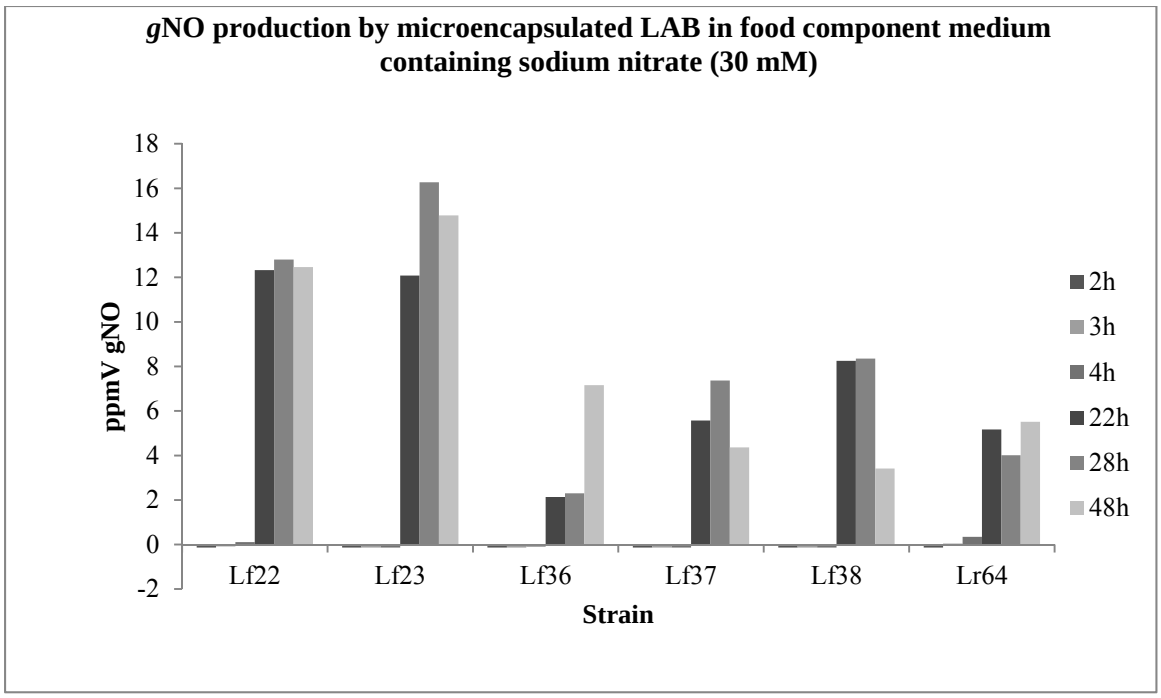


Figure 6.6.6. Microencapsulated LAB strains were tested for *g*NO production in a simulated food medium containing sodium nitrate (30 mM). Generation of *g*NO was significant up to 48 hours after inoculation. Lf = *Lactobacillus fermentum* and Lr = *Lactobacillus reuteri*.

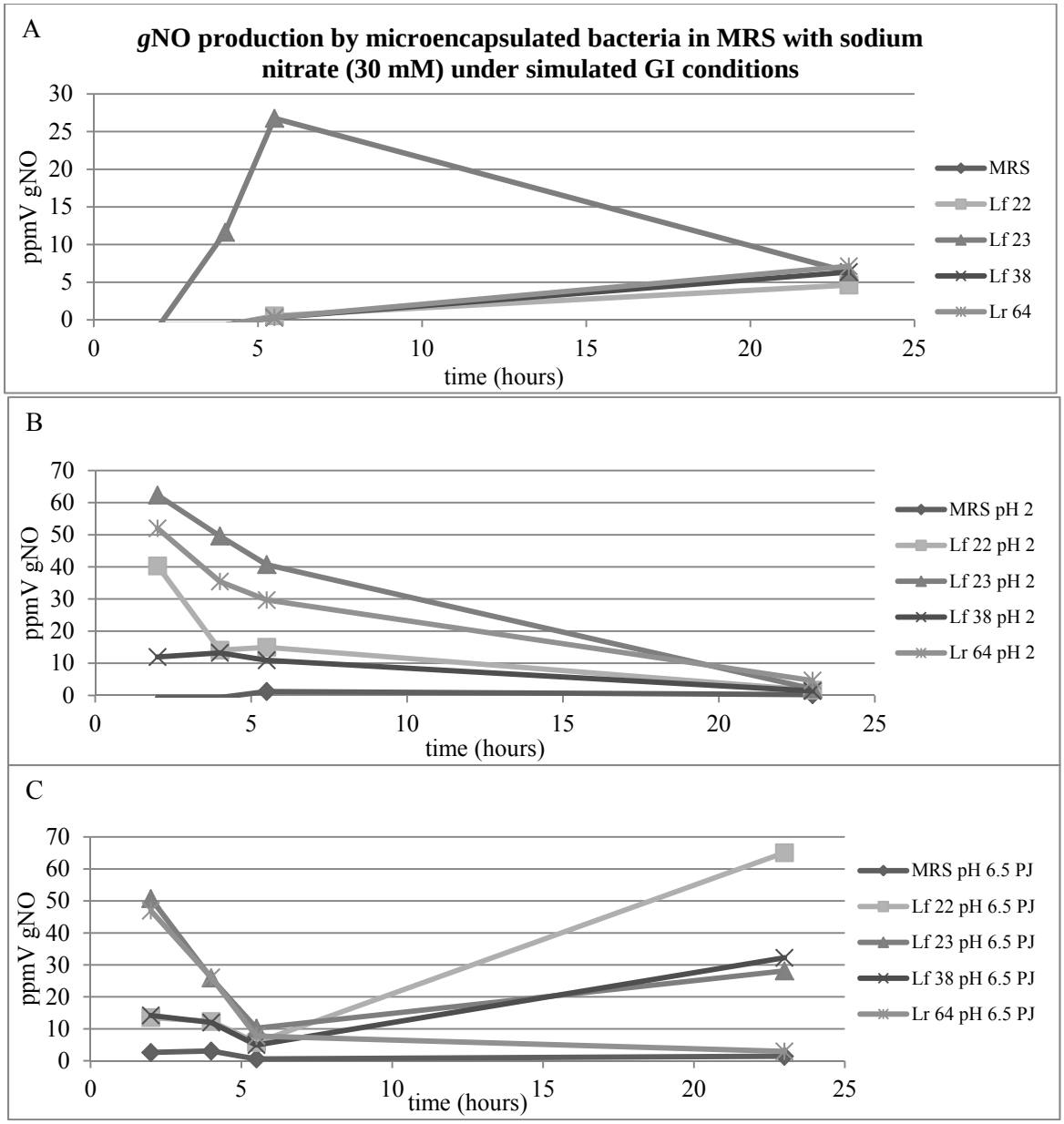


Figure 6.6.7. Microencapsulated LAB were tested for *g*NO production in MRS containing sodium nitrate (30 mM) under different gastrointestinal simulated conditions. A, No pH adjustments. Significant *g*NO production was observed for *Lactobacillus fermentum* 23 after 4 hours and up to 24 hours. B, simulated stomach conditions. Two hours after inoculation the pH was adjusted to 2 under anaerobic conditions. Very

significant production of NO was observed for every strain except for *Lactobacillus fermentum* 38. C, simulated full gastrointestinal conditions. Two hours after inoculation, the pH was adjusted to 2 under anaerobic conditions. Following two hours incubation, the pH was readjusted to 6.5 under anaerobic conditions and pancreatic juice simulate was added to the reaction container. The *g*NO production dropped 5 hours after inoculation but increased significantly by 24 hours for strains *Lactobacillus fermentum* 22, *Lactobacillus fermentum* 23, and *Lactobacillus fermentum* 38. Lf = *Lactobacillus fermentum* and Lr = *Lactobacillus reuteri*.

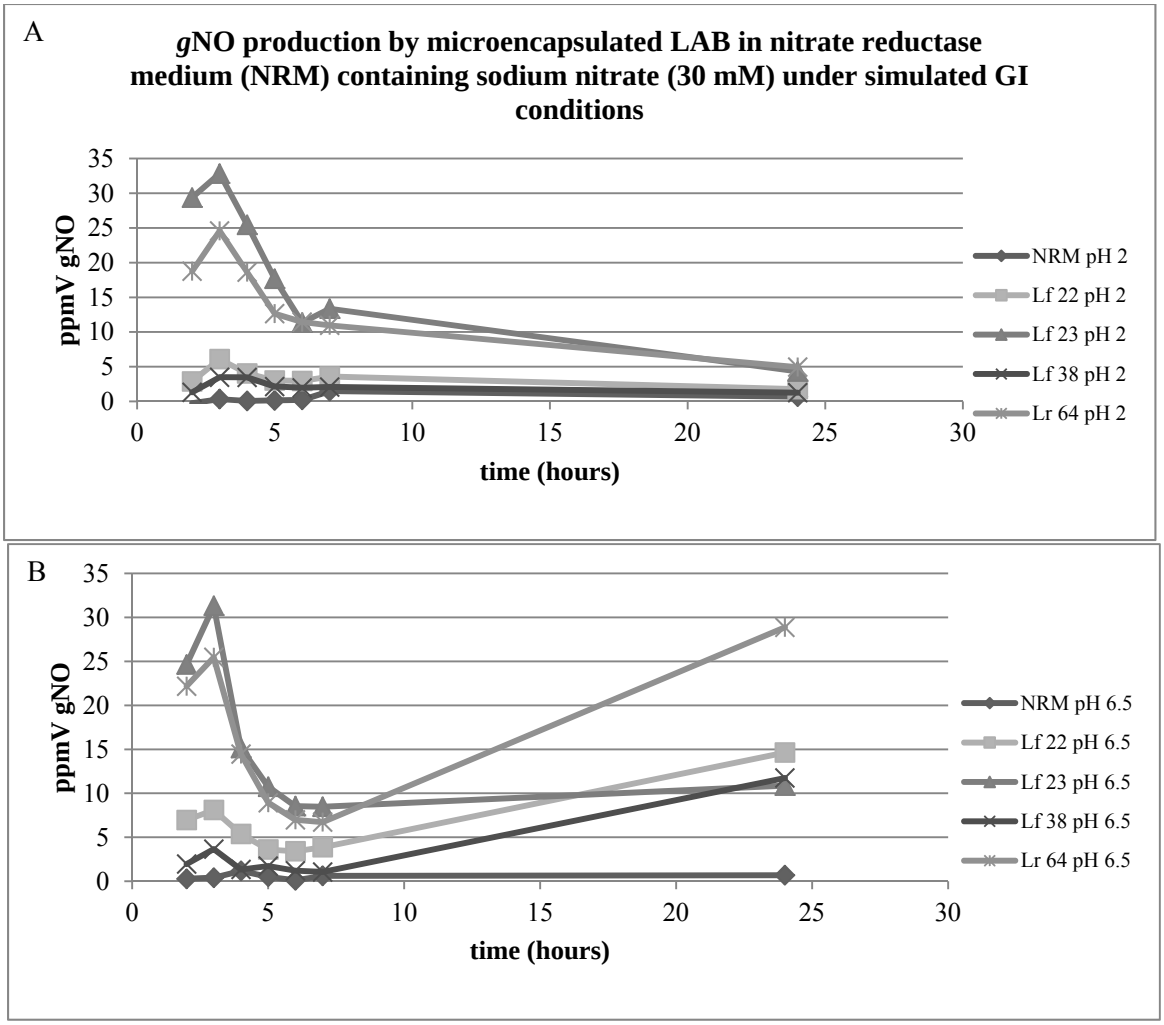


Figure 6.6.8. Microencapsulated LAB were tested for *g*NO production in nitrate reductase medium (sodium nitrate 30 mM) under different pH conditions. A, simulated stomach conditions. Two hours after inoculation the pH was adjusted to 2 under anaerobic conditions. *Lactobacillus fermentum* 23 and *Lactobacillus reuteri* 64 generated significant amounts of *g*NO 2 hours after inoculation. The *g*NO production reached a maximum 4 hours after inoculation. B, simulated gastrointestinal conditions. Two hours after incubation under stomach conditions, the pH was adjusted to 6.5 under anaerobic conditions. *g*NO production decreased initially after exiting stomach conditions due to the

loss of acidity, but kept increasing for at least 24 hours after inoculation for all the strains. Under these conditions, *g*NO production was maximal for *Lactobacillus reuteri* 64. Lf = *Lactobacillus fermentum* and Lr = *Lactobacillus reuteri*.

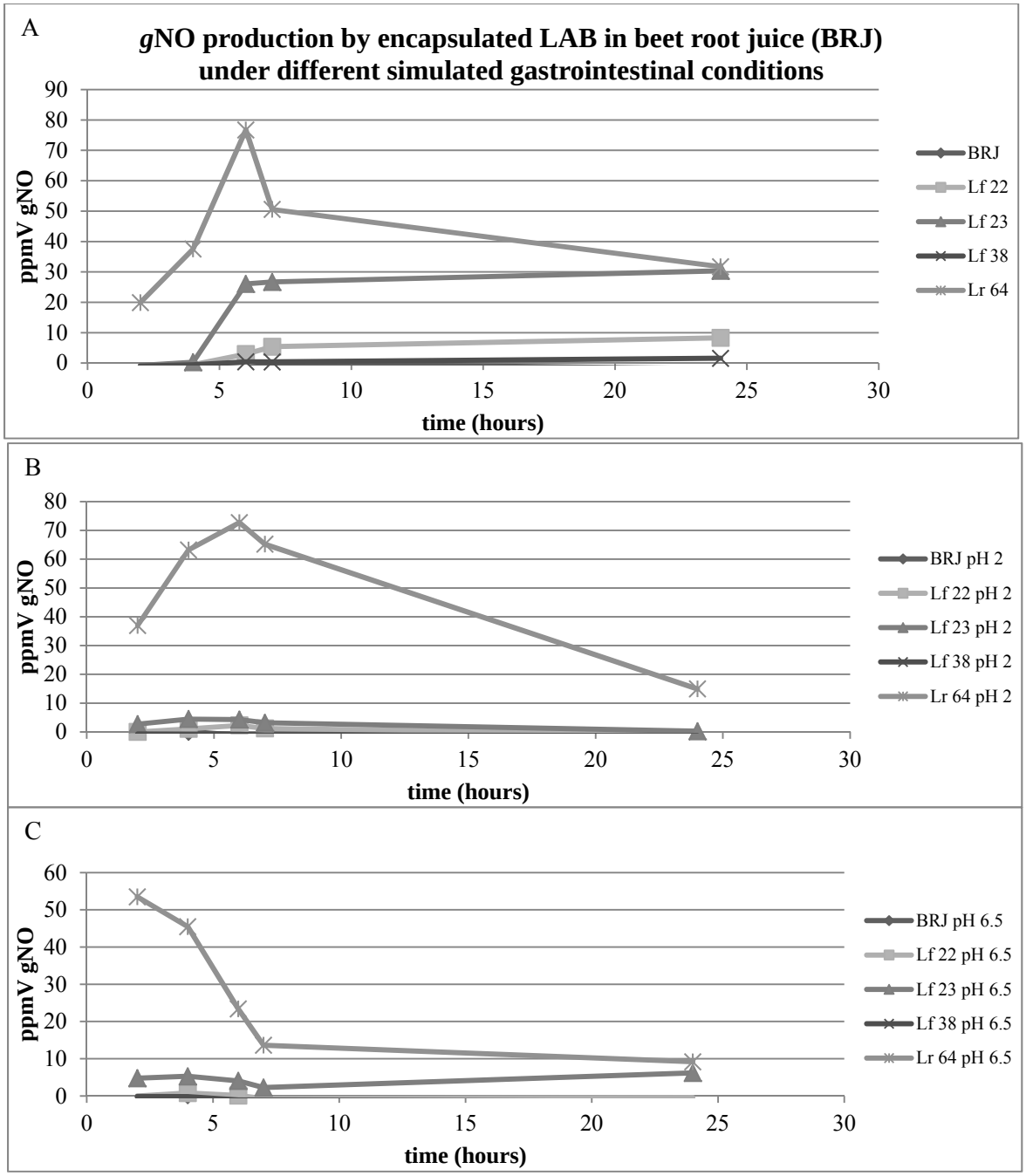


Figure 6.6.9. Microencapsulated LAB were tested for *g*NO production in beet root juice under different simulated gastrointestinal conditions. A, no pH adjustments. *g*NO production was very significant for *Lactobacillus fermentum* 23 and *Lactobacillus reuteri*

64 with a maximum at 6 hours after inoculation. B, simulated stomach conditions. The pH was adjusted to 2.0, 2 hours after inoculation. Generation of *g*NO was very significant for *Lactobacillus reuteri* 64 with a maximum at 6 hours after inoculation and lasted for at least 24 hours under these harsh pH conditions. C, simulated gastrointestinal conditions. The pH was adjusted to 2.0, 2 hours after inoculation for 2 hours and then readjusted to 6.5. *g*NO production was very significant for *Lactobacillus reuteri* 64 for up to 5 hours after inoculation. Lf = *Lactobacillus fermentum* and Lr = *Lactobacillus reuteri*.

CHAPTER 7: GENERAL DISCUSSION

7.1. General discussion

It is becoming increasingly clear that NO plays an important role in human specific and non-specific immunity, is an excellent broad spectrum antimicrobial agent, plays an important role in wound healing, and has a significant homeostatic role in the entero-salivary recirculation of nitrate regulating blood pressure and providing metabolic homeostasis. There is mounting evidence for endogenous NO production on skin, within the gut, and by the cellular immune system to protect against disease. Furthermore, the importance of systemic nitrate, nitrite, and nitrosylated compounds in blood is becoming better understood and the implications in maintaining homeostasis and preventing and fighting disease more apparent.

To address the need for devices and formulations that deliver *g*NO, treatment modalities using probiotic production of gaseous nitric oxide (*g*NO) applied topically for wound healing, antimicrobial, cosmetic, and dermatologic applications, as well as probiotic enzymatic production of *g*NO orally for metabolic disease applications were proposed. Microencapsulation or immobilization was used in some aspects to protect the biologic material, immobilize the active component, or improve handling and storage.

A review of the literature indicated that devices for the production and delivery of topical *g*NO utilizing the acidification of nitrite salts on skin, increasing endogenous production of NO from arginine, adsorbing the free gas under pressure, trapping *g*NO under pressure in silica metal chambers, and delivering free gas using a tank of compressed *g*NO have not yet overcome the design limitations including maintaining an anoxic environment, therapeutic concentrations, therapeutic durations and commercial

considerations. To address the need for topical devices for delivering *g*NO and address limitations of previous designs, probiotic patches, utilizing the metabolic activity of immobilized lactic acid bacteria (LAB), glucose, and nitrite salts, were designed to produce therapeutic levels of *g*NO (200ppmV) for therapeutic durations (24h).

To determine the antimicrobial efficacy of the *g*NO-producing probiotic patch device, patches were applied to several common bacterial and fungal pathogens growing on solid and in liquid media. It was demonstrated that the *g*NO-producing probiotic patches generated antimicrobial levels of *g*NO over therapeutically relevant periods and was antimicrobial against cultures of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Methicillin-Resistant Staphlococcus aureus (MRSA)*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum*. These results showed the novel use of probiotic bacteria in metabolizing glucose to lactic acid, generating protons, and dismutating nitrite salts for the production of *g*NO, confirmed the capacity of *g*NO to eradicate a wide spectrum of bacterial strains, and for the first time showed the use of low levels of *g*NO to kill fungal strains in liquid media. Thus, it was proposed that *in-vivo* studies demonstrating antimicrobial efficacy in wound healing were warranted to confirm the antimicrobial efficacy of *g*NO-producing probiotic patches, evaluate wound healing efficacy, and determine safety.

To determine the antimicrobial and wound healing efficacy of the *g*NO-producing probiotic patches, a New Zealand white rabbit model of ischemic and infected wounds was used in which ischemic wounds were surgically induced and wounds were infected with *S. aureus*. The efficacy of the probiotic *g*NO-producing patch on wound healing was measured by morphometric and time to closure analyses. Results indicated that wound

healing was significantly accelerated in ischemic wounds treated with *g*NO-producing probiotic patches. This accelerated closure was shown to be significant for both non-infected and infected ischemic wounds. Morphometric analysis did not reveal a significant effect of *g*NO on wound healing in non-ischemic wounds, both infected and non-infected. A non-significant improvement, however, was seen in treated ischemic and non-ischemic infected wounds when compared to vehicle control-treated wounds, whereas no such trend was evident in non-infected wounds. Thus, while morphometric analysis showed that ischemia plays a role in the pathophysiology of delayed wound healing, and that *g*NO-producing probiotic patches can be used to accelerate wound closure in infected or non-infected ischemic wounds, only a promising trend exists for treating non-ischemic infected wounds with *g*NO. However, based on the wide spectrum antimicrobial activity of nitric oxide, proven antimicrobial and antifungal activity of the probiotic patch, and known pathologic action of bacterial biofilms in slowing wound closure, a real difference may have been apparent in a larger population of wounds.

While reduced wound area over time was successfully revealed by morphometric analysis, the definitive endpoint for efficacy in chronic wound healing is complete wound closure. Thus, Log-Rank and Wilcoxon statistics for Kaplan-Meier survival probability plots and Cox proportional hazard regression analyses were performed to determine the increased or decreased likelihood of complete closure. Analysis of all wounds showed that treatment with *g*NO-producing probiotic patches significantly improved the likelihood of wound closure and that the presence of ischemia decreased the likelihood of closure. Also, analysis showed that ischemic wounds that were treated with *g*NO-producing probiotic patches were significantly more likely to close than vehicle control

patch-treated ischemic wounds. Finally, infected ischemic wounds were found less likely to close than non-infected ischemic wounds. These results corroborated those seen in analysis of morphometric data and strengthened the hypothesis that the pathophysiology of non-healing ischemic wounds is closely tied to a dysregulation of endogenous nitric oxide production and that the exogenous application of *g*NO may have a therapeutic effect.

Histological analyses showed improved scores for surface, epidermal and dermal observations and indicated a tendency towards improved wound healing in the *g*NO-producing probiotic patch treated wounds. These histological results provided growing support for the hypothesis that improved wound healing and maturity due to the topical application of *g*NO may be due to increased collagen deposition, increased stimulation of endothelial progenitor cells, stimulation of neovascularization, endothelial cell relaxation, and improved keratinocyte migration.

Determination of the safety and toxicity profile of the *g*NO-producing probiotic patches was performed by blood analysis showing no significant differences observed between *g*NO-producing probiotic patch treated and vehicle control patch treated animals in body weight, blood morphology, hematology, blood biochemistry, or methemoglobin levels. These results corroborate reports in the literature that the topical application and even inhalation of *g*NO is non-toxic and safe.

Thus, this study showed that *g*NO-producing probiotic patches can be designed and used for topical applications, including treating ischemic and infected full-thickness dermal wounds that approximate chronic non-healing ulcers, and that *g*NO-producing probiotic patches may provide an elegant solution to the obstacles to commercial

development. This study also provided further evidence that the dysregulation of NO may be intimately associated with the pathophysiology of chronic ischemic wounds and that exogenous application can accelerate wound healing. It was proposed that clinical efficacy should be evaluated.

While initial investigations utilized the metabolism of probiotic bacteria for the design of *g*NO-producing devices for topical application, it was also hypothesized that probiotic bacteria could be selected for their enzymatic capability to produce *g*NO from inorganic nitrates when delivered orally to prevent or treat hypertension, inflammatory bowel disease, gastric ulcers, diabetes, metabolic syndrome, and thrombosis. To investigate this research hypothesis, experiments were designed to select probiotic bacteria for the ability to reduce dietary nitrate *in-vitro*, using different substrate and nitrate sources, microencapsulated or delivered free, under simulated GI conditions, and in the presence of various food matrices.

Several strains of *Lactobacillus* were identified as capable of producing *g*NO in nitrate reductase media and for up to 24 hours. The production of *g*NO did not occur in the absence of nitrate; thus, it was hypothesized that the strains produced *g*NO due to an intrinsic ability to reduce nitrate to nitrite and through the further dismutation of nitrite to *g*NO. This capacity is likely due to the enzymatic activity of a nitrate reductase enzyme (NiR). Strains capable of producing *g*NO in the presence of nitrate were also found to produce significant amounts of *g*NO in the presence of another nitric oxide donor glyceryl trinitrate (GTN), indicating that the ability to reduce other nitric oxide donors was possible as well. Despite the presence of a more complex and more physiologically relevant food simulate media, it was found that all positive LAB produced significant

quantities of gNO indicating that the probiotic bacteria may be used orally to produce gNO in the complex milieu of the human GI.

As the reduction of nitrate to nitrite requires anoxic conditions, and probiotic bacteria are sensitive to upper GI conditions, the production of *g*NO from sodium nitrate was evaluated for alginate/poly-l-lysine/alginate (APA) microencapsulated strains. All microencapsulated strains proved capable of producing significant quantities of *g*NO in MRS media. Thus, it was hypothesized that the probiotic strains enzymatic capacity to reduce inorganic nitrates to *g*NO was maintained during APA microencapsulation and that microencapsulation may present an ideal solution for the protection of the strain and enzymatic machinery from storage conditions and the harsh upper GI conditions.

Finally, several microencapsulated strains were shown to produce *g*NO in simulated gastric and intestinal conditions. Under gastric conditions, significant production of gNO was observed for almost every strain showing *g*NO-producing efficacy when free. Once placed in simulated intestinal conditions only a few organisms produced *g*NO well for 24h. In nitrate reductase medium and under simulated gastric conditions only two generated significant amounts of *g*NO; however, *g*NO production was decreased after exiting gastric conditions, most likely due to the loss of acidity, but despite this loss in activity the strains produced *g*NO for at least 24 hours after inoculation. Finally, in the presence of a natural source of inorganic nitrates, and under simulated gastric conditions, the generation of *g*NO was significant for only one strain making it an excellent choice for the generation of *g*NO from dietary sources of nitrate in the conditions of the upper GI.

These results indicated that novel NiR-active probiotic bacteria can be selected for nitrate reductase (NiR) activity *in-vitro*, using different substrate and nitrate sources, and NiR activity can be maintained when microencapsulated or delivered free. Novel NiR-active probiotic bacteria can be selected for the ability to produce *g*NO *in-vitro*, under simulated GI conditions, and in the presence of various food matrices confirming the lab scale feasibility of the approach *in-vitro* and providing a starting point for *in-vivo* studies aimed at proving the efficacy of this potential therapeutic for treating hypertension, inflammatory bowel disease, gastric ulcers, diabetes and thrombosis.

Thus, it was shown that treatment modalities using probiotic and enzymatic production of gaseous nitric oxide (*g*NO) can be applied topically or orally for treating disease. The metabolic activity of probiotic bacteria can be used to design simple topical devices for the production of *g*NO and orally delivered probiotic bacteria can be selected for an enzymatic capacity to reduce dietary and salivary inorganic nitrates to *g*NO. These *g*NO treatment modalities can be applied topically for wound healing, antimicrobial, cosmetic, dermatologic disorders, and orally for treating metabolic disorders and metabolic syndrome.

CHAPTER 8: CONCLUSIONS, SUMMARY, CLAIMS TO THE CONTRIBUTION OF KNOWLEDGE, LIMITATIONS, AND RECOMMENDATIONS

8.1. Summary of observations

1. Probiotic patches, utilizing the metabolic activity of immobilized lactic acid bacteria (LAB), glucose, and nitrite salts, can be used for the production of gaseous nitric oxide (*g*NO) above therapeutic levels (200ppmV) and durations (24h) (Figure 5.8.3).

2. Probiotic *g*NO-producing patches applied to cultures of *E. coli, S. aureus,* and *P. aeruginosa* growing on solid media are highly bacteriostatic (Figure 4.7.3).

3. Probiotic *g*NO-producing patches applied to cultures of *E. coli, S. aureus, P. aeruginosa, MRSA,* are highly bactericidal, resulting in complete cell death at between 4 and 8 hours, and to cultures of *A. baumannii,* in fewer than 10 colonies detected per ml at 6 hours (Figure 4.7.4-7, right).

4. Probiotic *g*NO-producing patches applied to cultures of *T. mentagrophytes and T. Rubrum* are highly fungicidal, resulting in complete cell death at between 4 and 8 hours (Figure 4.7.9-10, right).

5. Treatment with probiotic *g*NO-producing patches, in a New Zealand White Rabbit model of wound healing, resulted in a significant decrease in morphometric wound area in all wounds ($P=0.0001$), a significant decrease in morphometric wound area for treated ischemic wounds ($P<0.0001$), a significant decrease in morphometric wound area for treated ischemic, non-infected wounds ($P=0.0006$), a significant decrease in

morphometric wound area for treated ischemic, infected wounds (P=0.015), but did not significantly decrease morphometric wound area for treated non-ischemic, non-infected wounds over the 21 day duration of the study (P=0.3539) (Figure 5.8.5).

6. Treatment with probiotic *g*NO-producing patches, in a New Zealand White Rabbit model of wound healing, showed a 2.45 fold increase in the likelihood of wound closure for treated wounds (Cox proportional hazard survival regression analysis, P=0.0533) and that treated ischemic wounds were 17.95 times more likely to close than non-treated ischemic wounds (Cox proportional hazard survival regression analysis, P=0.0302) (Table 5.7.3).

7. Treatment with probiotic *g*NO-producing patches, in a New Zealand White Rabbit model of wound healing, showed a 2.52 fold increase in the likelihood of wound closure for all treated wounds (Cox proportional hazard regression plot independent of co-variables, Hazard P=0.0375, Score P=0.032, and Likelihood ratio P=0.0355) (Figure 5.8.7).

8. Treatment with probiotic *g*NO-producing patches, in a New Zealand White Rabbit model of wound healing, showed increased likelihood of wound closure in all wounds (Kaplan-Meier plot and calculating the Log-Rank and Wilcoxon statistics, Log-Rank P=0.0225, and Wilcoxon P=0.0113) and that several of the control-treated wounds remained open (Figure 5.8.8).

9. Treatment with probiotic *g*NO-producing patches, in a New Zealand White Rabbit model of wound healing, showed that treatment of infected ischemic wounds significantly improves the likelihood of wound closure (Kaplan-Meier plot and calculating the Log-Rank and Wilcoxon statistics, Log-Rank P=0.04, and Wilcoxon P=0.046) (Figure 5.8.9).

10. Treatment with probiotic *g*NO-producing patches, in a New Zealand White Rabbit model of wound healing, showed a trend towards improved wound healing as evidenced histologically by less surface depression, less crusting and exudates, less inflammation/infiltration, greater epithelial coverage, reduced hyperplasia, greater dermal thickness, and improved overall epidermal and dermal maturity (Table 5.7.1).

11. Treatment with probiotic *g*NO-producing patches, in a New Zealand White Rabbit model of wound healing, appears to be safe, as no significant differences were observed between *g*NO-producing probiotic patch treated and vehicle control patch treated animals in body weight, blood morphology, hematology, blood biochemistry, or methemoglobin levels (Table 5.7.2).

12. Novel NiR-active probiotic bacteria can be selected for nitrate reductase (NiR) activity and capacity to produce *g*NO *in-vitro,* using different substrate and nitrate sources (Figure 6.6.1-6.6.4).

13. Novel NiR-active probiotic bacteria can be microencapsulated and selected for nitrate reductase (NiR) activity and capacity to produce *g*NO *in-vitro,* using different substrate and nitrate sources, and NiR activity can be maintained (Figure 6.6.5).

14. Microencapsulated NiR-active *Lactobacillus* strains produce different levels of *g*NO for up to 48 hours after inoculation in a food simulate medium containing sodium nitrate (30 mM) (Figure 6.6.6).

15. Microencapsulated NiR-active probiotic bacteria can be selected for nitrate reductase (NiR) activity and capacity to produce *g*NO *in-vitro*, under simulated GI conditions, and in the presence of various food matrices (Figure 6.6.7-9).

8.2. Limitations and recommendations

1. While *in-vitro* evidence is presented on the wide spectrum antimicrobial activity of *g*NO-producing probiotic patches and it is shown that when applied to a rabbit model of ischemic and infected wounds the patches improved wound healing, this evidence should be corroborated by a well conducted animal model of infected wounds. It is recommended that the antimicrobial activity of the *g*NO-producing probiotic patch be confirmed in an *in-vivo* model of infection, possibly a porcine model, and that clinical efficacy be determined in a human study for the prevention of catheter related infections, infected wounds, or topical skin infections such as visceral leishmaniasis.

2. The present results indicate the potential for antimicrobial and wound healing clinical applications and several other applications are suggested without direct evidence that the developed formulations and devices are efficacious. It is recommended that the potential efficacy of *g*NO-producing probiotic patches for topical applications such as dermatitis, psoriasis, topical infections, extensor tendon injury, extensor tendonitis, surgical flaps, wounds, and chronic wounds be investigated.

3. Current results indicate that *g*NO-producing probiotic patches are safe as determined by blood chemistry, hematology, histology, and general observations in a rabbit model of ischemic and infected wounds. It is recommended that the safety of the *g*NO-producing probiotic patches for treating wounds be confirmed through *in-vitro*

analysis of the presence and concentration of patch actives, excipients, and by-products within and outside of the patch as well as in a porcine model of wounds.

4. It is recommended that the mechanism of action for *g*NO in wound healing be investigated by monitoring the levels of *g*NO, nitrites, nitrates, nitrosylated compounds, and gene expression in keratinocytes, fibroblasts, and angiogenic cells in an appropriate animal model.

5. It is recommended that the strain safety of the identified NiR-active probiotics be determined prior to further development, including identification by 16s RNA DNA ID typing, antimicrobial resistance, biogenic amine production, hydrogen peroxide production, bacteriocin production, fermentation profile (API ferment), metabolic profile (API zyme), and the production of D/L lactate.

6. It is recommended that strains which are considered probiotic and safe be tested for safety and efficacy in an animal model of hypertension/Metabolic Syndrome (Zucker Diabetic Rat or high fat and sucrose fed rats) and/or an animal model of portal hypertension.

8.3. Conclusions

In the present project we proposed a treatment modality using probiotic and enzymatic production of gaseous nitric oxide (*g*NO) applied topically for wound healing, antimicrobial, cosmetic, dermatologic disorders, and orally for treating metabolic disorders and metabolic syndrome. Our general hypothesis was that bacterial metabolic activity can be exploited for the production of *g*NO and can be used for therapeutic purposes. Our specific hypothesis were that: bacteria can be used in the design of novel *g*NO-producing wound healing devices; novel *g*NO-producing wound healing devices can be used as topically applied antimicrobial dressings; novel *g*NO-producing wound healing devices can be used as dressings for ischemic and infected wounds; and that bacteria can be selected for a specific enzymatic activity that can be used to produce *g*NO when delivered orally for the prevention and treatment of metabolic disease. The project was undertaken, therefore, to prove the above hypothesis through our proposed research objectives. Our research objectives were to: design equipment and develop an assay to measure *g*NO by free bacteria and devices containing bacteria or enzyme by chemiluminescence; screen probiotic bacteria for lactic acid producing characteristics *in-vitro* and determine the minimal substrate required for acid production; design several probiotic adhesive patch formulations/devices and test *g*NO production *in-vitro*; evaluate the antimicrobial activity of probiotic patches *in-vitro*; evaluate wound healing efficacy and safety of probiotic patches for ischemic and infected wounds *in-vivo* in a New Zealand White Rabbit model; design several enzymatic adhesive patch formulations/devices and test *g*NO production *in-vitro*; screen probiotic bacteria for

nitrate reductase (NiR) activity *in-vitro*; and optimize an oral formulation for the production of *g*NO using different food substrate and nitrate sources under GI conditions.

After reviewing the results obtained the following conclusions can be made:

1. Probiotic patches, utilizing the metabolic activity of immobilized lactic acid bacteria (LAB), glucose, and nitrite salts, can be designed and used for the production of therapeutically relevant levels of gaseous nitric oxide (*g*NO) over therapeutically relevant durations.

2. Probiotic *g*NO-producing patches are bacteriostatic and bactericidal to a wide spectrum of virulent and drug resistant bacterial strains and fungicidal to tested fungal strains when tested *in-vitro* and may prove to be an effective wide spectrum antimicrobial agent in humans.

3. Probiotic *g*NO-producing patches can be used to improve wound healing and increase the likelihood of wound closure in ischemic and infected full-thickness dermal wounds in a New Zealand White Rabbit model for ischemic wound healing and may prove to be an effective treatment for chronic ischemic and/or infected wounds in humans.

4. Probiotic *g*NO-producing patches can be safely used in treating wounds in a New Zealand White Rabbit model of ischemic wound healing and may prove to be safely

applied topically in humans for wound healing, antimicrobial applications, dermatologic disorders, and cosmesis.

5. Novel NiR-active probiotic bacteria can be selected for nitrate reductase (NiR) activity *in-vitro,* using different substrate and nitrate sources, and NiR activity can be maintained when microencapsulated or delivered free.

6. Novel NiR-active probiotic bacteria can be selected for the ability to produce *g*NO *in-vitro*, under simulated GI conditions, and in the presence of various food matrices confirming the lab scale feasibility of the approach *in-vitro* and providing a starting point for *in-vivo* studies aimed at proving the efficacy of this potential therapeutic for treating hypertension, inflammatory bowel disease, gastric ulcers, diabetes and thrombosis.

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