McGill University

Faculty of Medicine

Department of Biomedical Engineering

Masters Thesis:

Microcapsules immobilizing activated charcoal and metabolically induced *Lactobacillus acidophilus* cells as potential oral renal failure therapy formulation.

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Abstract

Renal failure affects a wide global population; while acute renal failure may be reversed given prompt treatment, chronic renal failure often degenerates to end stage renal disease (ESRD). Dialysis is the main treatment modality for renal failure, but its long term use is associated with complications leading to morbidity and mortality events. In this thesis, a safe, economic and convenient adjunct consisting of microencapsulated active agents is formulated for oral administration. Probiotic bacteria *Lactobacillus acidophilus* and *Bifidobacteria longum* were metabolically induced for urea uptake, and the better adapted bacterium microencapsulated with activated charcoal in a calcium-alginate matrix with a chitosan membrane. The efficacy of the microcapsules was tested in-vitro in a simulated gastric media containing uremic analytes similar to pathological levels in ESRD patients. Results showed that the combination of probiotic bacteria and adsorbent not only trap uremic toxins such as creatinine and urea, but also, activated charcoal offer support in preserving the viability of the live bacterial cells in the gastric transit as well as in storage conditions for over a month.

Résumé

L'insuffisance rénale affecte une large partie de la population mondiale. Alors que l'insuffisance rénale aigue peut être soignée si elle est traitée rapidement, l'insuffisance rénale chronique évolue souvent vers une phase terminale (IRCT). La dialyse est le traitement principal en cas d'insuffisance rénale, mais sa pratique à long-terme est associée à des complications pouvant entrainer le décès du patient. Dans cette thèse, un adjunct peu couteux, pratique et sur d'utilisation, consistant en la micro-encapsulation d'agents actifs, est préparé pour une administration orale. Les bactéries pro-biotiques *Lactobacillus acidophilus* et *Bifidobacterium longum* ont été métaboliquement induites afin qu'elles absorbent l'urée, et la bactérie la mieux adaptée a été micro-encapsulée avec du charbon actif dans une matrice de calcium-alginate avec une membrane de chitosan. L'efficacité des microcapsules a été testée in-vitro dans un milieu gastrique reconstitué contenant des analytes urémiques à des taux similaires aux niveaux pathogènes chez des patients atteints d'IRCT. Les résultants ont montré que la combinaison des bactéries pro-biotiques et de l'agent adsorbant permet de capturer les toxines urémiques telles que la créatine et l'urée, mais aussi que le charbon actif offre un support pour la préservation de la viabilité des cellules bactériales au cours du transit gastrique ainsi que dans les conditions de stockage.

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Preface

In accordance with the McGill Thesis Preparation guidelines, I have taken the option of writing the experiment section of this thesis as a compilation of original papers appropriate for publication. These papers are presented in Chapters 3, 4 and 5 and are each divided into sections consisting of an abstract, introduction, materials and methods, results, discussion, and conclusion. A common abstract, introduction, literature review, summary of results, conclusions and references are included in this thesis in accordance to the guidelines.

In the course of the thesis, I have also made a legal change of name from "Trisnawati Halim" to "Trisna Lim", as will be reflected in the subsequent chapters for publication.

List of Abbreviations

MRS	Formulation by de Man, Rogosa, and Sharpe [1]
RCM	Reinforced Clostridial Medium
PLL	Poly-$_L$-lysine
APA	Alginate-PLL-alginate microcapsules
AC	Alginate-chitosan microcapsules

Units:

µL	Microliter
mL	Milliliter
dL	Deciliter
µm	Micrometer
mmol/L	Millimoles per liter
CFU	Colony Forming Unit

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1.0 General introduction

The kidneys play an important role in the maintenance of body health, particularly with respect to the removal of excess fluid, wastes, maintenance of electrolytic and acid-base balance, production of erythropoietin etc [2]. In the event of renal failure, nitrogenous wastes accumulate and if left untreated, the body enters a uremic state where all organ systems no longer function [3]. Renal failure may occur either suddenly in acute renal failure or gradually over a period of time in chronic renal disease.

Acute renal failure (ARF) is defined as the sudden and rapid drop in glomerular filtration rate (GFR) within hours to days. Such deterioration in parenchyma function is reversible although not definitively, and if untreated may result in uremia. ARF is defined by different etiologies; for example, prerenal ARF is due to the defective perfusion of the kidneys as a result of hypotension, hypovolemia, neurogenic shock due to spinal trauma, and blood vessel dilatation due to septic or anaphylactic shocks [2].

Irrespective of the etiology, chronic renal disease refers to the progressive loss in nephron number and function and in most cases lead to end stage renal disease (ESRD), where there is irreversible loss of kidney functions. When GFR drops to lower than 5% to 10% the patient is permanently dependent on renal replacement mechanisms for survival [3]. Chronic renal disease leading to ESRD are caused by a result of a number of causes; for example in the USA, top causes include diabetes, glomerulonephritis, interstitial nephritis, hypertensive disease etc [4].

Dialysis is the main treatment modality for renal failure. In essence it replaces the filtration properties of the diseased kidneys and removes accumulated metabolic toxins by connecting the patient to an external filtration system. This is done regularly to maintain serum composition within acceptable limits. As the chronic renal failure and ESRD population increases, the maintenance dialysis population increases accordingly, and is expected to reach one-half million in the United States by 2010 [5].

However, maintenance dialysis is associated with a wide range of complications, one of the most serious includes cardiomyopathy-related morbidities and mortalities [6-8]. Furthermore, patients on maintenance dialysis are plagued with increased infection [9] and inflammation [10] especially with the use of bioincompatible dialysis membranes such as Cuprophan (CU) membranes [11]; aged, synthetic membranes such as cellulose acetate membranes are also responsible for the increased inflammatory responses [10,12].

The need for an access site either to the vasculature or peritoneal cavity in hemodialysis and peritoneal dialysis respectively represents a constant threat of infection. In fact, infection-related mortality is considered as a serious consequence of maintenance dialysis [13-16], where peritonitis ranks one of the top cause of morbidity and loss of technique in peritoneal dialysis [17,18], and fungal peritonitis not only cause 1% - 5% of infection episodes, but also loss of technique in 40% peritoneal dialysis patients [19]. Endotoxin and bacterial contamination of dialysates is another common problem due to the formation of biofilm in silicone tubings of dialyzers [20], and is also recognized as a major contributor to high infection rates. Other complications of dialysis include iron overload [21] and dialysis related amyloidosis [22] etc.

Kidney transplantation is another choice of treatment, but such candidates are limited to a highly select population, largely due to eligibility issues including the rising age of ESRD patients, preformed antibodies from previously failed grafts, and a shortage of donors [23]. In the event of a successful graft transplant, the life long commitment to immunosuppression makes the patient vulnerable to opportunistic infection attacks.

With serious complications undermining the efficacy of current treatment modalities for renal failure, there is an active search for novel methods; one such area which has been the focus of research in various diseases and renal failure is "artificial cells". First developed by Chang in the 1960's [24], an artificial cell refers to man made structures typically of cellular dimensions for the compartmentalization of biologically active agents, to supplement or replace deficient cell functions. Entrapped within a semi-permeable membrane, the agents

are isolated from the host's defense system or external environments such as the gastrointestinal (GI) tract, yet survive within the microcapsules and perform the required biological functions.

One idea that emerged from the use of artificial cells is the formulation of microcapsules as an oral adjuvant to supplement conventional dialysis. Much research efforts have been invested in the use of encapsulated adsorbents or biologically active bacteria for the removal of metabolic toxins, especially urea and ammonia, from the GI tract such as oxidized starch (oxystarch) [25-27] and activated charcoal, but large doses of oxystarch are required and it lacked efficiency at physiological pH [27,28] while activated charcoal rapidly saturates [29].

In the 1970s, Setala et al [30,31] experimented with the encapsulation of enzymes isolated from soil bacteria for the degradation of nitrogenous compounds, but did not achieve optimal results in humans. Other similar attempts include using multienzyme systems for converting ammonia and urea into essential amino acids [32], or whole microorganisms for degrading nitrogenous metabolites [33-35].

Genetic technology was later incorporated where Prakash encapsulated the genetically engineered *Escherichia coli* DH5 cells containing the urea inducible *Klebsiella aerogenes* gene for in-vitro and in-vivo trials [36]. Within 30 minutes, there was almost complete removal of plasma urea and ammonia removal using only 100 mg bacterial cells. Unfortunately, such genetically modified systems encounter safety and environmental issues. It is only recently where a safer alternative is provided: using probiotic bacteria metabolically induced for urea uptake for urea removal [37]. Given increasing literature support of the numerous benefits of probiotic bacteria [38-40], as well as new evidence of their highly adaptive capacities [41,42], capitalizing on the inductive capacities of probiotic bacteria may prove a rewarding attempt.

This project is focused in the exploration of metabolic induction of probiotic bacteria strains in a dynamic fashion, as well as its combined encapsulation with activated charcoal for the removal of important metabolic wastes such as urea and creatinine.

1.1: Research Objectives

The current study investigates the efficacy of microcapsules containing probiotic bacteria adapted for urea metabolism and activated charcoal in lowering unwanted uremic metabolites such as urea, creatinine, phosphate, chloride, potassium and sodium to serve as an oral adjuvant to conventional dialysis. The research objectives are:

1. To metabolically induce probiotic bacteria strains for urea uptake, compare their responsiveness to the induction process, and to select the best conditioned probiotic bacteria.
2. To design a novel microcapsule composed of alginate with a chitosan membrane with strong mechanical properties, for the immobilization of activated charcoal particles and the probiotic bacteria.
3. To evaluate the efficacy of the microcapsules containing probiotic bacteria and activated charcoal in removing the above mentioned metabolites in-vitro.
4. To investigate if the co-encapsulation of activated charcoal with the probiotic bacteria affects bacterial viability or supports bacterial viability.

2.0 Literature review

2.1 Kidney function and diseases

The kidneys are two bean shaped organs about the size of one's fist, located below the rib cage, each on either side of the spine. While both kidneys weigh only about 0.5% of one's body weight, they receive about 20% of blood supply from the main circulation. This is because they play an indispensable role in the maintenance of body health, particularly with respect to the removal of excess fluid, nitrogenous and toxic wastes, maintenance of electrolytic and acid-base balance, regulation of blood pressure, production of erythropoietin and hydroxylation of Vitamin D [2]. Filtration is performed by the nephron unit, which is comprised of a renal corpuscle and a renal tubule (Fig. 2.1). Briefly, arterial pressure from the renal artery drives the filtration of numerous metabolites from the glomerulus into the Bowman's capsule. Useful substances are reabsorbed into the capillaries along the renal tubule by active transport and diffusion, while waste substances remain in the tubule, collected in collecting ducts and converted into urine. About a million nephron units are located in the cortex of each kidney and they work collectively to regulate fluid, acid-base, electrolyte and waste balance. Such vital and diverse functions performed by the kidneys mean that damage to the kidneys resulting in renal failure will adversely affect the rest of the body due to the accumulation of nitrogenous wastes. If left untreated, the body enters a uremic state, a syndrome where all organ systems no longer function [3]. Renal failure may result either suddenly in acute renal failure or gradually over a period of time in chronic renal disease. Acute renal failure (ARF) is defined as the sudden and rapid drop in glomerular filtration rate (GFR) within hours to days. Such deterioration in parenchyma function is reversible although not definitively, and if left untreated may result in uremia. ARF is defined by different etiologies; for example, prerenal ARF is due to the defective perfusion of the kidneys as a result of hypotension, hypovolemia, neurogenic shock due to spinal trauma, and blood vessel dilatation due to septic or anaphylactic shocks. A major renal cause of ARF is acute tubular necrosis, which is often due to renal ischemia, glomerulonephritis, or the accumulation of toxic drugs such as aminoglycosides,

lithium etc [2]. Post renal ARF is caused by obstructive nephropathy by renal calculi or pelvic tumors. ARF also occurs in 1% - 25% of intensive care patients [43] and are linked with high mortality.

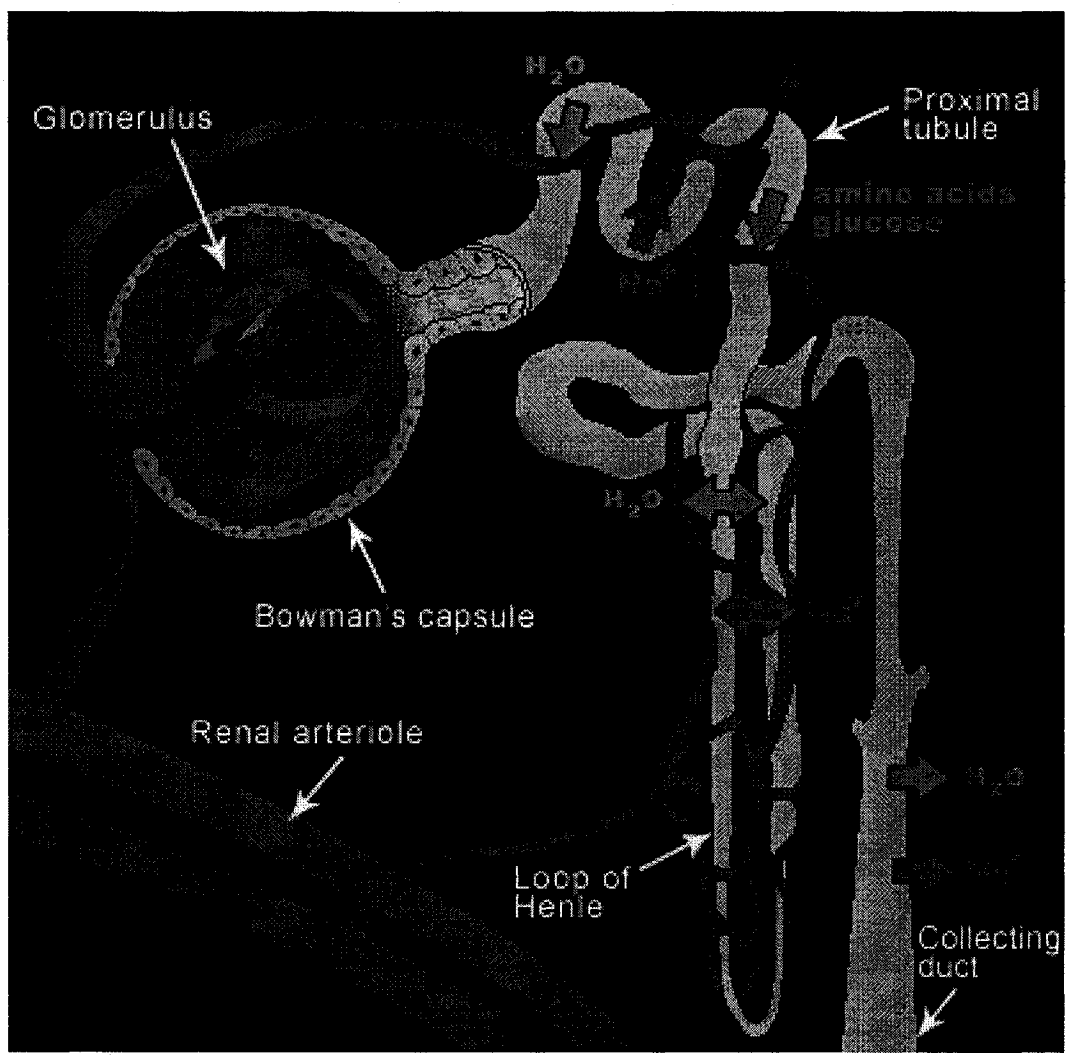

Figure 2.1: Diagram of a nephron unit. (Adapted from Greene [44])

Irrespective of the etiology, chronic renal disease refers to the progressive loss in nephron number and function and in most cases lead to end stage renal disease (ESRD), where there is irreversible loss of kidney functions. When GFR drops to lower than 5% to 10% the patient is permanently dependent on renal replacement therapies for survival [3]. Chronic renal disease leading to ESRD are caused by a number of causes; for example in the USA, top causes include

diabetes, glomerulonephritis, interstitial nephritis etc [4]. In fact the ESRD population was on a steady climb for the past 20 years despite increased awareness about healthy lifestyles and higher standards of living (Fig. 2.2).

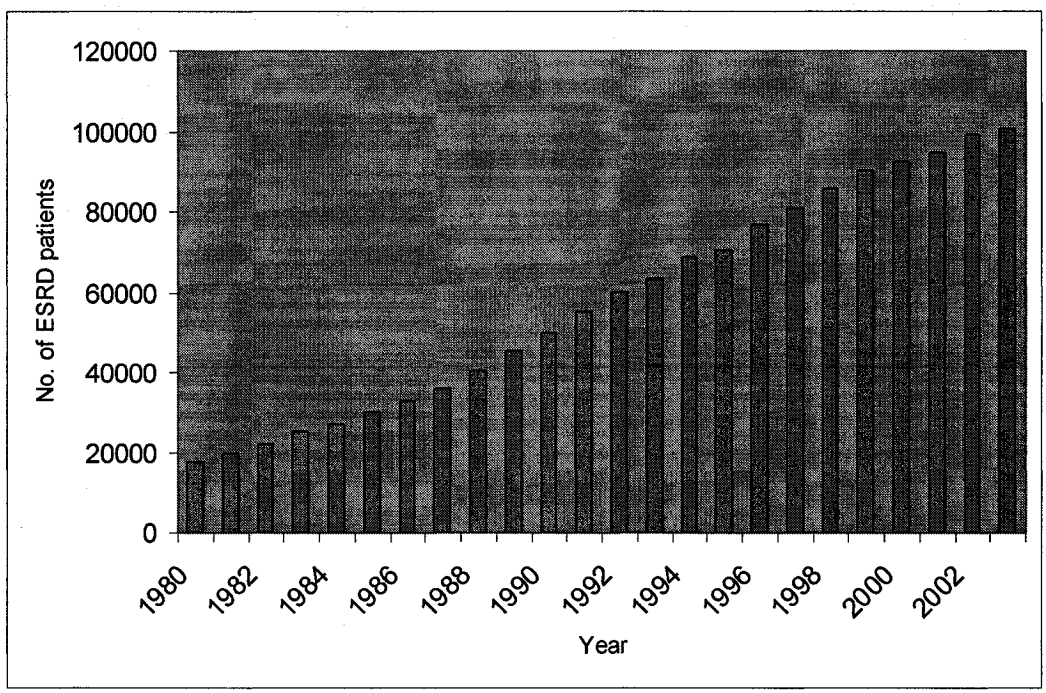

Figure 2.2: Incident counts of ESRD patients in the United States. (Adapted from the U. S. Renal Data System 2005 Annual Data Report (USRDS) [4])

2.2 Current treatment modalities for renal failure

Renal replacement therapies (dialysis) used today were originally described by Schreiner and Maher in 1961 [45]. Dialysis refers to a filtration technique where metabolic toxins accumulated in the body are removed by connecting the patient to an external filtration system at regular intervals, to maintain serum composition within acceptable limits. Since then, the technology has seen dramatic advancements and is most popular today in the form of hemodialysis and peritoneal dialysis. The continuous refinement in the technology is reflected in that the maintenance dialysis population is constantly increasing, and is expected to reach one-half million in the United States by 2010 [5].

Important principles such as diffusion, hemofiltration and convection are employed in hemodialysis. Waste metabolites diffuse from the patient's blood to

the dialysate fluid as a result of the concentration gradient set up by the composition of the dialysate. The hemofiltration process is manifested in the movement of large volumes of water from the patient's blood to the dialysate fluid, and in the process creates a convection current that "pulls" small solutes along. On top of this, hemodialysis utilizes the hemodynamic pressure in the arteries to drive blood through an external filtration circuit, and is efficiently accomplished within several hours (Figure 2.3). Hemodialysis is typically done three times a week to maintain serum composition within an acceptable range. A surgical procedure creating an arteriovenous (AV) fistula has to be done, which requires a latency period of weeks to months for wound healing before the start of hemodialysis. Therefore, hemodialysis may not be used to treat ARF cases, but instead a venous catheter connecting a large vein to the hemodialysis machine is employed. Alternatively, hemofiltration is used in ARF cases, especially in intensive care settings for emergency rescue of fluid overload or acidosis etc. Hemofiltration is done via a venous catheter and the low venous pressure mean that the procedure may take up to five hours for each cycle.

A gentler method of correcting the uremic state is peritoneal dialysis (PD), which consists of filling the peritoneal cavity with new dialysate. The dialysate contains high dextrose amounts to provide the concentration gradient for the diffusion of wastes from the blood into the peritoneal cavity. This is accomplished when the dialysate "dwells" or stays in the peritoneal cavity for several hours, after which it is removed. A catheter has to be surgically implanted and allowed to heal before PD is commenced, which also takes weeks to months. Two variations of PD are continuous ambulatory PD (CAPD) and continuous cyclic PD (CCPD). For CAPD, there is a 2.0 – 2.5 liters of dialysate dwelling in the peritoneal cavity at all times, but gives the patient freedom of movement and perhaps a higher quality of life. Dialysate exchanges are done without expert supervision, and commonly at the comfort of one's home. Alternatively, in CCPD, dialysate exchange is performed at night using an automated cycler, leaving fresh dialysate solution in the peritoneal cavity at the start of the day.

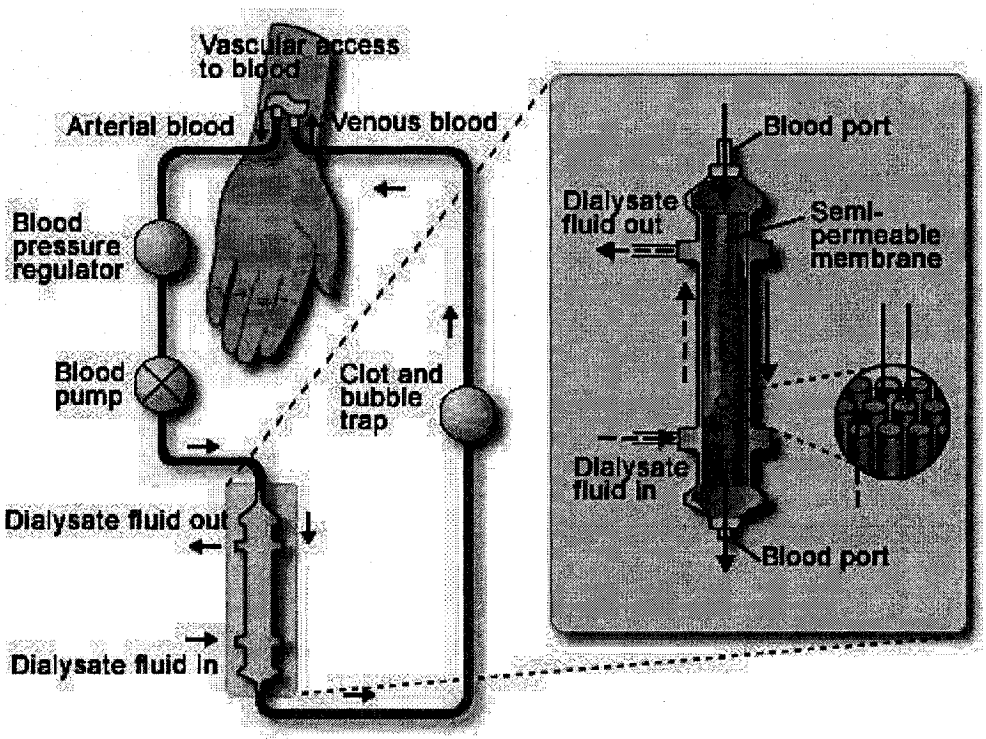

Figure 2.3: A hemodialysis system. (Adapted from Lippincott Williams & Wilkins [46].)

Dialysis only performs the filtration functions of the kidneys but not the synthetic functions, which explains the large number of ESRD patients on the organ transplant waitlist. For example in the United States, the number of patients on the waitlist for kidney transplant form more than 60% of total organ waitlist that includes organs such as intestine, pancreas, lung, heart and liver from 1994 to 2003 [47]. Kidney transplantation promises a lifestyle free of dialysis procedures but a life long commitment to immunosuppressants.

2.3 Limitations of current treatment modalities

Maintenance dialysis is associated with a wide range of complications, one of the most serious includes cardiomyopathy-related morbidities and mortalities [6-8]. Indeed, dialysis related cardiomyopathy has been linked to hypertension, anemia [48], increase in markers of inflammation and oxidative stress [49] etc. Furthermore, patients on maintenance dialysis are plagued with a defunct immune system, contributing to about fifteen percent of total dialysis mortality [50]. The

compromised capacity of the immune cells [10] is especially evident with the use of bioincompatible dialysis membranes such as Cuprophan (CU) membranes, which elicited increased oxidative responses from immune cells [11], while aged, synthetic membranes such as cellulose acetate membranes evoked increased inflammatory responses [10,12]. Increased susceptibility of dialyzed patients to malignancy or tumor growth may be explained by the reduced cytolytic abilities of natural killer cells associated with the use of the CU membrane in dialysis [51,52].

The need for an access site to either the vasculature or peritoneal cavity in hemodialysis and peritoneal dialysis respectively represents a constant threat of infection. In fact, infection-related mortality is considered as a serious consequence of maintenance dialysis [13-16], where peritonitis ranks as one of the top cause of morbidity and loss of technique in PD [17,18]. 1% - 15% infection are caused by fungal infections, which resulted in loss of technique in 40% of these patients [19]. Staphylococci, a skin dwelling bacteria, caused most of bacterial infections in PD; they evolve and develop antimicrobial-resistance to antibiotics such as penicillin, antistaphylococcal penicillin and vancomycin [53]. Furthermore, endotoxin and bacterial contamination of dialysates are another common problem due to the formation of biofilm in silicone tubings of dialyzers [20], and contributes to high infection rates. Other side effects of dialysis include iron overload causing bone disease [21] and atherosclerosis [54], and dialysis related amyloidosis [22].

Kidney transplantation is another choice of treatment, but such candidates are limited to a highly select population, largely due to eligibility issues including the rising age of ESRD patients, preformed antibodies from previously failed grafts, and a grave shortage of donors [23]. In the event of a successful graft transplant, the life long commitment to immunosuppression leaves the patient vulnerable to opportunistic infection attacks.

2.4 Artificial cells

With serious complications undermining the efficacy of current treatment modalities for renal failure, there is an active search for novel methods; one such area which is the focus of research in various diseases including renal failure is

"artificial cells". First developed by Chang in the 1960s [24], an artificial cell refers to a microscopic artificial structure that compartmentalizes biologically active agents to supplement or replace deficient cell functions. Entrapped within an ultra-thin semi-permeable membrane, these agents are isolated from the host's defense system or external harsh environments such as the GI tract, and deliver the desired cell derived therapeutic agents.

2.4.1 Applications of artificial cells

Utilizing the concept of artificial cells, Chang developed the first generation of artificial blood, producing soluble polyhemoglobin for the reperfusion of non-sustained ischemia. The advantages of having an unlimited supply of ready-to-use blood substitute without having to match blood types and without the risks of disease transmission made artificial blood an attractive choice over human blood. Dramatic improvements was made to this first generation artificial blood including the addition of essential enzymes such as catalase, superoxide dismutase etc [55]. This second generation blood substitute has a longer circulation time and is currently used in human clinical trials [56].

Immobilizing foreign cells in artificial cells or microcapsules enabled their isolation from the external environment and their escape from the policing of the immune system. This opened the doors to a new set of clinical applications such as the correction of in-born errors of metabolism or the replacement of dysfunctional organ functions in diseases. For example, the looming diabetic problem in the 1960s motivated the transplantation of encapsulated islet cells for Type 1 diabetes, leading to the development of the first artificial endocrine pancreas in the 1980s [57]. Xenografts of encapsulated rat islets were laterfound to maintain normoglycemia when transplanted into diabetic mice [58], although it was not only in the 1990s when the first successful clinical trial was reported [59].

There is also tremendous research interest in the encapsulation of hepatocytes [60-65] for the treatment of liver diseases, largely due to its diverse functionality. In fact, current research no longer focuses on the feasibility of hepatocyte encapsulation, but on its technique optimization to improve cell

viability and survival post transplantation. Examples include the development of a two-step encapsulation procedure, which totally eliminates the entrapment of cells in the capsule membrane matrix and their exposure to the external environment [66], and the co-encapsulation of hepatocytes with immunosuppressant producing Sertoli cells, which prolonged cell viability for four months [67].

The list of encapsulated therapeutic agents goes on, ranging from mammalian cells such as erythropoietin-secreting renal cells for anemic treatment [68], parathyroid cells for treating hypoparathyroidism [69], to biomolecules [70,71] and stem cells [72,73]. Table 2.1 summarizes the applications of artificial cells in the clinical field.

Disease	Immobilized Agent	Mode of Therapy	References
Liver Failure	Hepatocytes, or combined with stem cells	Implantation	60,63-65,67
Diabetes	Islet cells	Implantation	57-59
	Insulin	Oral delivery	70
	Insulin	Injection	71
Hyperbilirubinemia	Hepatocytes, or combined with bone marrow stem cells	Implantation	61,62,72
Ischemia	Polyhemoglobin, enzymes	Injection	56,74,75
Renal failure	Hepatocytes, or combined with stem cells	Implantation	67,73,76
	DH5 *E. coli* cells, soil bacteria, adsorbents	Oral delivery	28,31,36,37,77-80
	Adsorbents	Hemoperfusion	81,82
Crohn's disease, irritable bowel syndrome	Probiotic bacteria	Oral delivery	83-86
Toxin or medication overdose	Adsorbents	Hemoperfusion	87-91

Table 2.1: Applications of artificial cells as a mode of therapy.

The ultra-thin membrane of artificial cells, combined with their large surface-area-to-volume ratio meant that artificial cells containing bioadsorbents such as activated charcoal are many times more efficient in removing toxins compared to conventional dialysis [92,93], for example, in an extracorporeal shunt

system used in hemoperfusion. Before the invention of artificial cells, the use of bioadsorbents in such applications is limited by the release of undesirable particles upon direct contact with blood, such as the production of methyl guanidine from creatinine on the surface of activated charcoal, the production of lipoperoxide from fatty acids upon its chemical reaction of activated charcoal, the release of carbon fines leading to embolism etc [94,95]. It was only after the birth of the artificial cell concept that researchers develop a method of enveloping activated charcoal particles in polymeric membranes to prevent direct contact of these particles with blood in hemoperfusion [92]. Since then, the successful compartmentalization of bioadsorbents enabled artificial cells containing bioadsorbents to be used in the treatment of kidney failure, liver failure and toxin or medication overdose [87-91].

2.4.2 Common materials used in cell microencapsulation

Microcapsule designs depend on the type of the immobilized cells and the mode of therapeutic application, therefore microcapsules have to be custom designed to acquire specific properties necessary for the application. For example, artificial cells destined for transplantation require that their microcapsule membranes posses properties such as 1) biocompatibility and permeability [76,96], 2) mechanical strength for microcapsule integrity during preparation, production, handling [76] and long term storage [97] and 3) immunoprotectivity from the host's immune system [98]. As such, a variety of natural and synthetic materials suitable for microcapsule membranes were extensively studied and characterized. Examples of synthetic polyelectrolyte materials include acrylic/methacrylic acid (anionic) or dimethylaminoethyl methacrylate/diethylaminoethyl acrylate (cationic) or (meth)acrylates (less aqueous soluble). However these synthetic polyelectrolytes were generally more toxic than natural materials [99], and the processes involved in the production of several of these microcapsules are toxic to cells [100,101]. It is not surprising those milder and gentler methods of cell encapsulation such as the ionotropic gelation of the alginate polymer receive more

popularity; in fact calcium-alginate gel beads are considered a benchmark for cell encapsulation.

Alginate, one of the most abundant naturally occurring polymers extracted mainly from brown algae, is a well established, biocompatible and biodegradable material viewed most suitable for cell encapsulation [98]. Alginate encapsulation is a mild process for the immobilization of mammalian cells and the preservation of cell viability. It is used in numerous applications, ranging from the transplantation of encapsulated allo- and xenogenic cells, mammalian cells [97,100,102-104] to the encapsulation of microbial cells for drug delivery applications [36,37]. Alginate polymers compose of alternating mannuronic (M) and guluronic (G) sequences, where variation in these sequence and composition define different functional properties [99,105]. For the immobilization of live cells, alginate polymers typically gelated by ionotropic gelation, or the cross linking of the anionic alginate chains with divalent cations such as Ca^{2+}.

Alginate microcapsules are not without limitations however; their integrity and functionality may be compromised when positively charged proteins in the external surroundings are attracted to the carboxylic acid sites on the alginate matrix and compete with the Ca^{2+} ions [106]. In fact, long term studies involving cell encapsulation has reported cell protrusion and leakage using the conventional alginate configuration [107]. Therefore, enveloping the calcium-alginate matrix in a membrane layer is a popular and widely used technique to preserve the alginate core stability for an extended period of time [108].

The alginate-poly-$_L$-lysine-alginate (APA) microcapsule is one of the first microcapsule configurations investigated for cell encapsulation. Formed by the complexation of the polyanionic alginate and the polycationic PLL, the APA microcapsule possesses high biocompatibility and biodegradability due to the nature of sodium alginate [98], as well as immunoprotective properties attributed to the PLL layer [99], a third alginate layer neutralizes the cationic PLL to avoid attracting inflammatory cells [109]. The APA microcapsule was used in numerous applications, especially in the transplantation of encapsulated allo- and xenogenic islets cells in diabetic induced dogs [110], monkeys [111] and even a human patient [59].

Chitosan-alginate (AC) microcapsules are another popular choice of a biocompatible and biodegradable material for cell encapsulation. A naturally occurring cationic polysaccharide, chitosan is derived from chitin found in the exoskeletons of crustaceans. Compared to PLL, chitosan forms stronger ionic complexes with alginate as a result of the similar structure and therefore equal distance between the charges of the two molecules [112]. The interaction between chitosan and alginate binding was also studied in detail. It was reported that homogenous alginate gels bind as much as 6 times the amount of chitosan compared to nonhomogenous gels in 24 hours. The presence of calcium ions also facilitates higher amounts of chitosan binding within a shorter period of time, where higher calcium concentration corresponded to increased microcapsule porosity. Smaller chitosan chains of lower molecular weight were also found to bind to a higher extent compared to larger chitosan chains [113]. More detailed testing showed that alginate-chitosan microcapsules containing low molecular weight chitosan with Ca^{2+} ions during membrane formation have high mechanical strength to withstand high osmotic pressures [114].

Besides the commonly used materials for encapsulation described above, other materials studied include poly (ethylene glycol) (PEG), poly (methylene-co-guanidine) (PMCG), cellulose etc.

2.4.3 Common materials used in the bioadsorbent encapsulation

Besides live cell encapsulation, the encapsulation of adsorbents is also of major research interest. Of the many adsorbents developed, activated charcoal is still the most commonly used. This is especially the case in hemoperfusion, largely due to the fact that activated charcoal provides a rapid method of systemic detoxification resulting from drug or toxin overdose. To prevent embolism and other adverse reactions upon the direct contact with activated charcoal particles and blood, the particles were enveloped in polymeric membranes for use in extracorporeal shunts in hemoperfusion [92]. Essential properties of such polymeric coatings include: 1) biocompatibility with blood, 2) an ultrathin membrane, 3) permeability to toxins/metabolic products, 2) sufficient mechanical strength, 4)

adhesivity to the adsorbent, and 5) low costs [115]. Several polymers used in the encapsulation of activated charcoal include nylon, collodion, heparin-benzalkonium complex collodion, albumin-collodion, cellulose acetate membrane etc [24,93,116]. In particular, activated charcoal enveloped in an albumin coated cellulose nitrate was extensively studied for the hemoperfusive removal of phenols [117] and various toxins, and was reportedly efficient in removing toxins from aqueous media.

A study by Tijssen et al reported that a thin cellulose acetate coating over activated charcoal achieved minimal particle release [94]. However further research indicated that while a thin membrane reduce the leaching of carbon particles into the blood, it does not affect or improve the blood compatibility of the coated adsorbents. For example, the albumin based membranes may adhere less platelets, but γ–globulin and fibrinogen-adsorbing membranes may cause cellular adhesion leading to fibrin clot formation [118], forming a thick coating of cells and fibrin on the incompatible membrane surface and resulting in the subsequent failure of the hemoperfusion system. Therefore numerous studies were conducted to investigate the biocompatibility and efficacy of different coating materials. Agarose based membranes was investigated; the cross-linked agarose entrapped activated charcoal [119] were found relatively hemocompatible and satisfactory for clinical use. Another study with the use of poly-HEMA coated activated charcoal was tested in sheep and reported minimal platelet loss [120]. More recently, the chitosan coating was investigated in the search for a more biocompatible, inexpensive polymer. Adsorption capacities of activated charcoal remained similar as in other coatings used, and good adsorption was reported for creatinine, uric acid, bilirubin etc but lower adsorption for albumin, urea and inulin [81].

Unlike cell encapsulation, the encapsulation process of activated charcoal is comparatively harsher, and stronger sterilization techniques may be employed without losing the functional properties of activated charcoal. Examples include a phase-separation technique involving the heating of cyclohexane containing dispersed charcoal particles to 80 °C and vacuum ovening [78]; and the use of

chlorosulfonated polyethylene to fix charcoal granules on a tape for a fixed bed system [115].

2.5 Artificial cells as an alternative treatment in renal failure

The large surface area to volume ratio and the ultra-thin membrane of microcapsules meant that diffusion across the membranes of artificial cells is about a hundred times more efficient compared to a dialyzer membrane. It has in fact been estimated that about 10 mL of artificial cells each having a diameter of 20 μm have a total surface area of about 20 000 cm^2, equivalent to that of a hemodialysis machine [121].

As described above, the use of microcapsules in renal failure include those for implantation, hemoperfusion and oral delivery. In terms of practicality, implanted microcapsules containing hepatocytes does offer sustained functionality but only for several months, and extensive research still needs to be done before a fully functional and independent artificial kidney becomes accessible to the chronic renal failure and ESRD population. Hemoperfusion has a long history in the clinical practice however, like dialysis; hemoperfusion requires the need to break the skin barrier, apart from the fact that the procedure is time consuming and restricts the patients' movements. Since these renal replacement therapies are necessary for survival, patients suffering from chronic renal failure or ESRD need to commit a life long dependence to such therapies. On the other hand, the oral administration of microcapsules containing active bioreactors is not only a convenient method that allows for self administration, but will most likely result in higher patient compliance and acceptability [122].

2.5.1 Oral administration of artificial cells

The oral administration of artificial cells has practical advantages over other therapies, such as 1) no need for cannulation, 2) oral therapy may be applied to most patients without concern of existing diseases, extent of uremia, age etc, 3) no need for apparatus, 4) no need for trained personnel and 5) ease of supervision [78]. Researchers have extensively investigated the oral delivery of immobilized

agents such as adsorbents, biomolecules, microorganisms or their combination, to remove unwanted uremic metabolites from the GI tract, since it is where exogenous and endogenous nitrogen-containing compounds are metabolized.

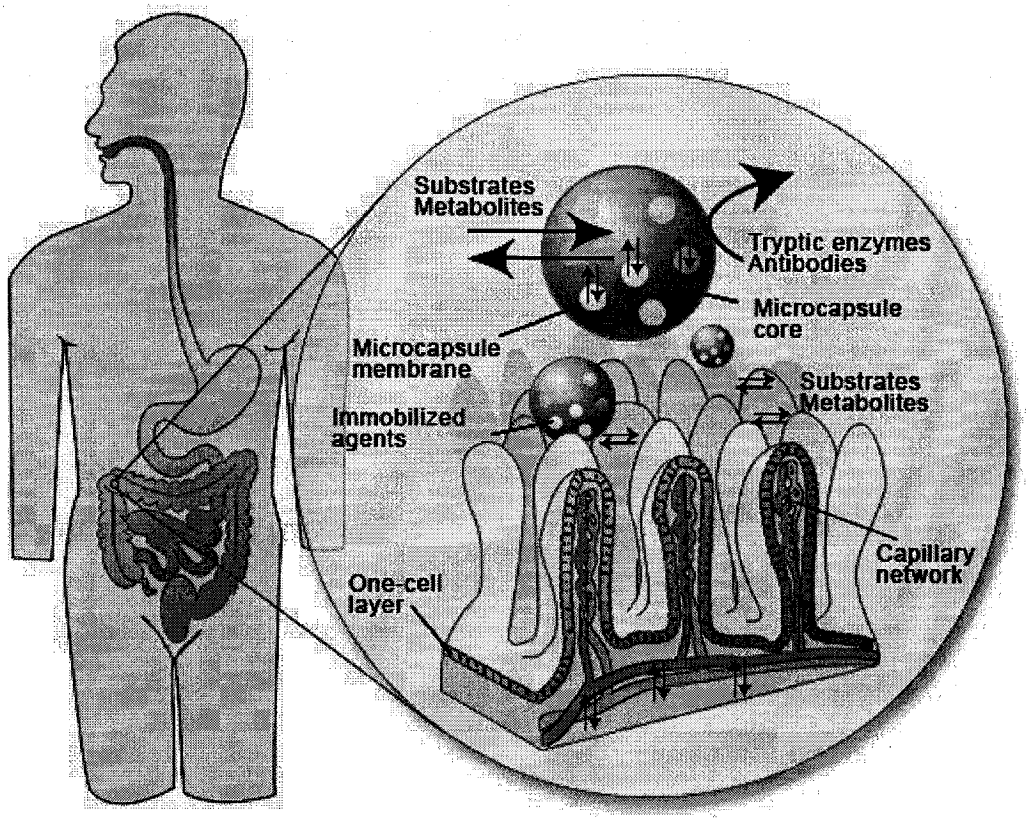

Figure 2.4: Theorized mode of action of orally administered microcapsules containing activated charcoal and metabolically induced probiotic bacterial cells.

The removal of uremic metabolites generally found in the blood circulation from the GI tract is possible due to several factors. An important factor is the exchange of uremic metabolites between the systemic circulation and the lumen of the GI tract [123,124]. This is made possible by the extensive capillary network surrounding the GI tract, which is in turn responsible for the rapid diffusion kinetics to and from the GI lumen. Efficient absorption of solutes from the GI tract is made possible by the large surface area of the small intestine, which is attributed to tiny protrusions called villi and microvilli. With such an intimately connected network, the removal of uremic metabolites by microcapsules in the GI

tract will set up a concentration gradient between the GI tract and its surrounding capillaries, and the diffusion of more uremic metabolites from the capillaries into the intestines (Fig. 2.4).

2.5.2 Adsorbents

A major research interest in renal failure is the formulation of microcapsules as an oral adjuvant to supplement conventional dialysis. In this regard, much research efforts was invested in the use of encapsulated adsorbents or biologically active bacteria for the removal of metabolic toxins, especially urea and ammonia, from the GI tract [25-27]. Two adsorbents that received much attention are activated charcoal and oxidized starch (oxystarch). Activated charcoal is a potent adsorbent and acts via non-specific electrostatic attraction for molecules. Equipped with a large internal surface area of about 1000 m^2/g, activated charcoal has a high affinity for non-specific polar and non-polar small molecular weight molecules, especially in aqueous solution and blood [94]. While the non-specificity may have contributed to its being a potent adsorbent for a number of uremic molecules, the non-specificity also results in rapid pore saturation [29]. Oxystarch refers to a $NaIO_4$-oxidized, aldehyde derivative of soluble starch prepared by Giordano et al [80]. Early research involved the oral administration of oxystarch to uremic patients, which resulted in the increase of fecal nitrogen content of these patients. This was accomplished by oxystarch binding of ammonia produced by urealysis in the intestines [28], but there was reduced efficacy at physiological pH and large dosages were required to remove sufficient nitrogen compounds in renal failure patients [27,28].

2.5.3 Microorganisms

In the 1970s, Setala et al [30,31] experimented with antiazotemic enzyme systems from soil bacteria for the degradation of nitrogenous compounds. Lyophilized bacteria strains were packed in gelatin capsules either in its free form or encapsulated, and given orally to uremic patients for several weeks. This regimen proved effective in lowering nitrogen containing compounds of the

patients, but results were not well quantified. This was the first realization of the oral administration of lyophilized bacteria to uremic patients as an adjunct therapy in renal failure.

Malchesky later cultured wild type bacteria *Pseudomonas alcaligenes, Diphtheroid bacillus* and *Alpha streptococci* in *in-vitro* batch reactors for the degradation of nitrogenous metabolites [33-35]. The batch reactors that were continuously supported for two months showed consumption of urea, uric acid and creatinine, producing ammonia and sludge mass.

Genetic technology was incorporated more recently, where Prakash encapsulated the genetically engineered *Escherichia coli* DH5 cells containing the urea inducible *Klebsiella aerogenes* gene for in-vitro and in-vivo trials [36]. Within 30 minutes, there was almost complete removal of plasma urea and ammonia removal from 975.14 μM/L to about 81.15 μM/L using 100 mg bacterial cells. Unfortunately, such genetically modified systems encounter safety and environmental issues. It is only recently where a safer alternative is provided: using probiotic bacteria metabolically induced for urea uptake for urea removal [37].

2.5.4 Biomolecules

Instead of using whole microorganisms, several researchers investigated the possibility of isolating the enzyme urease for the degradation of urea in the GI tract. Urease was encapsulated in combination with zirconium phosphate for the break down of urea and the subsequent removal of ammonium ions [125]. This combination microcapsule was used in in-vivo studies involving dogs, but zirconium phosphate showed limited capacity for the ammonium ions [126]. Other attempts to immobilize urease in microcapsules were Ristau et al [127] and Elcin [128]. The disadvantage of using isolated enzymes is that they are highly specific, and intermediates have to be removed with additional methods or other enzymes. To overcome this problem, Gu and Zhang designed a multienzyme system aimed at converting urea and ammonia into essential amino acids, namely L-leucine, L-valine, and L-isoleucine. Shortly after, this multienzyme system was encapsulated

in artificial cells and a complete characterization of this system was performed in-vitro [32,129].

Issues related to enzyme isolation and high costs for isolation procedures still cloud the practicality of using enzyme systems. Therefore, whole microorganisms were considered an attractive alternative due to the fact that they produce the required enzymes to complete the whole metabolic pathway and do not require extensive isolation procedures. It was theorized that the most effective method for the degradation of a target substance was to utilize it as a growth requirement for a particular microorganism [130], and that naturally occurring strains of microorganisms could be conditioned to be effective at breaking down nitrogenous compounds when cultivated in a media supplemented with urine. The potential of metabolic induction and conditioning of wild type microorganisms forms the basis of this thesis, where microorganisms beneficial to humans such as probiotic bacteria could be adapted to metabolize uremic metabolites such as urea and creatinine.

2.6 Proposed Microcapsule Design: Probiotic bacteria and activated charcoal

2.6.1 Probiotic bacteria

There are 10^{13-14} bacteria from about 400 different species colonizing the human's GI tract, and the existence of different bacteria species at different part of the GI tract depend on factors such pH, peristalsis, redox potential, bacterial adhesion, bacterial cooperation, mucin secretion, nutrient availability, diet, and bacterial antagonism.

There is increasing evidence that probiotic bacteria confer numerous benefits to the human host (Table 2.2). While their specific mode of action is still under investigation, it is known that one of the more important ways probiotic bacteria contribute to the health of the GI tract is to maintain the integrity of the mucosal barrier [38] by colonization resistance, or the mass colonization of the GI tract by the indigenous microflora thus making it more difficult for pathogenic bacteria to colonize [131]. It was recently proven in-vitro that the preincubation of

Lactobacillus plantarum lowers the adherence of enteropathogenic *Escherichia coli* [132,133]. It was also found that *Lactobacillus rhamnosus* GG enhanced the growth of the epithelial cells of the GI tract, which is an important function since the epithelial cells are continuously removed during peristalsis [134]. Furthermore, probiotic bacteria are also associated with other important benefits to the GI tract such as nutrient processing and adsorption [135], regulation of fat storage [136] etc.

Recently, it is found that probiotic bacteria also play a role in the treatment of infections and diseases. For example, probiotic bacteria are associated with the efficient treatment of Helicobacter pylori infection [83,84], irritable bowel syndrome [137,138], inflammatory bowel disease [85,86] etc. In particular, probiotic strains that are the focus of much research efforts include *lactobacilli* and *bifidobacteria*, both of which have been labeled as a functional food [139-141]. Of particular interest are their highly adaptive capabilities, which were recently illuminated in the evolutionary conserved genome sequence of *Bifidobacteria longum* [142]. Another such evidence of adaptation is the fact that *Bifidobacterium longum* expresses different levels of protein when exposed to different levels of bile salts [143]. An attempt to utilize the adaptive abilities of probiotic bacteria is reported in a recent paper where *Lactobacillus delbrueckii* exposed to metabolic induction for urea uptake removed urea in-vitro [37].

Fights diseases	References	Benefits to humans	References
H. pylori infection	83,84	Reinforces mucosal barrier	132,133
Inflammatory bowel disease	85,86	Enhances epithelial growth	134
Irritable bowel syndrome	137,138	Aids nutrient processing	135
		Regulates fat storage	136

Table 2.2: Beneficial effects of probiotic bacteria on the GI tract

2.6.2 Activated charcoal

As described above, activated charcoal and oxystarch are two potential candidates for the removal of uremic toxins from the GI tract. Oxystarch reacts

with urea to form a solution but precipitates when it reacts with ammonia. When orally administered to uremic patients, oxystarch binds the ammonia produced by urealysis in the intestines, and is reported to increase the fecal nitrogen content from these patients. Issues that arise from the use of oxystarch include: the opposing reactions of oxystarch with urea and ammonia presents some degree of variability to the system since urea and ammonia levels are not easily controlled in the GI tracts of different individuals. Secondly, urealysis in the GI tract of different individuals is also an uncontrollable variable and could not be relied upon for quantitative studies. Furthermore, different sources of oxystarch were reported to have different properties that may affect its use as a sorbent. Despite initial findings by Giordano et al that oxystarch is not hydrolyzed in the GI tract [144], Meriwether et al reported that oxystarch prepared from commercial sources of starch and cellulose contains low molecular weight fractions and were reported to penetrate dialysis membranes, raising the question of its possible absorption from the GI tract into the blood circulation [27] when orally administered. This is compounded by the fact that as much as 30 - 40 g of oxystarch should be administered to a patient to obtain 1 g of nitrogen excretion daily [144].

On the other hand, activated charcoal has a long history of medical applications. Its uses range from systemic detoxification due to drug or toxin poisoning [92,94,117,145] to the treatment of intestinal disorders [146]. Indeed, activated charcoal has been extensive studied in hemoperfusion as well as oral administration applications. It is regarded as a well characterized, nontoxic bioadsorbent, and is an excellent candidate for this project. The effectiveness of activated charcoal lies in its large internal surface area, formed from irregular, branched and interconnected passages. Activated charcoal is characterized by its pore size distribution, where the source materials, method and extent of activation will yield pore sizes from less than 10 Å to more than 100, 000 Å (0.01 cm). Therefore, it is important to select activated charcoal particles of the appropriate pore size distribution, for example; the adsorption of high molecular weight molecules requires activated charcoal particles with larger pore sizes and vice versa. In regard to the removal of uremic toxins in renal failure, it was reported

that activated charcoal has a high affinity for small molecules such as creatinine and uric acid but not urea [115]. Although activated charcoal has been used in oral administration for the removal of creatinine and uric acid in renal failure patients, the overall performance of using only activated charcoal is limited by its low affinity for urea. For the first time, this thesis shows a proof-of-concept combination of activated charcoal and probiotic bacteria for the dual function of removing creatinine as well as urea.

2.6.3 Proposed mode of action of combination microcapsules

The goal of this thesis is to formulate microcapsules containing active agents for orally administration to chronic renal failure or ESRD patients. The microcapsules will serve as mini bioreactors during the GI transit, inactivating or metabolizing important uremic metabolites such as creatinine and urea and entrapping these molecules within the microcapsules for their complete removal in fecal excretion. As such, it is expected that regular ingestion of these microcapsules will act as an adjunct therapy to conventional dialysis, and help to reduce the frequency and duration of dialysis, thereby improve the quality of life of ESRD patients.

The immobilized agents chosen are probiotic bacteria and activated charcoal. Not only do both agents have to be safe, but also efficient in the removal of uremic metabolites. For many decades, activated charcoal is considered as a classical detoxificant used to treat drug overdose, food poisoning etc. In fact, its use in oral administration or hemoperfusion is accepted as common practice and is currently employed in numerous clinical settings. Activated charcoal is used in this project as a non-specific adsorbent to remove small molecules such as creatinine, ammonia etc from the GI tract. In particular, activated charcoal with the smallest diameter distribution will be selected so as to increase the surface-area-to-volume ratio for efficient adsorption.

However, activated charcoal does not have a high affinity for urea [115], an important uremic toxin; therefore a second agent is necessary for its effective removal. Probiotic bacteria was chosen for urea removal largely due to their

ability to respond to conditioning methods for the uptake of a chosen substance, and the fact that they are considered safe for consumption having undergone much scrutiny and testing by the research field. The resulting urea-adapted probiotic bacteria will serve as an economic and readily available source of oral adjuvant for chronic renal failure and ESRD patients. As such, several probiotic bacteria strains will be selected to undergo dynamic metabolic induction to urea, a continuous process of urea adaptation and colony selection. This process will be repeated with small and gradual increase of urea added to the media culture, so as to yield a highly efficient strain to be microencapsulated and tested in-vitro.

A brief research plan includes adapting several probiotic bacteria strains for urea uptake, testing the effect of activated charcoal on the bacteria, optimization of the encapsulation process to produce microcapsules containing a combination of activated charcoal and probiotic bacteria, in-vitro studies of the microcapsules immersed in simulated gastric media containing known concentrations of the uremic analytes, bacterial viability in storage conditions etc.

2.6.4 Microcapsule material considerations

Unlike cell encapsulation, the encapsulation of activated charcoal is comparatively a harsher process, and the resultant beads withstand stronger sterilization techniques such as autoclaving without losing the functional properties of activated charcoal. For example, to make the fixed-bed charcoal system, charcoal granules are fixed on tapes pre-wetted with chlorosulfonated polyethylene [115]. Since live bacterial cells are to be encapsulated together with activated charcoal, conventional methods of coating activated charcoal cannot be used. Instead a mild and gentle microencapsulation method is needed to preserve the viability and functionality of the bacterial cells.

The microcapsules formulated in this project are meant for oral administration; it is no longer pertinent to keep to stringent immunoprotective properties, although biocompatibility of the microcapsule core is necessary to maintain the viability of the immobilized bacterial cells. Since alginate is a well established polymer used in the encapsulation of a wide variety of cell types, it

will be a first choice for the immobilization of the bacterial cells and activated charcoal.

It is also necessary to coat the calcium-alginate gel beads with a membrane layer to prevent or reduce the swelling of the beads. This destabilization occurs because the Ca^{2+} ions in the alginate gel are exchanged by chelators such as phosphate, lactate, citrate or non-gelling cations such as Na^{+} or Mg^{2+} ions [147] present in the GI tract. Furthermore, the proposed formulation requires the immobilization of activated charcoal, which is a non-specific adsorbent and rapidly saturates. Immobilizing these particles in the uncoated porous calcium-alginate gel beads may result in pore saturation. Therefore, a membrane layer enveloping the calcium-alginate core may help limit the entry of larger molecules into the microcapsule; such well established and biocompatible choices include PLL and chitosan.

As previously mentioned, chitosan forms a stronger membrane around the alginate core, which will improve mechanical properties of the microcapsule. Since the incorporation of activated charcoal particles within the microcapsule design results in the formation of larger sized calcium-alginate beads, these gel beads have reduced mechanical strength and will require a strong membrane. Although the APA microcapsule is a suitable microcapsule configuration due to its high biocompatibility, it has been reported to display weakened mechanical strength [98]; this mechanical strength will be further weakened with the larger sized microcapsule configuration used in this project. Therefore, chitosan will be used and tested as a suitable choice for the encapsulation of the bacteria and activated charcoal.

2.7 Research justification

As indicated above, although current renal replacement therapies alleviate the uremic syndrome in renal failure patients they are not affordable and are associated with life threatening complications. Therefore a safe, convenient, and affordable oral adjunct is needed to help reduce the frequency and duration of dialysis procedures. It is with this goal that this thesis investigates the efficacy of

a combination microcapsule containing the well established adsorbent activated charcoal, and metabolically induced probiotic bacteria in the removal of important metabolic wastes in-vitro.

Preface for Chapters 3, 4, 5 and 6

Results of the experiments performed are presented in Chapters 3, 4, 5 and 6. Chapter 3 compares the dynamic metabolic induction profiles of *Lactobacillus acidophilus* and *Bifidobacterium longum* (cultivated in both RCM and MRS media) at both high and low levels of urea, and investigates the effect of encapsulation within an alginate matrix with a chitosan membrane on bacterial viability. The most responsive bacterium chosen is *Lactobacillus acidophilus.* Chapter 4 describes in-vitro studies of the effect of bacterial microcapsules as well as combination microcapsules containing both bacterial cells and activated charcoal on creatinine levels over a period of 48 hours. The effect of activated charcoal on the viability of the bacterial cells during stomach transit is also investigated. Chapter 5 describes the effect of the aforementioned microcapsules on urea levels in a period of 48 hours. Viability studies in storage conditions were also investigated. Finally, Chapter 6 shows other not published results obtained from the in-vitro experiments. The microcapsules removed creatinine and urea as shown in Chapters 4 and 5, but not potassium, sodium and chloride. Phosphate levels however showed change and the effects of the microcapsules on phosphate were discussed.

Research articles presented in Chapters 3, 4 and 5:

1) **Trisnawati Halim**, Arun Kulamarva, Fatemeh, Afkhami and Satya Prakash. Metabolic Induction of Probiotic Bacteria for Urea Uptake – Applications in Encapsulation and Oral Administration as an Adjunct for Kidney Failure. Proceedings of the International Federation for Medical and Biological Engineering (IFMBE), Volume 12, 2005. Published.

2) **Trisna Lim**, Ouyang Wei, Christopher Martoni and Satya Prakash. Encapsulated *Lactobacillus acidophilus* and activated charcoal for creatinine reduction in-vitro. Biotechnology Letters, 2005. Under revision.

3) **Trisna Lim**, Hongmei Chen, Arun Kulamarva and Satya Prakash. Effect of metabolic induction of *Lactobacillus acidophilus* on urea reduction – combination with activated charcoal for applications in renal failure. Enzyme and Microbial Technology. Under consideration.

Contributions related to the current research that is not included in the thesis:

1) Satya Prakash, Ouyang Wei and **Trisna Lim,**. Applications of artificial cells in renal failure. Book chapter. Under revision.

Abstracts:

1) **Trisnawati** Halim, Ouyang Wei, Hongmei Chen, Fatemeh Afkhami and Satya Prakash. Activated charcoal and probiotic bacteria in microcapsules for creatinine reduction - adjunct therapy for kidney failure. European Society for Artificial Organs (ESAO) XXXII Congress, October 5-8, 2005, Bologna, Italy.

2) **Trisnawati Halim**, Arun Kulamarva, Fatemeh, Afkhami and Satya Prakash. Metabolic Induction of Probiotic Bacteria for Urea Uptake – Applications in Encapsulation and Oral Administration as an Adjunct for Kidney Failure. Annual 12th International Conference on Biomedical Engineering (ICBME), December 7-10, 2005, Singapore.

Contribution of authors:

I am the first author for several of the above mentioned articles and there I was responsible for the design of the research, experiment procedure and data analysis. All co-authors have contributed significantly to the research. Dr. Satya Prakash, the last author, is the research author as well as correspondence author for all manuscripts, abstract, and proceedings written during the course of the Masters project.

Contributions to other research:

I am listed as co-author in the following articles and have contributed significantly in these areas:

1) Fatemeh Afkhami, Ouyang Wei, Hongmei Chen, Jasmine Bhathena, **Trisnawati Halim** and Satya Prakash. In-vitro analysis of APPPA microcapsules for oral delivery: impact of microcapsules on GI model flora. European Society for Artificial Organs (ESAO) XXXII Congress, October 5-8, 2005, Bologna, Italy.

2) Hongmei Chen, Ouyang Wei, Bisi Lawuyi, **Trisnawati Halim** and Satya Prakash. A new method for microcapsule characterization: fluorogenic genipin can be used to characterize polymeric microcapsule membranes. Applied Biochemistry and Biotechnology. In Press.

3) Terrence Metz, Mitchell L. Jones, Hongmei Chen, **Trisnawati Halim**, Tasima Haque, Amre Devendra, Sujata K. Dass and Satya Prakash. A new method for targeted drug delivery using polymeric microcapsules: implications for treatment of Crohn's disease. Cell Biochemistry and Biophysics, Volume 43, Issue 1, August 2005.

Chapter 3 :

Metabolic Induction of Probiotic Bacteria for Urea Uptake – Applications in Encapsulation and Oral Administration as an Adjunct for Kidney Failure

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Chapter 3: Metabolic Induction of Probiotic Bacteria for Urea Uptake – Applications in Encapsulation and Oral Administration as an Adjunct for Kidney Failure

3.1 Abstract

Probiotic bacteria *Lactobacillus acidophilus* ATCC 314 and *Bifidobacterium longum* ATCC 55813 have been metabolically induced for urea uptake over 180 culture passages. *Lactobacillus acidophilus* responded better to the induction, with a urea uptake of 70 mg/dL at the 100th induction day to 200 mg/dL after 142 days of dynamic urea induction. *Bifidobacteria longum* profiled a decreasing trend despite increasing urea levels, with 150mg/dL urea uptake at the 100th day and 120 mg/dL after 142 days. The results of this study will allow for the use of probiotic bacteria in encapsulation and its oral administration for use as an adjunct therapy in renal failure.

3.2 Introduction

In recent years, probiotic bacteria have been attributed to maintenance of host health by nutrient processing and absorption [135], homeostasis of the intestinal epithelium [148], regulation of fat storage [136] etc. Not only has probiotic bacteria been shown to prevent and ameliorate diarrhoeal illnesses, its efficacy has been reported in the treatment of irritable bowel syndrome [137], Helicobacter pylori infection [39,83,84], inflammatory bowel disease [86], or as a potential alternative therapy to broad spectrum antibiotics and the resulting microbial infections.

In particular, *lactobacilli* and *bifidobacteria* have been associated with host benefits and were recently labeled as a functional food. Indigenous to the human GI tract, the bacteria have evolved to tolerate stress factors. In fact, there are increasing evidence of the adaptive mechanism of *Bifidobacterium longum*, such as its different levels of protein expression when exposed to bile salts [42,143]. Previous research has also demonstrated the feasibility of decreasing urea concentration in-vitro by exposing *Lactobacillus delbrueckii* to urea rich media for 20 cycles [37]. As such, the potential role of probiotic bacteria in renal failure lies in their ability to regulate catabolic and anabolic pathways in response to a

particular stimulus. In this study, *Bifidobacterium longum* and *Lactobacillus acidophilus* have been chosen as candidates for urea removal by dynamic metabolic induction where both bacteria will be subjected to a series of selection and comparative process.

Given the financial strains of dialysis [149], novel adjunctive therapies such as this may be valuable in providing a convenient, affordable treatment for a larger renal failure population. For the first time, this study will describe a method of dynamic metabolic induction where probiotic bacteria will be adapted for the removal of the uremic toxin urea. The main part of this study compares the inductive capacities of two probiotic candidates, using increasing urea levels as a stimulus, followed by a screening process. The selected bacterium was then harvested for encapsulation in the next part of the study for initial viability studies.

Encapsulation technology has found extensive use in medical applications for the controlled release of active therapeutic agents from within the capsule, such as treatments for tuberculosis [150], diabetes [151], Alzheimer's disease [152] etc. This technology has seen much advancement in the search for a biocompatible, non-toxic polymer for a variety of applications. In particular, the extensively studied calcium-alginate matrix is known to support cell growth and will be used in this study, together with the chitosan membrane, for the encapsulation of live bacterial cells. Bacterial viability studies will be conducted to compare colony forming units (CFUs) before and after the encapsulation procedure. The microcapsules containing metabolically induced probiotic bacterium will act as mini bioreactors that transit temporarily in the GI tract for 24 hours before being excreted. The microcapsule offers protection against extreme environments in the GI tract while the porous membrane allows the diffusion of nutrients and uremic toxins, urea in particular, for consumption by the induced bacterium.

The goal of this study is to test the feasibility of inducing probiotic bacteria for toxin uptake, and its encapsulation for oral administration to serve as a safe and convenient adjunct to renal failure patients.

3.3 Materials and Methods

L. acidophilus ATCC 314 and *B. longum* ATCC 55813 were obtained from the American Type Culture Collection, USA. Both strains were cultivated in Lactobacilli MRS Broth (Difco 218081). *B. longum* was also cultivated in Reinforced Clostridial Media (RCM) Broth (Difco 234234) as was specified in the ATCC instructions. Urea (Sigma Chemical Co., USA) was filtered through 0.22 μm filter before adding to the sterilized media. Both media were sterilized in Castle Labclaves for 15 minutes at 121°C. Incubation was done overnight at 37°C in anaerobic conditions, constituting one passage.

L. acidophilus and *B. longum* were cultivated in MRS medium, and *B. longum* also cultivated in RCM medium, at a 1:15 inoculum to media ratio. Urea was added at an initial concentration of 50 mg/dL, repeatedly increased by 0.3 g/dL steps, to about 2.7 g/dL. The bacteria were adapted at each urea increment level for several passages, before screening for visible colonies on agar plates containing additional 0.3 g/dL urea. The visible colonies were re-inoculated in MRS medium with the increased urea concentration of the urea-enriched (UE-) agar plate. This process was stopped at 2.7g/dL urea concentration (corresponding to 140 days or p140), after which further increase of urea resulted in a bacterial growth too scant for practical use. The bacteria cultures were subsequently grown at 2.7 g/dL UE-media until p180 to examine the effect of a prolonged consistent urea concentration on their ability to consume urea. Control tests included cultivating the bacteria in un-modified media at similar anaerobic conditions and temperature.

Sampling of the media was done every 20 days, in which 1.0 mL bacterial culture was centrifuged for 10 minutes at 10 000 g at 4 °C. The supernatant was collected and tested for urea levels using a direct colorimetric assay (Stanbio BUN kit, USA). Where appropriate, the supernatant was diluted to allow for the quantification of urea in the linear range of the test kit.

The two bacteria *L. acidophilus* and *B. longum* cultivated UE-MRS medium was selected for re-adaptation to lower urea levels - 150 mg/dL - similar to pathological levels in renal failure patients. This is the final selection stage to

select for the best candidate for in-vitro experiments. Briefly, the bacterial pellet was washed three times in physiological solution (PS) (0.85 % (w/v) sodium chloride) then re-suspended in 150 mg/dL UE-MRS medium for 12 hour induction cycles for 5 cycles. The induction process showed *L. acidophilus* as the best candidate for urea removal and was used for further encapsulation and viability studies.

Calcium-alginate beads containing *L. acidophilus* bacterial cells were prepared based on microencapsulation procedures described elsewhere [153]. Cultivated in 150 mg/dL UE-MRS medium, *L. acidophilus* was harvested at late log phase, which had been determined turbidimetrically at 660 nm. The bacterial pellet was collected by centrifugation at 10 000 g for 20 minutes at 4 °C. The cell mass was washed three times in PS, and re-suspended in PS, followed by addition of 0.22 μm filtered sodium alginate solution 1.65% (w/v) dissolved in PS at a 1:9 ratio. The bacterial suspension was extruded through a 600 μm diameter nozzle with pressurized nitrogen using an INOTECH encapsulator that was adjusted to output pressure at 0.2 bar, frequency 450 Hz and voltage 0.25 kV. The droplets were stirred gently in calcium chloride solution, 1.0 M (w/v) for 30 minutes and allowed to gel at 4 °C for 2 hours. Chitosan-alginate microcapsules were prepared by immersing the alginate beads in a 0.5 % (w/v) chitosan solution in 0.5 % acetic acid (w/w), pH adjusted to 4.6, for 30 minutes. The resulting microcapsules were washed three times and stored in PS at 4 °C until further use.

For viability studies, 0.1 mL of bacterial culture was plated on 150 mg/dL UE-MRS agar plates before encapsulation. After encapsulation 0.1 mL of microcapsules was immersed for 10 minutes in 3 % (w/v) sodium citrate solution to liquefy the capsules, after which they were manually ruptured using a rubber pestle for serial dilution and plating.

3.4 Results

The control groups - *L. acidophilus* and *B. longum* in their respective un-modified media – did not show differences in urea levels in the media before and after bacterial cultivation (data not shown).

The urea consumption profile from p100 to p180 (earlier data not available) was plotted as a function of time in Figure 1. Except *B. longum* in UE-RCM medium, *L. acidophilus* and *B. longum* that were cultivated in UE-MRS medium consumed urea. Of these two bacteria, *B. longum* started consuming more urea than *L. acidophilus*, then its intake decreased despite increasing urea levels. From p140, both's urea consumption decreased although urea levels were constant. These two bacteria were re-adapted to 150 mg/dL UE-MRS media. *L. acidophilus* showed significantly higher consumption than *B. longum*, especially at the third induction cycle (Fig. 2).

In order to determine the late log phase of *L. acidophilus* for encapsulation, its growth profile was obtained by turbidimetric measurement (Fig. 3). This corresponds to 10 hours after inoculation.

Encapsulation of the live bacterial cells results in microcapsules of with an average dimension of 630 μm/ 767 μm (minor/major diameter), ± (24 μm /27 μm), n=30. The majority of the microcapsules formed "egg" shaped or oblate ellipsoidal capsules, and about 1 % capsules are formed larger and more spherical. This is due to the irregularity of the applied air pressure.

0.1 mL microcapsules contain $2.9x10^8$ CFU/mL in 300 mL UE-MRS medium before encapsulation, and $1.2x10^9$ CFU/mL in 30 mL alginate solution after encapsulation. Taking into account dilution factor of 10, there is a slight decrease of CFU counts in the same order of magnitude.

3.5 Discussion

The first part of this study is to compare and select the probiotic bacterium best adapted for urea uptake. The first round of selection consists of exposing three bacteria groups to extreme levels of urea in graduated steps. The lack of response by *B. longum* in UE-RCM medium demonstrated the importance of culture media in the activation of particular genes. While both *L. acidophilus* and *B. longum* cultivated in UE-MRS medium consumed urea, their inductive capacities decreased when urea levels stayed constant (Fig. 1). An implication of this may be to utilize the bacteria at a specific passage while its inductive capacity

is at its peak, before inductive genes are switched off. Directly exposing the bacteria from high to low urea levels elicited no immediate response (data not shown), indicating a need for re-adaptation to the pathological urea range, where a high end concentration of 150 mg/dL was chosen. Besides re-adaptation, the second round of selection also allowed for the comparison of both bacteria at the pathological level, which is necessary in case of possible changes in their inductive potentials at a much lower urea concentration. It is certain that *L. acidophilus* demonstrated better adaptation in 150 mg/dL UE-MRS medium (Fig 2), and was selected for further studies.

Harvesting *L. acidophilus* after 10 hours of incubation ensures the viability and functionality of the majority of the cells for encapsulation. The mix of smaller "egg" shaped capsules and larger spherical capsules are attributed a combination of the 600 μm nozzle and the air pressure that is needed to extrude beads of such large size. Regular, spherical capsules are obtained with smaller 300 μm nozzles where a mechanical pump is sufficient to produce the required extrusion pressure. The large nozzle is used in this study as there is a possibility of co-encapsulating the probiotic bacteria with larger sorbent particles.

The viability of the encapsulated bacteria dropped slightly possibly due to the stresses that the bacteria are under from the encapsulation and coating process. Furthermore there may be an underestimation of the bacterial viability within the capsules due to erroneous packing in the 0.1 mL volume as a result of the large size of the microcapsules. Therefore, this decrease is considered an acceptable one.

3.6 Conclusion

This study reports the feasibility of metabolically inducing probiotic bacteria *L. acidophilus* and *B. longum* by increasing the urea concentrations in their media in a controlled manner. Their different urea consumption profiles at two different urea levels is indicative of how external urea levels affect their inductive capacities; however this pathway is not well understood and needs further experimentation.

Viability studies showed that encapsulation does not drastically lower viability of the encapsulated bacteria. Further studies are needed to ascertain the potential of encapsulated induced bacteria for urea removal.

3.7 Acknowledgements

This work was supported by research grants (to Prakash) from Natural Sciences and Engineering Research Council (NSERC) of Canada and Canadian Institute of Health Research (CIHR). NSERC post graduate scholarship to Halim and postgraduate scholarship from Iranian Ministry of Health and Medical Education to Afkhami are acknowledged.

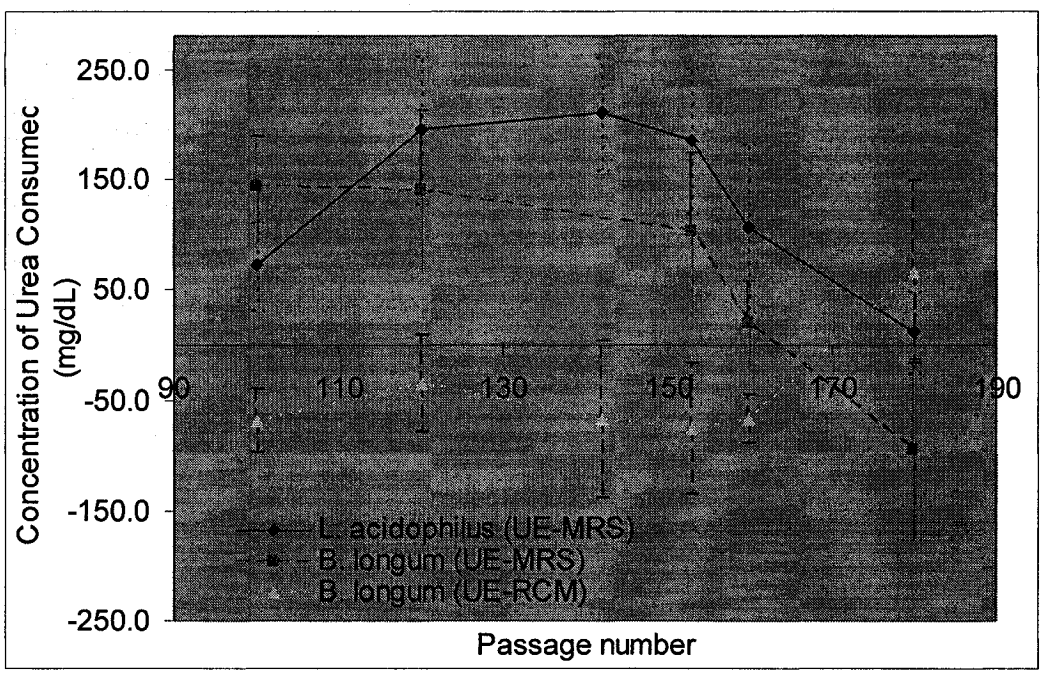

Figure 3.1: Urea consumption profile by *L. acidophilus* (urea-enriched - MRS media), *B. longum* (urea-enriched – MRS media), and *B. longum* (urea-enriched - RCM medium) from p100 to p180 of metabolic induction with increasing urea levels. Urea levels were increased until p140 to 2.7 g/dL, and kept constant at 2.7 g/dL from p140 to p180.

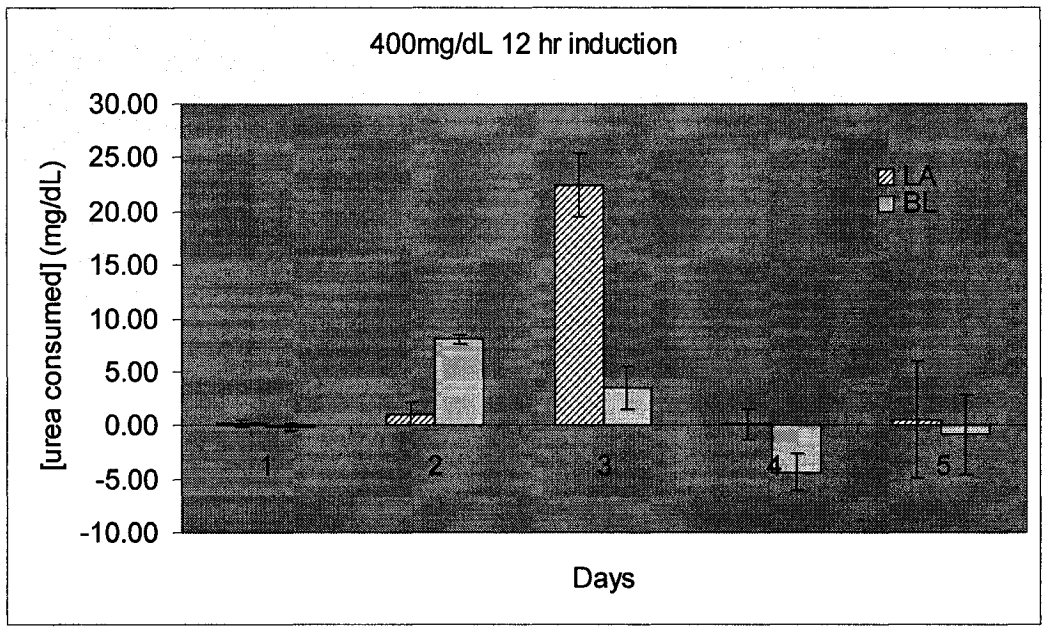

Figure 3.2: Urea consumption profiles for 5 12-hour induction cycles to 150 mg/dL urea-enriched MRS medium. Average cell mass responsible for urea consumption shown is *L. acidophilus*: 23.33±2.05 mg, *B. longum*: 28.63±1.31 mg.

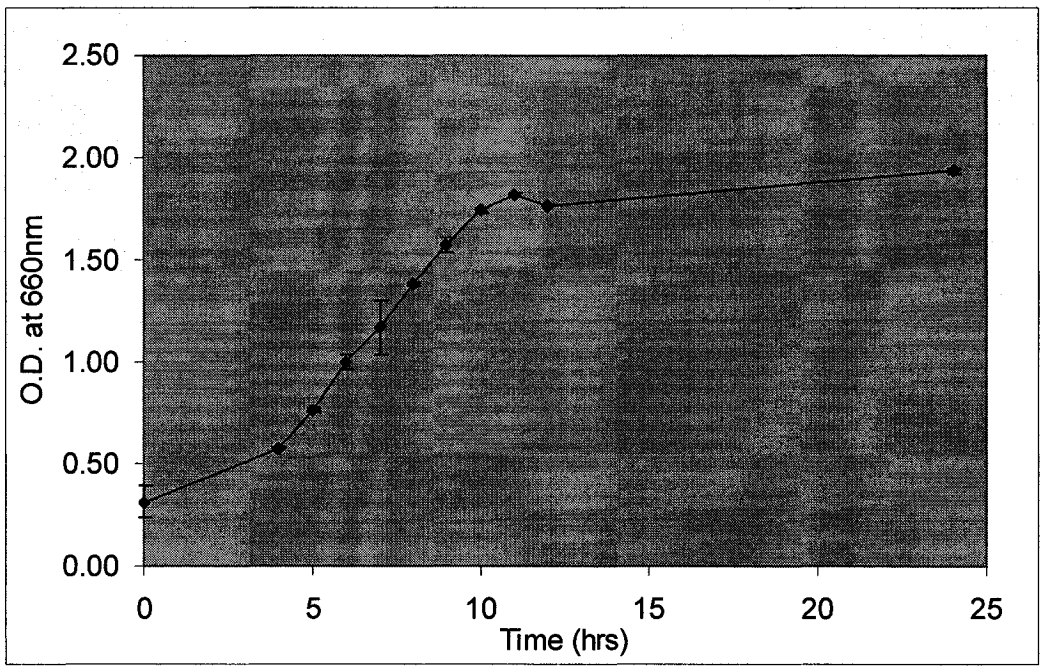

Figure 3.3: 24 hour growth profile of *L. acidophilus* in UE-MRS medium. Optical density measurements were taken at 660 nm (n=3).

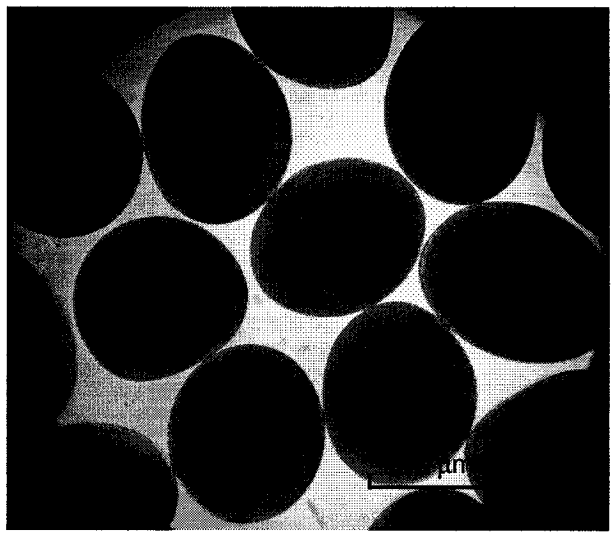

Figure 3.4: Photomicrograph of live *L. acidophilus* cells encapsulated in Chitosan-Alginate microcapsules in physiological solution directly after encapsulation. Microcapsule cell density is at $1.2x10^8$ CFU/mL. Minor diameter = 630 μm ± 24 μm (n=30). Magnification x47.

Chapter 4:

Encapsulated *Lactobacillus acidophilus* and activated charcoal for creatinine reduction in-vitro

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Chapter 4: Encapsulated *Lactobacillus acidophilus* and activated charcoal for creatinine reduction in-vitro

4.1 Abstract

Activated charcoal was microencapsulated with probiotic bacteria *Lactobacillus acidophilus* previously adapted for urea uptake. The creatinine removal capacity of this combination microcapsule was evaluated in-vitro in media simulating the small intestine. Results show that microcapsules containing activated charcoal or *L. acidophilus* demonstrated potential for decreasing creatinine; a reduced creatinine lowering capacity was observed when activated charcoal and *L. acidophilus* were co-encapsulated. There was also enhanced bacterial cell viability due to activated charcoal co-encapsulation. These results suggest the feasibility of using microcapsules containing activated charcoal and probiotic bacteria as an oral adjuvant for creatinine removal.

Key words: activated charcoal, creatinine, microencapsulation, oral therapy, renal failure

4.2 Introduction

Creatinine, a product constantly produced in muscles from the breakdown of creatine, is accumulated in the blood when renal excretory functions are impaired. This build up is indicative of the late stages of chronic renal failure and must be removed. Currently, dialysis offers an excellent renal replacement therapy for chronic renal failure and end stage renal disease (ESRD) patients by the removal of unwanted metabolites such as creatinine. Dialysis, however, has substantial limitations. For example, it is associated with complications such as cardiomyopathy-related deaths [6], peritonitis [154], dialysis related amyloidosis [155] etc. The staggering cost of dialysis also places a tremendous burden on the health care sector in developed countries, and the majority of the population in developing countries cannot afford this treatment. Furthermore, synthetic kidney functions, such as vitamin D and erythropoietin production, are not achieved in dialysis. Kidney transplantation answers these needs, but this treatment does not

reach the majority of the ESRD population due to shortage of donors and eligibility issues.

The oral administration of encapsulated bacterial cells was proposed for renal failure treatment [36,37] but this approach was not realized due to the insufficient efficacy of the bacterial cells. Alternatively, the oral administration of encapsulated indigestible adsorbents was proposed for the removal of uremic toxins such as urea, uric acid and creatinine from the GI tract [27,78,156], but adsorbents such as oxystarch and zirconium phosphate-urease were limited by either large dosages or the increase of other undesirable molecules [27,28,125]. On the other hand, activated charcoal is a well established, nontoxic adsorbent for creatinine and uric acid, but its use is limited due to its low affinity for urea [78,157]. This article for the first time explores the combination of live bacterial cells and activated charcoal. Specifically, the aim of this study is to examine the efficacy of creatinine removal by microcapsules containing activated charcoal and metabolically induced *L. acidophilus* cells in in-vitro conditions simulating those of the small intestine.

The ultimate goal is to orally administer these microcapsules as mini bioreactors for the entrapment of uremic toxins in the GI tract, followed by their removal in fecal excretion. Such a method of removing systemically circulating uremic toxins from the GI tract is feasible due to evidence of the exchange of uremic metabolites between the lumen of GI tract and its surrounding capillaries [123,124]. The therapeutic potential of such a phenomenon was capitalized by researchers in the 1960s, who reported on the feasibility of intestinal perfusion as a means to remove uremic toxins to manage renal failure [158,159]. In particular, it was reported that the clearance of urea [160], creatinine and uric acid [161] was highest in the jejunum. As intestinal perfusion procedures need to be done at frequent intervals to prevent the build up of uremic toxins such as creatinine, they are not only expensive but also lower the quality of life of these patients.

In this study we have encapsulated activated charcoal and probiotic bacteria, and evaluated their creatinine removal efficacy for potential applications in renal failure therapy. If successful, this approach would provide a model where,

by varying the type of bacterial cells for the metabolism of specific unwanted metabolites, microcapsules containing probiotic bacteria and activated charcoal may be developed as an oral adjunct therapy in renal failure treatment.

4.3 Materials and Methods

Chemicals

Sodium alginate (low viscosity), creatinine and urea were purchased from Sigma-Aldrich, USA. Chitosan (low viscosity) was obtained from Wako Chemicals, Japan. Activated charcoal (Norit E Supra USP) was obtained from Norit Americas Inc., USA. Oxgall (dehydrated Fresh Bile) was obtained from Difco, USA, and pancreatin purchased from Acros Organics, USA. All other chemicals were of analytical reagent grade and not purified further before use.

Metabolic Induction of L. acidophilus

Lactobacillus acidophilus 314 was obtained from ATCC. Bacterial cultures were maintained in Lactobacilli MRS Broth (Difco, USA) overnight at 37 °C in anaerobic conditions. *L. acidophilus* was cultivated in 1.5 mL MRS broth, with urea levels starting from 50 mg/dL, and increments of 0.3 g/dL, to 2.7 g/dL. The urea solution was filtered through 0.22 μm filter and added to autoclaved MRS broth. At each urea increment, the bacterium was cultivated for several passages for adaptation, before screening for healthy colonies on 0.3 g/dL higher urea-enriched MRS agar plates. The colonies were then re-inoculated into MRS broth at the urea concentration of the urea-enriched agar plate. This process was stopped at 2.7 g/dL urea concentration (corresponding to 120 days), after which further increase of urea resulted in a bacterial growth too scant for practical use. The induced *L. acidophilus* was re-adapted to a lower urea level – 150 mg/dL – similar to pathological levels in renal failure patients and used for in-vitro experiments.

Microcapsule Preparation

Chitosan-alginate microcapsules containing bacterial cells and/or activated charcoal were prepared based on microencapsulation procedures described in a previous report [162]. *L. acidophilus* was grown in 150 mg/dL urea-enriched MRS solution, harvested at late log phase, and collected by centrifugation at 10 000 *g* for 20 minutes at 4 °C. The media solution was discarded and the cell mass washed three times with physiological solution (0.85 % (w/v) sodium chloride). 1.65 % (w/v) sodium alginate in physiological solution (PS) was sterile filtered through a 0.22 μm filter. 0.9 g *L. acidophilus* cells were re-suspended in alginate/physiological (PS) (9:1, v/v) reaching a final volume of 30.0 mL. The bacterial suspension was extruded through a 600 μm diameter nozzle with pressurized nitrogen using an INOTECH encapsulator that was adjusted to a frequency of 450 Hz, a voltage of 0.25 kV and an output pressure of 0.2 bars. The droplets were stirred gently in chilled calcium chloride solution, 1.0 M (w/v), for 30 minutes and allowed to gel at 4 °C for 2 hours. Chitosan-alginate microcapsules were prepared by immersing the alginate droplets in a 0.5 % (w/v) chitosan solution in 0.5 % acetic acid (w/w) that is pH adjusted to 4.6, for 30 minutes. Microcapsules formed were washed and stored in PS at 4 °C until further use.

Microcapsules containing a combination of bacterial cells and activated charcoal were prepared according to the procedure described above except that activated charcoal was added to the bacterial-alginate mixture prior to droplet extrusion. The encapsulator settings differ for the extrusion of different groups of droplets and are shown in Table 4.1.

4.4 Results and Discussion

The well established adsorbent properties of activated charcoal and the inductive potential of bacteria have motivated their encapsulation for the removal of the uremic toxin creatinine in the GI tract. In-vitro studies were focused on two pHs (pHs 6.4 and 7.8) representative of the proximal and distal sections of the small intestine. Since exchange of molecules between blood capillaries and the GI

cavity is the highest in the small intestine, the encapsulated bioreactors are likely to be most effective within this section at the pHs of 6.4 and 7.8. All values represented in the graphs (Fig. 4.1) are averages of triplicates or more set of experiments and the bars indicate standard deviation.

To visualize the efficacy of the microcapsules in decreasing creatinine, it may be beneficial to include values in terms of the number of moles of creatinine removed per gram microcapsules, as shown in Table 4.2.

Effect of capsules on creatinine exposure at pH 6.4 (proximal section of small intestine)

Experiments were designed to evaluate the effect of encapsulated *L. acidophilus*, activated charcoal, and microcapsules containing both *L. acidophilus* and activated charcoal, in a reaction medium containing creatinine. These three groups were compared against two negative controls: the reaction media without microcapsules, and empty microcapsules containing neither bacteria nor adsorbent. After incubating the microcapsules in simulated GI media of the proximal and distal sections of the small intestine for 48 hours in anaerobic conditions, it was shown that *L. acidophilus* microcapsules removed 204 μmol/L creatinine compared to creatinine removal of 103 μmol/L by empty microcapsules. This suggests that *L. acidophilus* has the ability to metabolize creatinine, and that the empty capsules were able to trap creatinine following its diffusion into the alginate matrix, thus ensuring the complete removal of creatinine from the GI tract in fecal excretion after 24 hours. The largest removal of creatinine was achieved by activated charcoal microcapsules, where 379 μmol/L creatinine was removed in the same period.

The combination of *L. acidophilus* and activated charcoal is not a superposition of their individual effects, where only 309 μmol/L creatinine was removed compared to 379 μmol/L by activated charcoal microcapsules. This may be due to physical obstruction of the pores on activated charcoal particles by *L. acidophilus,* resulting in the inability to utilize the full capacity of the adsorption area.

From Table 4.2, the creatinine removal capacities of the microcapsules are compared per gram microcapsules, and these values support the discussion above. Creatinine was removed at the capacities of 1.26 μmol creatinine/g activated charcoal microcapsules, and at 0.71 μmol creatinine/g *L. acidophilus* microcapsules. The combination microcapsules showed a creatinine removal capacity of 0.97 μmol creatinine/g combination microcapsules. This decreased creatinine removal capacity is due to obstruction of the activated charcoal pores by the bacterial cells.

Effect of capsules on creatinine exposure at pH 7.8 (distal section of the small intestine)

When the experiment was repeated at pH 7.8, *L. acidophilus* microcapsules did not display the same ability to remove creatinine as was observed at pH 6.4; 250 μmol/L creatinine was removed after 48 hours, which was comparable to the 253 μmol/L creatinine removed by the empty microcapsules in the same period. This is due to the fact that the pH range for *L. acidophilus* survival is 4.0 – 6.4; therefore the slight basicity at pH 7.8 may have ceased *L. acidophilus* activity. As was observed at pH 6.4, activated charcoal microcapsules showed the largest creatinine decrease of 533 μmol/L. This suggests that activated charcoal's affinity for creatinine is not pH dependent within this range. Physiologically this means that activated charcoal will function throughout the length of the small intestine, and is only limited by saturation effects.

As was observed at pH 6.4, the combination microcapsules removed 438 μmol/L creatinine compared to 533 μmol/L removal by activated charcoal microcapsules despite similar amounts of activated charcoal used for both groups. This also hints at the physical obstruction of activated charcoal pores by the bacteria. Since this is a physical process the reduced efficacy of the combination group is reflected at both pHs to similar extents. The exact mechanism of the interaction between activated charcoal and *L. acidophilus,* as well as, the

metabolic process of creatinine uptake by the *L. acidophilus* cells is not fully understood and further exploration in this area is needed.

A comparison of the creatinine removal capacities can be made from Table 4.2 and supports the discussion above. Creatinine was removed at the capacities of 1.78 μmol creatinine/g activated charcoal microcapsules and 0.83 μmol creatinine/g *L. acidophilus* microcapsules. The creatinine removal capacity between *L. acidophilus* microcapsules (0.83 μmol creatinine/g microcapsules) and empty microcapsules (0.84 μmol creatinine/g microcapsules) were comparable, indicating that *L. acidophilus* did not remove creatinine. Similar to the experiment at pH 6.4, the combination microcapsules removed creatinine at a capacity of 1.40 μmol/g combination microcapsules, lower than that compared to activated charcoal microcapsules.

Comparing the creatinine removal capacity of 1 g microcapsules from the same test group between the two pHs, a higher removal capacity was observed at pH 7.8. This phenomenon is observed across the four microcapsule test groups. This is may be due to the change in properties of the alginate core or the chitosan membrane. However, more studies are needed to examine this change in properties at different pHs.

Effect of activated charcoal on bacteria viability

The addition of activated charcoal to the bacteria culture did not affect bacterial viability. Both *L. acidophilus* capsules and capsules containing activated charcoal and *L. acidophilus* contained 10^9 CFU/mL at the start of the experiment. At the end of 48 hours, microcapsules containing both activated charcoal and *L. acidophilus* decreased CFU counts by one order of magnitude, while *L. acidophilus* microcapsules showed a decrease of 2 orders of magnitude (data not shown).

Acid tolerance test on bacteria viability

Initially *L. acidophilus* microcapsules showed comparable CFU counts compared to microcapsules containing activated charcoal and *L. acidophilus*,

while the latter showed higher CFU counts as time progressed. At the end of 120 minutes, bacterial CFU decreased 1 ½ orders of magnitude in the combination capsule, compared to a reduction of about two orders of magnitude in the *L. acidophilus* capsule (Fig. 4.2). This small but significant difference suggests supportive functions undertaken by activated charcoal to preserve bacterial viability. Depending on food intake, the microcapsules may transit in the stomach for a maximum of 2 hours, and these results indicate the rate of decrease of bacterial viability which will help determine time and dosage administration.

Microcapsule morphology evaluation

This experiment was designed to compare how efficient the microcapsules were at removing creatinine from a simulated GI medium. It is therefore pertinent to standardize microcapsule sizes across the test groups for comparable uptake kinetics. To accommodate the larger activated charcoal particles, a 600 μm nozzle was used for all groups of microcapsules. Extruding the droplets through this larger sized nozzle requires the use of pressurized nitrogen (instead of the mechanical process used with smaller nozzles) to provide sufficient force. While the air pressure may be constant, minute irregularities in the air stream resulted in the formation of two different sizes of microcapsules: 99 % egg shaped or oblate ellipsoidal microcapsules and 1 % larger, spherical microcapsules. The average diameters (minor diameters) of the 99 % microcapsule population are as follows: empty microcapsules: 619 μm $\pm$ 36 μm, *L. acidophilus* microcapsules: 628 μm $\pm$ 24 μm, activated charcoal microcapsules: 629 μm $\pm$ 28 μm and microcapsules containing both *L. acidophilus* and activated charcoal: 640 μm $\pm$ 23 μm, n=30.

4.5 Conclusion

The efficacy of creatinine removal by microcapsule containing *L. acidophilus* and activated charcoal was evaluated in this study. Results show that combination microcapsules were not as efficient as the microcapsules containing activated charcoal. Activated charcoal, however, in the combination microcapsules help preserves bacterial viability in media simulating that in the

stomach, indicating their use in protecting bacterial cells during the gastrointestinal transit when given orally. More studies are needed to understand the metabolic pathway of creatinine in *L. acidophilus* to maximize the probiotic's potential for creatinine uptake. This study may provide a model for the metabolic induction of different bacteria strains for the uptake of unwanted metabolites; also, microcapsules containing activated charcoal and probiotic bacteria may potentially serve as an oral adjuvant to reduce the frequency and duration of dialysis; further research is still required.

4.6 Acknowledgements

This work was financed by Canadian Institute of Health Research (CIHR) Canada. Ouyang received postdoctoral fellowship from Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT) and Lim received postgraduate scholarship from Natuaral Science and Engineering Research (NSERC), Canada.

Table 4.1: InoTech Encapsulator settings for the encapsulation of a) empty capsules, b) capsules containing activated charcoal, c) capsules containing *Lactobacillus acidophilus*, d) capsules containing both activated charcoal and *Lactobacillus acidophilus.*

Parameters	Microcapsules containing			
	Empty	Activated charcoal	*L. acidophilus*	*L. acidophilus* & activated charcoal
Frequency (Hz)	450	420	430	420
Voltage (kV)	0.25	0.197	0.25	0.197
Output pressure (bar)	0.3	0.25	0.2	0.25
Drop Height (inch)[a]	2.0	1.0	1.5	1.0

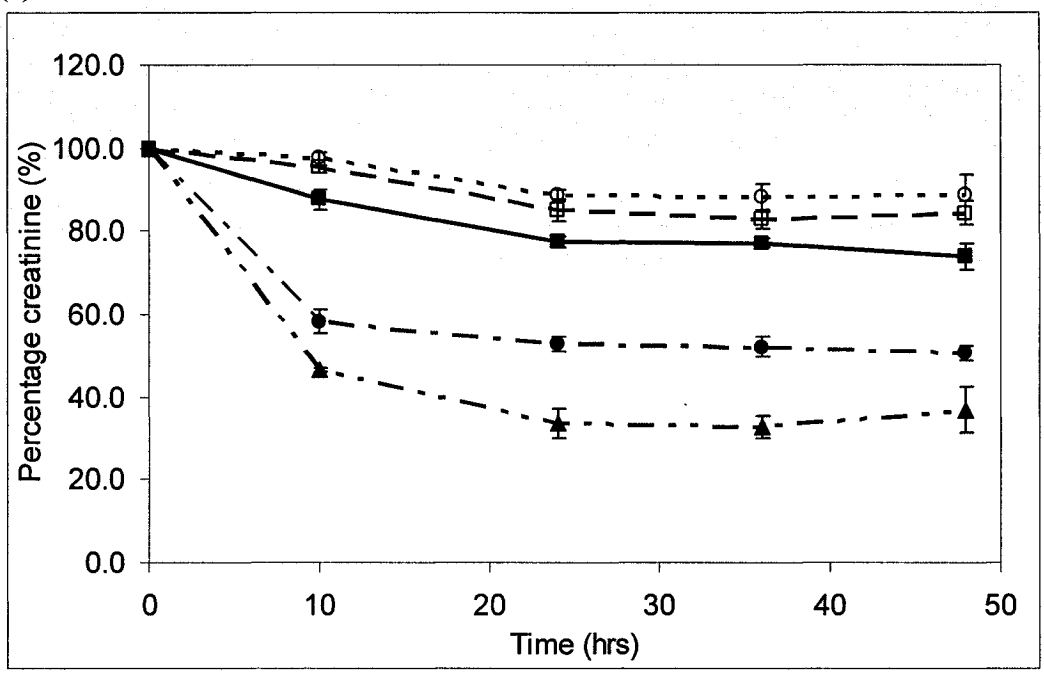
[a]The height refers to the drop height measured from the nozzle tip to the surface of the calcium chloride solution.

Table 4.2: Calculated values[b] showing moles of creatinine removed per gram microcapsules.

Microcapsule groups	Creatinine removed per gram microcapsules	
	pH 6.4 (µmol/g)	pH 7.8 (µmol/g)
Empty microcapsules	0.34	0.84
Activated charcoal microcapsules	1.26	1.78
L. acidophilus microcapsules	0.71	0.83
L. acidophilus & activated charcoal microcapsules	0.97	1.40

[b]The calculation is as follows: The number of moles of creatinine in the 10.0 mL reaction media is first calculated by the equation: Y moles/1000 mL x 10 mL = y moles. The number of moles of creatinine at the end of the 48 hour experiment is also calculated (x moles). Their difference gives the total amount of creatinine reduced (($y - x$) moles). When divided by the wet weight of microcapsules used, this yields the moles of creatinine removed per gram microcapsules.

(a)



(b)

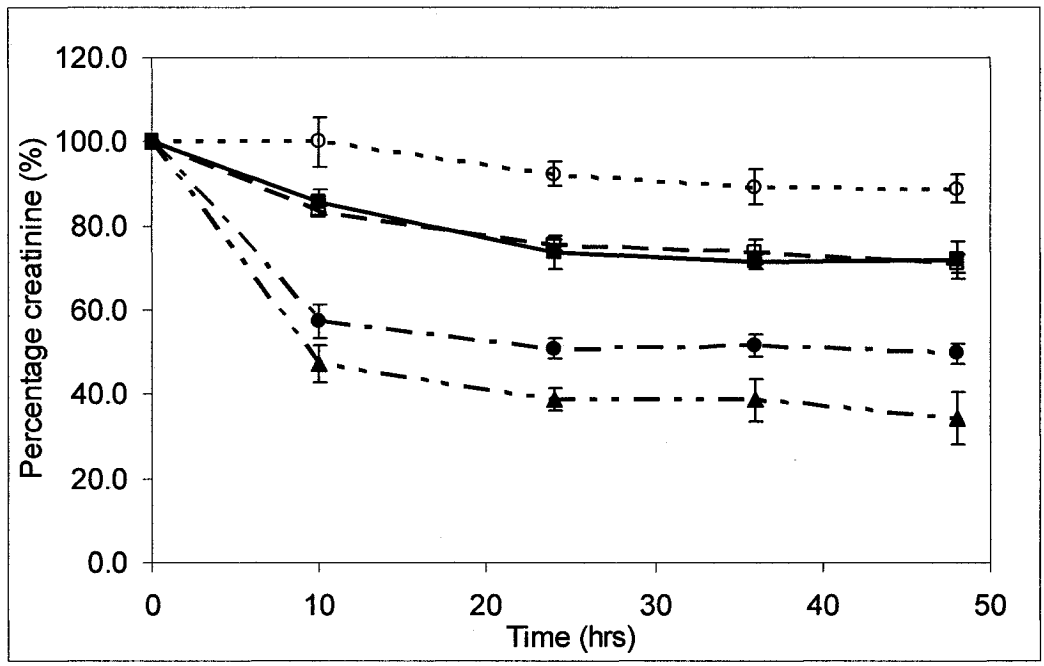


Figure 4.1: Effect of microcapsules on creatinine in simulated GI media over 48 hours.

Panel A: Creatinine profile in simulated GI media at pH 6.4 simulating conditions in the proximal section of the small intestine, for a) no capsules – ○- -, b) empty capsules — □ —, microcapsules containing c) activated charcoal —▲- -,

d) *L. acidophilus* —■— and e) activated charcoal and *L. acidophilus* — ● -. Absolute creatinine concentrations at time zero are a) 927 µmol/L, b) 657 µmol/L, c) 600 µmol/L, d) 782 µmol/L, e) 626 µmol/L.

Panel B: Creatinine profile in simulated GI media at pH 7.8 simulating conditions in the distal section of the small intestine, for a) no capsules - ○- -, b) empty capsules — □ —, microcapsules containing c) activated charcoal —▲- -, d) *L. acidophilus* —■— and e) activated charcoal and *L. acidophilus* — ● -. Absolute creatinine concentrations at time zero are a) 1039 µmol/L, b) 875 µmol/L, c) 812 µmol/L, d) 883 µmol/L, e) 871 µmol/L. *Procedure:* Microcapsules were immersed in simulated GI media (consists of a carbohydrate-based diet with 1.0 g arabinogalactan/L, 2.0 g pectin/L, 1.0 g xylan/L, 3.0 g starch/L, 0.4 g glucose/L, 3.0 g yeast extract/L, 1.0 g peptone/L, 4.0 g mucin/L and 0.5 g cystein/L. Pancreatic juice (containing 6.0 g oxgall/L, 0.9 g pancreatin/L and 12.0 g sodium bicarbonate/L) is added at 27.8 mL/100.0 mL simulated food mixture and is pH adjusted to simulate different sections of the small intestine. 12.5 mg creatinine is added per 100.0 mL simulated GI media. The microcapsules in each test group were immersed in 10.0 mL of the simulated GI media and incubated at 37 °C and anaerobic conditions for 48 hours. 1.0 mL of the synthetic media was sampled at 0, 10, 16, 24, 36 and 48 hours. The samples were centrifuged at 10 000 *g*, 4 °C for 10 minutes to obtain the supernatant for further analysis. *L. acidophilus* was standardized across the test groups that contain the bacterial cells at 1.1×10^9 CFU/mL and 1.2×10^9 CFU/mL for the studies at pH 6.4 and pH 7.8 respectively; and the activated charcoal standardized by weight across the test groups containing it at 54 mg for the studies at both pH. Creatinine concentrations were determined using the 911 Hitachi Blood analyzer.

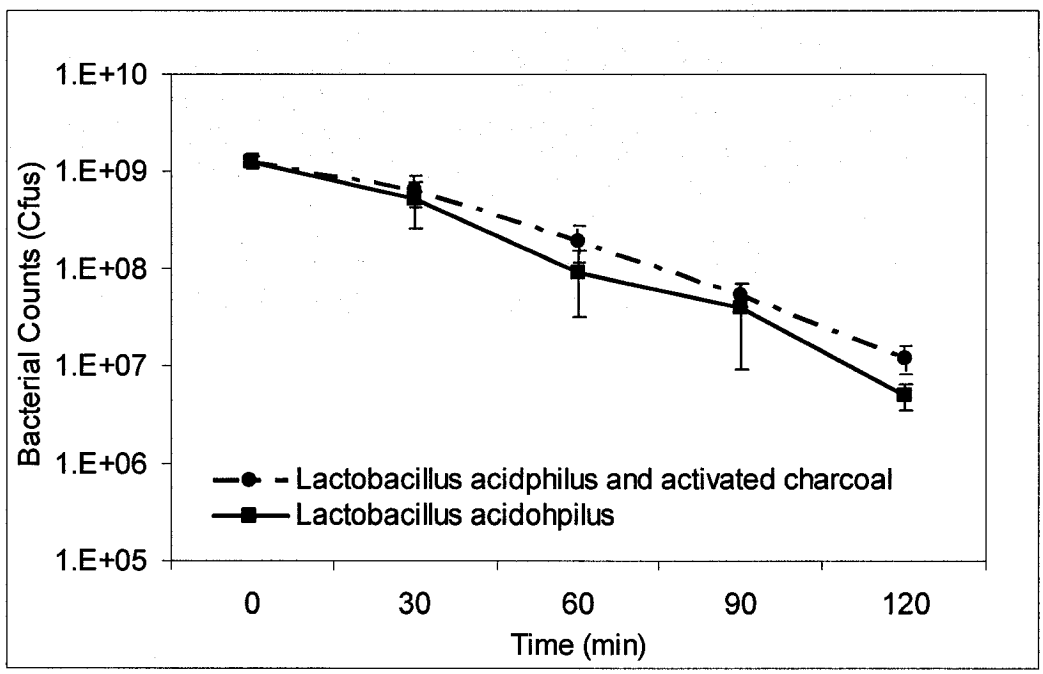


Figure 4.2: Viability profile of microencapsulated *Lactobacillus acidophilus* in pH 1.9 simulated GI media, n=3. *Procedure:* Microcapsules containing a) *L. acidophilus* and b) activated charcoal and *L. acidophilus* are immersed in simulated GI media pH adjusted to 1.9 at a volume ratio of 1:9, and incubated at 37 °C for 120 minutes. Sampling is done at 30 minute intervals, where the microcapsules were immersed in 3 % (w/v) sodium citrate for 10 min, crushed with a capsule crusher and diluted for colony plating.

Chapter 5 :

Effect of metabolic induction of *Lactobacillus acidophilus* on urea reduction – combination with activated charcoal for applications in renal failure

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Chapter 5: Effect of metabolic induction of *Lactobacillus acidophilus* on urea reduction – combination with activated charcoal for applications in renal failure

5.1 Abstract

Probiotic bacterium *Lactobacillus acidophilus* ATCC 314 was metabolically induced over 180 passages for urea uptake. Its urea metabolism peaked at the 142th passage, where 200 mg/dL urea was removed from the culture medium. The adapted bacterium was encapsulated within a calcium-alginate matrix and chitosan membrane. These microcapsules removed a maximum of 57 mg/dL urea when exposed to a simulated gastric intestinal (GI) reaction medium containing 150 mg/dL urea. A second group of microcapsules containing the urea-conditioned probiotic together with activated charcoal reported a lower urea removal capacity. The effect of activated charcoal on bacterial viability in storage conditions was evaluated; the combination microcapsules containing activated charcoal and *L. acidophilus* not only showed significantly higher survival of bacterial cells, and for a longer period of time compared to those containing *L. acidophilus* cells only. Microcapsules containing probiotic bacteria and activated charcoal show potential in lowering urea, and may serve as an oral adjunct therapy to conventional renal replacement therapies.

Keywords: activated charcoal, microencapsulation, probiotics, renal failure

5.2 Introduction

Urea, a major nitrogenous waste produced by the deamination of amino acids, is continuously excreted by healthy kidneys to maintain plasma urea levels within an acceptable range. It is typically measured in blood urea nitrogen (BUN) levels, and normal BUN levels range from 18 mg/dL – 25 mg/dL depending on factors such as sex, age, and existing diseases such as diabetes etc. In renal failure patients, urea is accumulated in the body and must be removed. In fact, BUN tests are commonly used together with creatinine tests to diagnose renal problems.

Maintenance dialysis is the main mode of renal replacement therapy for chronic renal failure or end stage renal disease (ESRD) patients and plays a major role in removing unwanted metabolites including urea. However, long term dialysis is associated with side effects, such as cardiovascular related morbidity and mortality [6], infections [9], inflammation [10] etc, that seriously undermine its benefits. Therefore, there are research interests for the search of new alternatives to existing renal replacement therapies. In particular, the advancement of encapsulation technology has contributed to renal replacement research in many ways. The human nephron filter [163] and bioartificial kidneys [164] are but two of the most recent and sophisticated examples of encapsulation technology. These elaborate and innovative research inventions still need to be optimized and tested before they are made accessible to the renal failure population. It is of concern that such new treatment protocols may not reach the larger ESRD population due to their prohibitive costs, leaving renal failure patients little choice but to depend on current dialysis treatment. In an effort to reduce the duration and frequency of dialysis, this paper evaluates the efficacy of microcapsules containing active agents for oral administration. The exchange of uremic metabolites such as urea between the GI tract and the blood circulation [123,124] forms the basis of oral therapy, where undesirable uremic metabolites are removed by indigestible microcapsules containing therapeutic agents as they pass through the GI tract. Such attempts to alleviate uremia have been made in the past using oxystarch, zirconium phosphate-urease, and activated charcoal; the former two were not realized due to the large dosages required and the increase of other molecules [27,28,125], while activated charcoal saturates rapidly and does not adsorb urea efficiently [78,157]. In recent years, a new system using genetically engineered *E. coli* bacteria that lowered systemic urea when orally administered to rats [165] was reported. However, the associated environmental and safety issues thwarted its potential.

In this study, probiotic bacterium *Lactobacillus acidophilus* was metabolically induced for urea uptake. Not only will the use of a probiotic remove problems linked to genetically engineered agents, but also probiotic bacteria is

associated with benefits such as maintenance of a healthy mucosal barrier [166], treatment of irritable bowel syndrome [167], and treatment of Helicobacter pylori infection [168]. Furthermore, recent research evidence of the large adaptive capacities of probiotic bacteria made it an excellent candidate for metabolic induction [169].

The efficacy of the probiotic bacterium is tested in the presence of activated charcoal, which is a well established adsorbent for exogenous and endogenous toxins. The bacteria and activated charcoal are encapsulated together in microcapsules for the removal of uremic metabolites including urea. Ultimately, the goal of this study is to formulate effective mini bioreactors for the capture and removal of undesirable uremic metabolites such as urea as they pass through the GI tract. This study also presents a model for the metabolic induction of bacteria strains to specific metabolites, for potential therapeutic applications.

5.3 Materials and methods

Materials

Sodium alginate (low viscosity) and urea were purchased from Sigma-Aldrich, USA. Chitosan (low viscosity) was obtained from Wako Chemicals, Japan. Activated charcoal (Norit E Supra USP) was supplied by Norit Americas Inc., USA. Oxgall (dehydrated Fresh Bile) was obtained from Difco, USA, and pancreatin purchased from Acros Organics, USA. All other chemicals were of analytical reagent grade and not purified further before use.

Metabolic induction of L. acidophilus

Lactobacillus acidophilus ATCC 314 was cultured in Lactobacilli MRS Broth (Difco, USA) overnight at 37 °C in anaerobic conditions. The induction procedure was initiated with 50 mg/dL urea that was filtered through 0.22 μm filter and added to autoclaved MRS broth. The urea level was increased by 0.3 g/dL stepwise, to a final concentration of 2.7 g/dL urea enriched MRS broth. *L. acidophilus* was adapted to each urea increment for several passages, after which colonies were screened on urea enriched MRS agar plates containing 0.3 g/dL higher concentration of urea. Visible colonies were re-inoculated into MRS broth

with the increased urea concentration similar to the urea-enriched agar plate. This process continued until an upper limit of 2.7 g/dL urea was reached (corresponding to 120 days), beyond which the bacterial cell mass was too scant for practical use. The induction process continued to passage 180 (p180) at a constant urea level of 2.7 g/dL to observe the effect of a constant urea concentration on urea consumption. The induced *L. acidophilus* was then re-adapted to 150 mg/dL urea levels, similar to pathological levels in renal failure patients, then encapsulated and used for in-vitro experiments.

Microcapsule preparation

Encapsulation of the bacterial cells and/or activated charcoal in a calcium-alginate matrix was based on microencapsulation procedures described elsewhere [162]. *L. acidophilus* was cultivated in 150 mg/dL urea enriched MRS solution, harvested at late log phase, and collected by centrifugation at 10 000 *g* for 20 minutes at 4 °C. Discarding the media solution, the cell mass was washed three times with physiological solution (0.85 % (w/v) sodium chloride). Sodium alginate (1.65% (w/v)) dissolved in physiological solution (PS) had been sterile filtered through a 0.22 μm filter. 0.9 g *L. acidophilus* cells were re-suspended in the alginate solution with additional physiological solution (PS) at a 9:1 ratio attaining a total volume of 30.0 mL. Using an INOTECH encapsulator that was adjusted to output pressure at 0.2 bars, frequency 450 Hz and voltage 0.25 kV, the bacterial suspension was extruded through a 600 μm diameter nozzle with pressurized nitrogen. The process produced calcium-alginate droplets, which were gently stirred in chilled 1.0 M $CaCl_2$ solution for 30 minutes and allowed to gel at 4 °C for 2 hours. The droplets were then immersed in a 0.5 % (w/v) chitosan solution dissolved in 0.5 % acetic acid (w/w) and pH adjusted to 4.6, by immersion for 30 minutes. Microcapsules formed were washed and stored in PS at 4 °C until needed.

Combination microcapsules containing bacterial cells and activated charcoal were prepared according to the procedure described above, except that activated charcoal was added to the bacterial-alginate mixture prior to droplet extrusion. To test the reaction of the microcapsules in a simulated GI medium

containing urea, the test groups include two negative controls: a) without microcapsules and b) empty microcapsules containing neither bacteria nor activated charcoal, and three test groups: c) *L. acidophilus* microcapsules, d) activated charcoal microcapsules and e) combination microcapsules containing both *L. acidophilus* and activated charcoal. Depending on the contents of the alginate mixture, the encapsulator settings differ for the extrusion of droplets and are summarized in Table 5.1.

In-vitro studies in gastric simulated reaction media

The reaction medium is a simulated GI medium of the small intestine that consisted of a carbohydrate-based diet with 1.0 g/L arabinogalactan, 2.0 g/L pectin, 1.0 g/L xylan, 3.0 g/L starch, 0.4 g/L glucose, 3.0 g/L yeast extract, 1.0 g/L peptone, 4.0 g/L mucin and 0.5 g/L cystein sterile autoclaved. To simulate conditions of the small intestine, pancreatic juice (containing 6.0 g/L oxgall, 0.9 g/L pancreatin and 12.0 g/L sodium bicarbonate added to autoclaved water) was added at 27.8 mL/100.0 mL simulated food mixture and pH adjusted to 6.4 and 7.8 (corresponding to the proximal and distal sections of the small intestine respectively), after which 150 mg/dL urea was added.

The microcapsules from each test group was added to separate 10.0 ml of the simulated GI medium and incubated at 37 °C in anaerobic conditions for 48 hours. 1.0 mL of the simulated GI medium was sampled at 0, 10, 16, 24, 36 and 48 hours. These samples were centrifuged at 10 000 *g*, 4 °C for 10 minutes to obtain the supernatant for further analysis. Across the test groups, *L. acidophilus* was standardized in the microcapsules containing the bacterial cells at 1.1 x 10^9 CFU/mL (colony forming units/mL) and 1.2 x 10^9 CFU/mL for the studies at pH 6.4 and pH 7.8 respectively; while activated charcoal was standardized by weight in the microcapsules containing it at 54 mg for the studies at both pHs.

Quantitative determination of urea

Urea was quantified using the Stanbio Urea Nitrogen kit, USA, which is based on a diacetylmonoxime methodology. Briefly, 1.0 mL BUN color reagent,

2.0 mL BUN acid reagent and 20 μL of urea sample were mixed well and heated at 100 C for 12 minutes before cooled in cold water for 5 minutes. 0.1 mL of the final solution was read at 520 nm. Tests were performed at the very least in triplicate, together with a blank and Stanbio urea standards for each run.

Bacterial viability in storage conditions

Using the microcapsules immediately after its production as in this study is not always feasible, therefore it is important to test for bacterial viability in storage conditions, specifically in media at 4 °C. This study involved two test groups: *L. acidophilus* microcapsules and the combination microcapsules containing *L. acidophilus* and activated charcoal. 4 g of microcapsules was immersed in 18 mL storage medium, which was either a) 10 % MRS medium enriched with 150 mg/dL urea 90 % PS solution or b) PS solution. 0.1 mL microcapsules was sacrificed and plated at various dilutions to determine CFU counts in 1.0 mL at 0, 1, 2, 4, and 8 weeks.

5.4 Results and discussion

Induction profile

While activated charcoal has a high affinity for uremic toxins such as creatinine, this adsorbent has a low affinity for urea. In this study, *L. acidophilus* was metabolically induced to utilize urea as a nitrogen source, and combined with activated charcoal in microcapsules to evaluate its urea removal efficacy.

The urea consumption profile of *L. acidophilus* from p100 to p180 (earlier data not available) is shown in Figure 5.1. While urea concentration in MRS broth stayed constant at 2.7 g/dL from p120 onwards, urea uptake by the bacteria continued to rise, from 195 mg/dL at p120 to reach a maximum of 210 mg/dL at p142, indicating an adaptive mechanism. From this point however, urea consumption decreased to a minimum of 11 mg/dL at p180, reasons were not known. Although this data reflects the fact that *L. acidophilus* has the ability to adapt to an increasing external urea stimulus, the exact mechanism of metabolic induction is not known and a more detailed examination is needed. It is clear

however, that *L. acidophilus* may possibly play a role as a mini bioreactor for urea uptake in the GI tract.

Effect of activated charcoal on bacteria

Since the combination of *L. acidophilus* and activated charcoal in microcapsules place the two agents in close proximity, it is essential to determine if activated charcoal exhibits detrimental effects on bacteria viability. Bacterial CFU of the combination microcapsules containing both *L. acidophilus* and activated charcoal at the start of the experiment was 1.1×10^9 CFU/mL. At the end of the 48 hour study in the simulated GI medium, the combination microcapsules showed a decrease of bacterial CFU by two orders of magnitude to 1.3×10^7 CFU/mL; in contrast, *L. acidophilus* microcapsules showed a CFU decrease of three orders of magnitude from 1.0×10^9 CFU/mL to 4.7×10^6 CFU/mL. While this finding does not illuminate the interactions of activated charcoal on bacteria metabolism, it proves that activated charcoal does not decrease bacterial viability and may even provide support.

Effect of microcapsules on urea exposure

Experiments were conducted to evaluate urea removal capacities from a simulated GI medium by microcapsules containing either *L. acidophilus*, activated charcoal or a combination of both bacteria and charcoal. They were compared against two negative controls - simulated GI media without microcapsules and simulated GI media with empty microcapsules. Shown in Figure 5.2, the urea consumption profiles of activated charcoal microcapsules at both pHs 6.4 and 7.8 are similar to both negative controls in that they did not significantly affect urea levels in the media. This observation of activated charcoal is similar to literature findings that it does not have a high affinity for urea.

On the other hand, *L. acidophilus* microcapsules removed 57 mg/dL and 30 mg/dL urea at pHs 6.4 and 7.8 respectively, when urea levels were stabilized. This shows that urea removal by *L. acidophilus* cells is not only possible in a urea enriched MRS broth, but also in a simulated GI medium. Urea was removed to a

higher extent at pH 6.4 than at pH 7.8; this is due to the fact that the optimum pH range for *L. acidophilus* survival is pH 6.0 – 6.4. At pH 7.8, urea removal is almost halved, meaning that about half of the bacterial cells were inactivated.

To give a better perspective of the urea removal capacity of these microcapsules, the amount of urea removed per gram microcapsules was calculated. At pH 6.4, 2.0 mg urea/g *L. acidophilus* microcapsules were removed, where as at pH 7.8, 1.0 mg urea/g *L. acidophilus* microcapsules were removed.

The combination microcapsules demonstrated similar urea reduction profiles compared with *L. acidophilus* microcapsules regardless of pH. This shows that the addition of activated charcoal does not affect the ability of the probiotic to utilize urea in a simulated GI environment.

Microcapsule morphology

The aim of this study is to compare the efficiency of different microcapsules in reducing urea concentrations in the simulated GI medium, and it is necessary to standardize capsule sizes across the test groups for comparable diffusion kinetics. This required the use of the larger sized nozzle (600 μm) to compensate for the activated charcoal particles, which produced egg-shaped or oblate ellipsoidal microcapsules as shown in Figures 5.3 a-d. The average diameters (minor diameter) of the microcapsules from the different test groups for n=30 are: 619 $\pm$ 36 μm (empty microcapsules), 628 $\pm$ 24 μm (*L. acidophilus* microcapsules), 629 $\pm$ 28 μm (activated charcoal microcapsules), 640 $\pm$ 23 μm (microcapsules containing activated charcoal and *L. acidophilus*). These dimensions apply to 99 % of the microcapsules formed, while 1 % of the microcapsules produced were larger and spherical. This irregularity could be caused by the need for larger pressure which was provided by a nitrogen gas source, where minute irregularities in the molecular movement of the gas resulted in disturbances of the extrusion force leading to the different sized droplets formed.

Bacterial viability in storage conditions

The aim of this study is two fold: to determine if storage media containing urea affects bacterial viability, and whether the addition of activated charcoal provides additional support in prolonging bacterial viability. Results show that microcapsules containing activated charcoal preserved bacterial viability at least 5 weeks longer than microcapsules containing only bacteria; *L. acidophilus* microcapsules showed 0 % bacterial viability at week 3. In contrast, the combination microcapsules showed bacterial viability until the end of week 8 when the experiment was concluded. This supports the observation that activated charcoal provides support for bacterial viability.

Upon comparison of the two storage media it is observed that the PS storage resulted in higher bacterial viability compared to the storage in 10 % urea enriched MRS broth. Comparing only *L. acidophilus* microcapsules, the CFU count for the bacterial microcapsules stored in PS was 6.7×10^8 CFU/mL at week 2 compared to the 1.1×10^7 CFU/mL CFU count for the bacterial microcapsules stored in 10 % urea enriched MRS broth. Further comparison of the combination microcapsules show that those stored in PS reflected a slower decrease of bacterial CFU counts than the urea enriched broth; the combination microcapsules stored in PS reported a CFU count of 1.0×10^7 CFU/mL compared to a CFU count of 2.0×10^3 CFU/mL for those stored in 10 % urea enriched MRS medium. This shows that the additional urea may have caused stress to the survival of the bacteria at low temperatures. As a side note, MRS broth without urea was not tested as a storage medium because of the possibility of compromising the inductive capacities of the bacteria.

5.5 Conclusion

L. acidophilus was metabolically induced for urea uptake and was encapsulated either individually or with activated charcoal in chitosan-alginate microcapsules. Results show that the encapsulated bacteria (whether encapsulated with or without activated charcoal) removed 18 % to a maximum of 34 % urea from a simulated GI medium of the small intestine. Furthermore, viability studies

show that activated charcoal provides support in the maintenance of bacterial viability during the stomach transit and in storage conditions. These results ascertained the feasibility of combining bacteria and adsorbent in a formulation for oral consumption, and presented a model for the metabolic induction of bacteria strains for therapeutic applications. However, further studies are still required.

5.6 Acknowledgements

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Figures

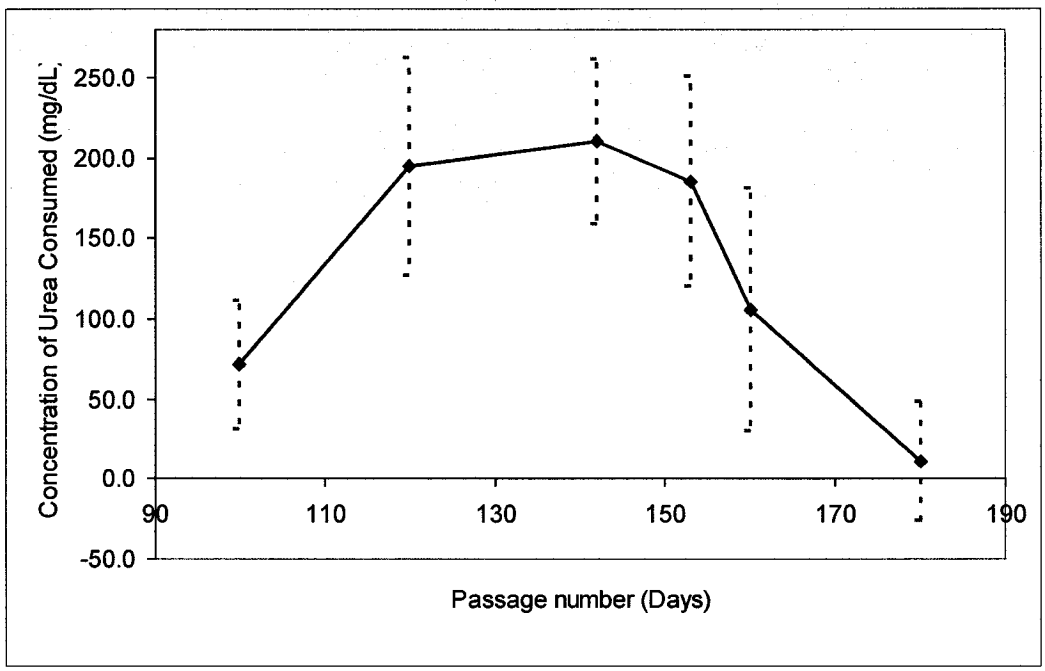
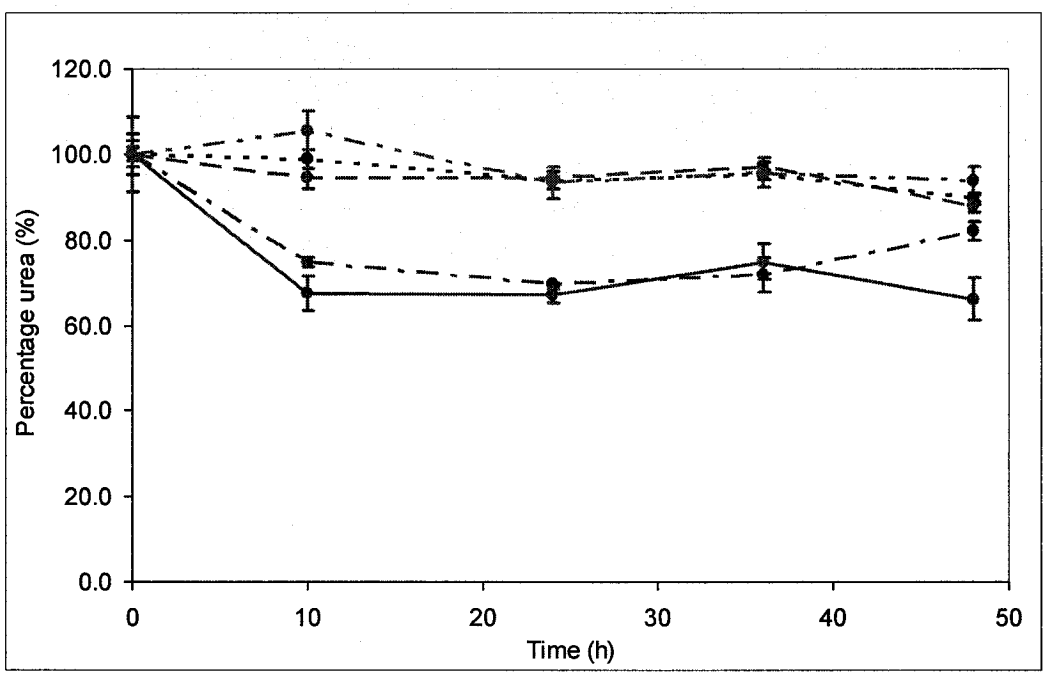


Figure 5.1: Urea consumption profile by *L. acidophilus* in urea-enriched - MRS media, at induction cycles p100 - p180. Urea levels were increased until p120 to 2.7 g/dL, and kept constant at 2.7 g/dL from p120 to p180.

(a)



(b)

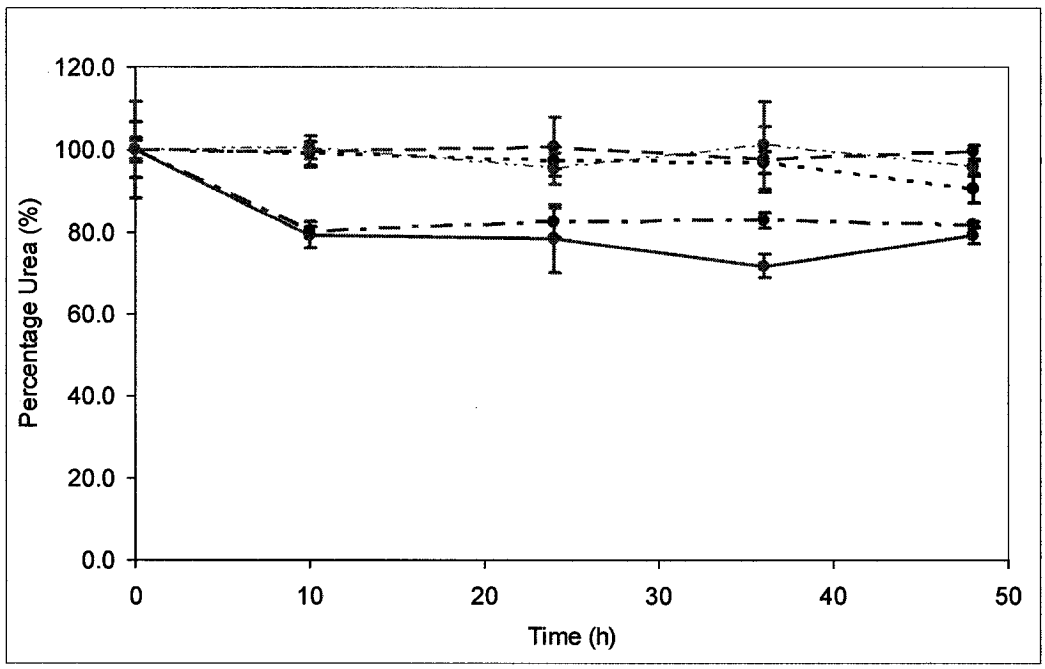


Figure 5.2: Effect of microcapsules on urea in simulated GI media over 48 hours. Urea profiles in simulated GI media at (a) pH 6.4 simulating conditions in the proximal section of the small intestine, and (b) pH 7.8 simulating conditions in the

distal section of the small intestine for i) no capsules -•-, ii) empty capsules —•—, microcapsules containing iii) activated charcoal —•-, iv) *L. acidophilus* —•— and v) activated charcoal and *L. acidophilus* —•-.

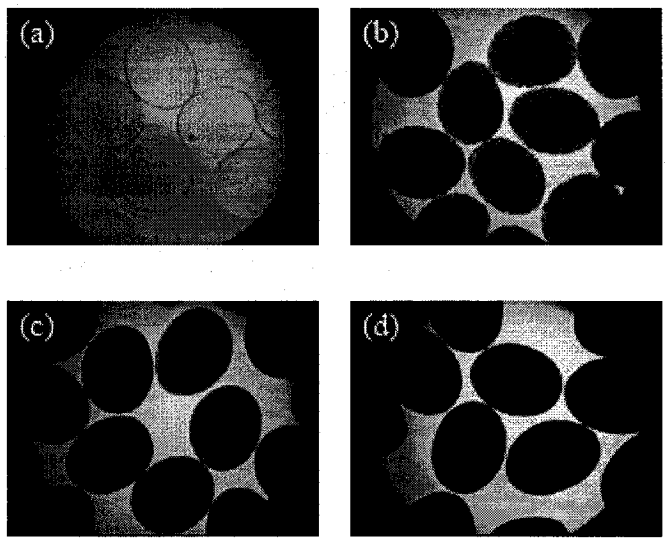


Figure 5.3: Photomicrographs of (a) "empty" microcapsules, microcapsules containing (b) activated charcoal, (c) *L. acidophilus* (d) activated charcoal and *L. acidophilus*. Magnification: 47X. Diameters across the microcapsule minor (short) sides are (a) 619 $\pm$ 36 μm, (b) 629 $\pm$ 28 μm, (c) 629 $\pm$ 24 μm, (d) 640 $\pm$ 20 μm.

CHAPTER 6:

Probiotic and charcoal microcapsules and their potential for managing other renal failure ions

In this section, the effect of the microcapsules containing a) *L. acidophilus,* b) activated charcoal, and c) *L. acidophilus* and activated charcoal on sodium, potassium, chloride, and phosphate is investigated in simulated GI media of the small intestine at pH 6.4 and 7.8 for 48 hours. They were compared against negative controls: d) reaction media without microcapsules and e) empty microcapsules containing neither bacteria nor adsorbent. The microcapsules and the simulated GI media were prepared as described in Chapters 4 and 5. The uremic analytes were added to this simulated GI media; a detailed breakdown of the analytes added per 100 mL reaction media, and the amount of ions yielded in 100 mL reaction media was shown in Table 6.1.

Analytes (compound form)	Concentration of compounds (mg/dL)	Analytes (ion form)	Concentration of ions (mg/dL)
Urea	150	-	-
Creatinine	20	-	-
KH_2PO_4	69.6	K^+	20.0
		PO_4^{2-}	48.6
NaCl	1070.0	Na^+	420.9
		Cl^-	648.7

Table 6.1: Analytes added to simulated GI media for in-vitro studies.

First, the concentrations of the ions to be added was determined by looking up the pathological values in renal failure literature; the compounds containing these ions were then selected, and their concentrations calculated. For example, to have a final concentration of 20 mg/dL K^+ ions in the reaction media, the number of moles of K^+ ions is first calculated: its concentration in g/L (which is 0.2 g/L) is divided by its molar mass 39.098 g/mol to obtain 5.115 mmol/L.

This is multiplied with the molar mass of the compound KH_2PO_4 (136.069 g/mol) to obtain the concentration of the compound needed, which is 69.6 mg/dL.

Quantitative analysis of the ions was performed using the 911 Hitachi blood analyzer. The profiles of the Na^+, K^+, and Cl^- ions showed no significant change for all four microcapsule groups throughout the 48 hour experiment at both pHs; the profiles of the PO_4^{2-} ions on the other hand, revealed definitive trends for four groups.

At pH 6.4, there was an initial decrease in PO_4^{2-} levels, followed by a gradual increase back to the starting value for empty microcapsules, activated charcoal and the combination microcapsules (Fig. 6.1a). This may be explained by diffusion kinetics; PO_4^{2-} ions diffuse into the microcapsules down a concentration gradient to about 85 %. As time passes the PO_4^{2-} ions inside the microcapsules and the free PO_4^{2-} ions in the reaction media equilibrate, leading to the slight increase of PO_4^{2-} levels to about 90 % towards the end of the experiment. The combination microcapsules showed an increase in PO_4^{2-} levels beyond the starting value; the reason is not known.

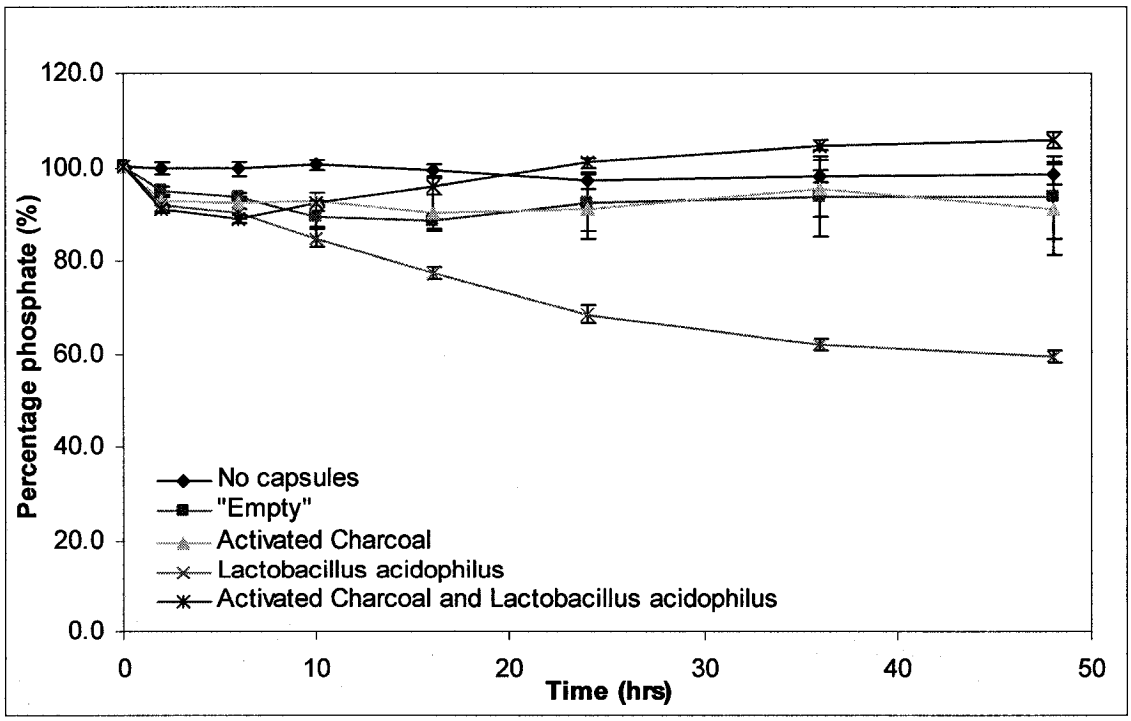


Figure 6.1a: Phosphate profiles in simulated GI media of the small intestine at pH 6.4.

On the other hand, *L. acidophilus* microcapsules showed a steady decreasing trend of PO_4^{2-} levels to about 60 %. This may be due to the fact that *L. acidophilus* cells metabolize PO_4^{2-} ions but this pathway is not understood. Furthermore, the PO_4^{2-} profile of the combination microcapsule is completely different from that of *L. acidophilus* microcapsules; more research is needed to explain the relationship between the PO_4^{2-} ions and the probiotic.

At pH 7.8, PO_4^{2-} levels decreased to about 80 % within the first 16 hours then gradually increase for empty microcapsules, activated charcoal and the combination microcapsules, similar to the observations made at pH 6.4. Similarly, this trend can be explained by diffusion kinetics. The rebound of the PO_4^{2-} profile by the combination microcapsules to a value slightly higher than the starting point is also observed at pH 7.8, however little is known about the interaction between the probiotic and the activated charcoal that leads to such a high increase.

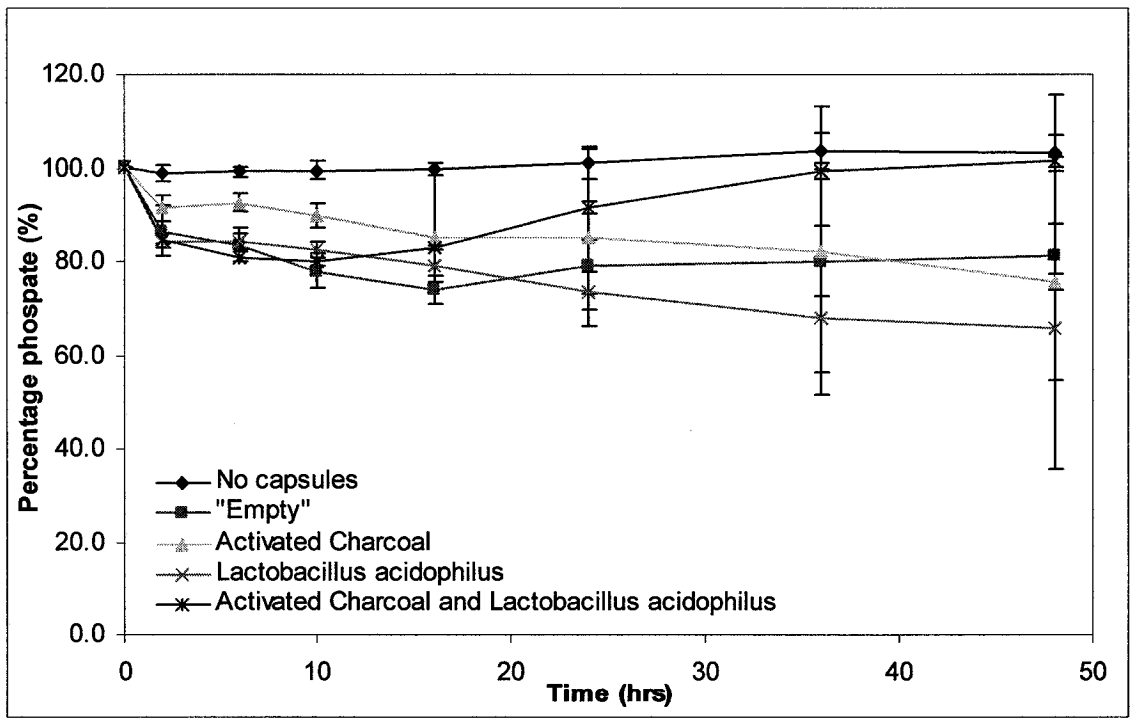


Figure 6.1b: Phosphate profiles in simulated GI media of the small intestine at pH 7.8.

At pH 7.8, *L. acidophilus* microcapsules decreased PO_4^{2-} ions to about 66 %, slightly less than that at pH 6.4. If this decrease is indeed due to the metabolism of the PO_4^{2-} ions by the bacterial cells, the slight decrease is explained by the inactivation of the metabolic activity of *L. acidophilus* at pH 7.8. However the difference in PO_4^{2-} levels between the 2 pHs is small, leading to the possibility of a different interaction between the bacteria and PO_4^{2-} ions.

To summarize, there is the indication of a diffusion related process for the empty microcapsules and activated charcoal microcapsules. The steady decrease of PO_4^{2-} levels depicted by *L. acidophilus* microcapsules at both pH hint at bacterial metabolism, but does not exclude the possibility of a different relationship. The combination microcapsules do not exhibit similarity compared with the *L. acidophilus* microcapsules, and further studies is needed to better understanding the relationship between PO_4^{2-} ions and the probiotic bacteria, and with activated charcoal.

CHAPTER 7:

Summary of Observations

The metabolic induction capacities of probiotic bacteria *Lactobacillus acidophilus* and *Bifidobacterium longum* were compared. The more responsive *L. acidophilus* was co-encapsulated with activated charcoal in chitosan-alginate microcapsules. These combination microcapsules were compared against microcapsules containing either probiotic or adsorbent for their effect on uremic metabolites such as urea, creatinine, phosphate, sodium, chloride and potassium. Viability studies in low pH and storage conditions were also performed to investigate the effect of the additional activated charcoal on the viability of the *L. acidophilus* cells. The study can be summarized as the following:

1) Metabolic induction of probiotic bacteria strains:

Probiotic bacteria *Lactobacillus acidophilus* ATCC 314 and *Bifidobacterium longum* ATCC 55813 were metabolically induced for urea uptake over 180 culture passages. *B. longum* profiled a decreasing trend of urea metabolism despite increasing urea levels in the MRS broth. On the other hand, *L. acidophilus* metabolized increasing amounts of urea when urea levels in the MRS broth were increased correspondingly, resulting in a maximal removal of 200 mg/dL urea after 142 days of induction. The culture media was also found to affect the metabolic induction process; *B. longum* cultivated in its ATCC recommended RCM broth showed no response to the urea induction compared to its cultivation in the MRS broth (Fig. 3.1). Although both *L. acidophilus* and *B. longum* cultivated in the 2.7 g/dL urea-enriched MRS broth showed potential for urea removal, they were both non-responsive when tested in 150 mg/dL urea-enriched MRS broth, the urea level more similar to pathological levels of renal failure patients. For the probiotic to be effective at this lower urea levels, they were re-adapted to 150 mg/dL urea enriched MRS broth for several culture

passages. Of the two probiotic, *L. acidophilus* responded better and was selected for microencapsulation and in-vitro experiments.

2) Preparation of microcapsules containing L. acidophilus and/or activated charcoal:

The microcapsule groups used in this thesis include: microcapsules containing a) *L. acidophilus*, b) activated charcoal, c) both *L. acidophilus* and activated charcoal and d) neither bacteria nor activated charcoal, all of which were encapsulated in a calcium-alginate matrix and a chitosan membrane. Microcapsule sizes were standardized across the different groups by extruding the alginate droplets through a 600 μm nozzle (to accommodate the activated charcoal particles), which require the use of pressurized nitrogen. This process resulted in egg-shaped (oblate ellipsoidal) gel droplets that posses reduced mechanical properties compared to spherical gel droplets. To compensate for this, a chitosan coating which possess strong mechanical properties was used to form the microcapsule membrane. The correlation of a stronger chitosan coating with calcium ions also prompted the direct immersion of the calcium-alginate cores from the gelling solution ($CaCl_2$) into the coating solution without the conventional wash step. Upon microscopic analysis, smooth microcapsules were observed with the diameters (across the short side): 619 $\pm$ 36 μm (empty microcapsules), 628 $\pm$ 24 μm (*L. acidophilus* microcapsules), 629 $\pm$ 28 μm (activated charcoal microcapsules), 640 $\pm$ 23 μm (microcapsules containing activated charcoal and *L. acidophilus*), n=30. Viability studies revealed that bacterial viability was not drastically lowered by this encapsulation process.

3) Effect of the microcapsules on creatinine:

The microcapsules were exposed to simulated GI media of the small intestine containing additional creatinine at pHs 6.4 and 7.8 for 48 hours. A comparison made between the two pHs revealed that the microcapsules possess higher creatinine removal capacities at pH 7.8. This phenomenon was observed for all microcapsule groups, and may be due to the change in the properties of the

alginate core or the chitosan membrane. The following comparisons were made keeping this difference in mind. At both pHs, empty microcapsules were found to remove small amounts of creatinine, as shown in that the slightly decreased creatinine levels were sustained throughout the 48 hour study. *L. acidophilus* cells demonstrated the ability to metabolize creatinine at pH 6.4 (at a capacity of 0.71 μmol/g microcapsules) but not at pH 7.8, due to the inactivation of acidophile's metabolism at this basicity. At both pHs, the combination microcapsules showed lower creatinine removal capacities compared to activated charcoal microcapsules, (pH 6.4: 0.97 μmol creatinine/g combination microcapsules compared to 1.26 μmol creatinine/g activated charcoal microcapsules; pH 7.8; 1.4 μmol creatinine/g combination microcapsules compared to 1.78 μmol/g activated charcoal microcapsules). This was due to the obstruction of the activated charcoal pores by the bacterial cells in the combination microcapsules. The adsorption capacity of activated charcoal at both pHs was also found to be similar.

4) Effect of the microcapsules on urea:

The microcapsules were exposed to simulated GI media of the small intestine containing additional urea at pHs 6.4 and 7.8 for 48 hours. Unlike creatinine, urea was not removed by the empty microcapsules at both pHs. The low affinity of activated charcoal for urea [78,157] was ascertained in this study, where activated charcoal microcapsules did not remove a significant amount of urea. At pH 6.4, *L. acidophilus* microcapsules removed almost twice the amount of urea (2.0 mg urea/g microcapsules) compared to at pH 7.8; this is due to the fact a portion of the bacterial cells (most likely half) ceased to be metabolically active. The urea removal profiles of the combination microcapsules were very similar to those of the *L. acidophilus* microcapsules (Fig. 5.2); this showed that activated charcoal did not affect the urea metabolism of the probiotic.

5) Effect of the microcapsules on other molecules:

The microcapsules were exposed to simulated GI media of the small intestine containing additional phosphate, potassium, sodium and chloride at pHs

6.4 and 7.8 for 48 hours. All four groups of microcapsules showed no effect on potassium, sodium and chloride levels. On the other hand, the phosphate profiles hinted at a diffusion related process, particularly for empty microcapsules and activated charcoal microcapsules, where the initial decrease of the phosphate profiles was followed by a stabilization stage. In contrast, the steadily decreasing phosphate profiles associated with *L. acidophilus* microcapsules indicated possible phosphate metabolism by the probiotic. This trend was however not observed in the combination microcapsules possibly due to the influence of activated charcoal.

6) Effect of activated charcoal on bacterial viability:

Bacterial viability at the start and end of the 48 hour experiments in simulated GI media was compared. At the end of 48 hours, *L. acidophilus* microcapsules showed a lower CFU count than the combination microcapsules, showing that activated charcoal do not decrease bacterial viability and may even provide support. Similarly in acid tolerance studies, the combination microcapsules showed an order of magnitude CFU counts higher than *L. acidophilus* microcapsules, indicating that activated charcoal may provide support in the preservation of bacteria viability during the stomach transit. While the mechanism is not known, the combination group is more effective in delivering a higher percentage of functional bacterial to the small intestine for urea and creatinine removal.

A comparison of bacterial viability in storage conditions was performed between *L. acidophilus* microcapsules and the combination microcapsules, to determine if storage media containing urea affects bacterial viability, and whether the addition of activated charcoal provides additional support in prolonging bacterial viability. Results show that that microcapsules containing activated charcoal preserved bacterial viability at least 5 weeks longer than microcapsules containing only bacteria, which showed 0 % bacterial viability at week 3. Upon comparison of the storage media, it was found that the addition of urea was detrimental to the bacterial viability. Bacterial microcapsules in PS showed higher

bacterial viability compared to those stored in 10 % urea-enriched MRS broth. Furthermore, the combination microcapsules stored in PS showed a slower decrease of bacterial CFU counts compared to those stored in 10 % urea-enriched MRS broth. The additional urea may have caused stress to the survival of the bacteria at low temperatures. (MRS broth without urea was not tested as a storage medium because of the possibility of compromising the inductive capacities of the bacteria.)

CHAPTER 8:

Conclusions

Current renal replacement therapies such as dialysis and kidney transplantation do not answer the needs of the ESRD and chronic renal failure population. Microcapsules containing probiotic bacteria adapted for urea metabolism and activated charcoal were formulated with the goal of providing these patients with an oral adjuvant. It was hypothesized that these mini bioreactors will entrap unwanted uremic metabolites such as creatinine and urea as they pass through the GI tract, and potentially help reduce the frequency and duration of dialysis routines. The objectives of this project was to compare the metabolic adaptivity of two probiotic bacteria strains, design a suitable microcapsule for its encapsulation with activated charcoal, and assess the efficacy of the combination microcapsule in removing urea and creatinine in-vitro and investigate the effect of activated charcoal on bacterial viability. After reviewing the results, the following conclusions can be drawn:

1) Probiotic bacteria exhibit capacity for metabolic induction; this induction capacity was influenced by different culture media. The *L. acidophilus* and *B. longum* cells conditioned in high urea media were no longer responsive when exposed to low urea media that was more comparable to pathological urea levels in renal failure patients. As such, further adaptation to low urea levels was performed. *L. acidophilus* responded better compared to *B. longum* in the induction process at both high and low urea levels.

2) The AC microcapsule design was suitable for the encapsulation of both *L. acidophilus* and activated charcoal. The chitosan membrane was not toxic to the bacterial cells. Furthermore, it exhibited excellent mechanical strength to compensate for the reduced mechanical stability of the oblate ellipsoidal shaped microcapsules, as shown by good microcapsule integrity and lack of swelling for all microcapsule groups, in the experiments performed in simulated GI media.

3) The combination microcapsules showed lower creatinine removal capacities compared to activated charcoal microcapsules due to the physical obstruction of the activated charcoal pores by the probiotic in the combination microcapsules. *L. acidophilus* also exhibited the ability to metabolize creatinine, although this metabolism was arrested at the basic conditions of pH 7.8. Furthermore, empty microcapsules trap creatinine, and larger amounts of creatinine were removed at pH 7.8 compared to pH 6.4, possibly due to changes in the properties of the alginate core or chitosan membrane.

4) As was shown in the literature, activated charcoal did not adsorb urea efficiently. Furthermore, empty microcapsules did not exhibit significant capacity for trapping urea; urea removal was dependent on *L. acidophilus* cells, where only microcapsules containing *L. acidophilus* showed urea removal. *L. acidophilus* metabolized urea more efficiently at pH 6.4 than at pH 7.8. The combination of activated charcoal and *L. acidophilus* did not affect the bacteria's ability to utilize urea. As such, the combination microcapsules demonstrate potential for the removal of both creatinine and urea.

5) All four microcapsules groups exerted no effect on the levels of potassium, sodium and chloride. Changes in the phosphate profiles for the empty microcapsules and activated charcoal microcapsules showed diffusion-related trends, while *L. acidophilus* showed possible phosphate metabolism. The co-encapsulation of activated charcoal may have influenced the bacteria in its phosphate uptake but this is not fully understood, and more research in this area is warranted.

6) Activated charcoal supports bacterial viability in the acid tolerance studies, simulated GI media of the small intestine as well as in storage conditions. The combination microcapsules demonstrated higher bacterial viability compared to *L. acidophilus* microcapsules, and showed potential for delivering the largest amount of viable bacteria to the small intestine.

CHAPTER 9:

Recommendations and Future Applications

1) The oral administration of encapsulated therapeutic agents for the removal of unwanted uremic metabolites such as creatinine and urea has been investigated by numerous researchers. Such removal of uremic metabolites found in the systemic circulation from the GI tract is possible because of the exchange of these molecules between the capillaries and the lumen in the small intestine [123,124]. Activated charcoal is an excellent adsorbent for creatinine and uric acid but not urea [78,157], and its efficacy is limited by rapid pore saturation as a result of its non-specificity. Recently, probiotic bacteria were reported to posses capacities for adaptation [169]. Their adaptation potential was maximized in this thesis for urea uptake. For the first time, a probiotic bacterium conditioned for urea uptake was co-encapsulated with activated charcoal to increase their efficacies in toxin removal. The current study has shown the feasibility of lowering creatinine and urea in-vitro by microcapsules containing *L. acidophilus* and activated charcoal, as well as the benefits of activated charcoal in preserving the viability of the bacteria in the harsh conditions of the GI tract. This study shows the possibility of formulating an oral adjuvant for renal failure patients, to remove unwanted metabolites and potentially reduce the frequency and duration of dialysis.

2) Although the combination microcapsule formulated in this thesis was designed for oral delivery, the combination of the urea-adapted probiotic and activated charcoal may also be used in the hemoperfusion of renal failure patients for the rapid removal of target uremic metabolites. The use of a chitosan membrane for the co-encapsulation of *L. acidophilus* cells and activated charcoal was found suitable for maintaining microcapsule integrity in this thesis, but it may not be suitable for hemoperfusion applications. The membrane for hemoperfusion should be hemocompatible [118]. Furthermore, the design of such a membrane should ensure no release of bacterial toxins into the blood stream in hemoperfusion.

Therefore, while the combination microcapsule formulated in this thesis have potential in hemoperfusion applications, the microcapsule membrane will have to be custom designed to achieve better hemocompatibility properties.

3) The success in the metabolic induction of the probiotic bacteria in this thesis presents a model for the metabolic induction of other bacteria for the removal of unwanted metabolites. Compared to genetically engineered bacteria, the metabolically induced bacteria may be less efficient; the higher efficacy of the genetically engineered *E. coli* DH5 cells [36] which removed almost all plasma urea using 100 mg bacterial cells in 30 minutes. However the use of genetically engineered microorganisms faces regulatory obstacles related to safety and environmental issues which may delay or restrict the genetically engineered agent to the patient population. The metabolic induction of bacteria circumvents these problems. Furthermore, the versatility and rapid multiplying properties of bacteria compensate for the time spent on metabolic induction, making them excellent choices for in-vitro studies and metabolite conditioning. If successful, this opens the door to numerous possibilities and potential applications for bacteria adapted to specific metabolites.

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