A novel microencapsulated probiotic yogurt formulation for oral delivery in the suppression of intestinal tumorigenesis in *Apc*Min mice

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A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of

Doctor of Philosophy

Daj mi, proszę, dobry Boże,
Zamiast drogi - to bezdroże,
Które prosto poprowadzi
Poprowadzi mnie w nieznane.
Innym, dobry Boże, zostaw
Wstęgi ulic i autostrad,
A ja niech już na manowcach pozostanę!

Pozwól mi się, Boże, błąkać
Po gęstwinach i po łąkach,
W niepewności wydeptywać tysiąc ścieżek.
Wydeptywać tysiąc ścieżek.
Nie bój się, że gdzieś przepadnę,
Dokąd iść mam - nie odgadnę...
Uwierz we mnie, jak ja w ciebie, Boże, wierzę!

Spełnij, proszę, o czym marzę!
Niech pod tęczy twej ołtarzem
Wyspowiadam gorzkie żale, słodkie grzechy!
I niech sercu siły doda
Darowana mi swoboda,
Co nie szczędzi mi ni smutku, ni pociechy!

Choć niejeden dasz mi, Boże,
Do zgryzienia twardy orzech,
Daj też iskrę, żeby radość z niej rozniecić!
I nadzieję mi pozostaw,
Że gdy drodze swej nie sprostam,
Ty, mój dobry Boże, wyjdziesz mi naprzeciw!

- ***Edyta Geppert - Modlitwa do dobrego Boga.***

Everything should be made as simple as possible, but not simpler.

Albert Einstein (1879-1955)

To my parents

ABSTRACT

There is a direct relationship between the development of colon cancer and exogenous factors, such as diet. The immunomodulatory effect of probiotic bacteria has been postulated earlier; however, due to low clinical efficacy and associated limitations they have not been devised as cancer theraphy and preventative procedures. One of the main obstacles is the efficient delivery of live bacterial cells to targeted sites. The work contained in this dissertation aims to develop and evaluate a yogurt based formulation containing microencapsulated live probiotic bacterial cells for use in colon cancer prevention and theraphy.

Alginate – poly-L-lysine – alginate (APA) and alginate-chitosan (AC) microcapsule systems have been optimized, prepared and characterized. Survival of *Lactobacillus acidophilus* cells and mechanical stability of the microcapsules were evaluated in various pH conditions in presence of yogurt and simulated gastrointestinal fluids. The physical properties of the microcapsules were investigated using a computer controlled dynamic simulated human gastrointestinal (GI) model. Results show that the APA microcapsule system was more robust, had higher retention capacity, preserved better bacterial viability and had greater resistance to GI environment.

The *Apc*Min mouse was used to investigate the potential of a microencapsulated probiotic yogurt formulation for clinical efficacy and safety. As *Apc*Min mice are genetically predisposed to spontaneously develop multiple intestinal and colonic adenomas, they provide a practical and genetically relevant model system for cancer prevention studies. Using various biomarkers such as interleukin - 1β, interleukin - 6, interleukin - 12, immunoglobulin A, prostaglandin E_2, thromboxane B2, C - reactive protein, tumor necrosis factor - α, interferon - γ and cyclooxygenase – 2. In-vivo studies show an overall reduction of inflammation in animals receiving a microencapsulated probiotic yogurt formulation. Moreover, parameters such as the animals' body weight and the presence of blood in the fecal samples were monitored. In addition, histological evaluation revealed a significant reduction in tumor multiplicity after oral administration

of APA microencapsulated probiotic yogurt formulation. Immunohistochemical analysis of intestinal samples was performed using a custom designed MatLab program.

This research introduces a new concept in treatment and prevention of inflammation leading to intestinal tumorigenesis. Further studies, however, are required.

RÉSUMÉ

Le cancer colorectal est l'une des malignités les plus répandues dans le monde occidental. La majorité des cancers colorectaux sont soit localement ou régionalement invasifs au moment du diagnostic, limitant les options de traitements et réduisant les taux de survies. Plusieurs études épidémiologiques et laboratoires suggèrent une relation entre le développent du cancer du colon et des facteurs exogènes, tel que le régime alimentaire. L'effet immunomodulateur des bactéries probiotiques a été postulé; cependant, en raison de l'efficacité clinique réduite et d'autres limitations qui y sont associées, elles n'ont pas été conçues comme une thérapie préventive du cancer. L'un des plus grands obstacles reste l'administration efficace des bactéries vivantes aux organes ciblés. Le travail décrit dans cette dissertation vise à développer et à évaluer une formulation à base de yogourt contenant des cellules bactériennes probiotiques vivantes pour l'atténuation de l'inflammation gastro-intestinale qui cause le développement du cancer du colon, par la conduite d'études in vitro et in vivo.

Des systèmes microcapsules Alginate – poly-L-lysine – alginate (APA) et alginate-chitosan (AC) ont été optimisés, préparés et caractérisés. La survie de cellules *Lactobacillus acidophilus* et la stabilité mécanique des microcapsules ont été évaluées sous diverses conditions de pH et en présence de yogourt et de fluides gastro-intestinales simulées. Les propriétés physiques des microcapsules ont été investiguées en utilisant un model gastro-intestinale (GI) humain dynamique, simulé et contrôlé par ordinateur. Les résultats démontrent que le système microcapsule APA est plus robuste, a une plus grande capacité de rétention, préseruee une meilleure viabilité bactérienne et a une plus grande résistance à l'environnement gastro-intestinal.

La souris *Apc*Min a été utilisée afin de conduire les études in vivo. Comme les souris *Apc*Min sont génétiquement prédisposées à développer spontanément de multiples adénomes intestinaux et du colon, elles offrent un moyen pratique et un système modèle génétiquement approprié pour les études de prévention du cancer. À l`aide de différents biomarqueurs tels que interleukin - 1β, interleukin - 6, interleukin - 12, immunoglobulin A, prostaglandin E_2, thromboxane B2, protéine réactive C, facteur nécrose tumorale-α, interféron - γ and cyclooxygenase – 2 les études in vivo démontrent une réduction

générale de l'inflammation chez les animaux qui reçoivent une formulation yougourt probiotique microencapsulée. De plus, des paramètres tels que le poids de l'animal et la présence de sang dans les échantillons fécaux ont été surveillés. L`évaluation histologique a révélé une réduction significative dans la multiplicité des tumeurs après l'administration orale d`une formulation yougourt probiotique APA microencapsulée. L'analyse immunohistochimique d`échantillons intestinales a été accomplis à l`aide d'un programme a MatLab créé sur mesure.

Cette recherche introduit un nouveau concept dans la prévention de l'inflammation qui mène à la tumorigénèse intestinale. De plus amples études doivent êtres faites pour élucider son potentiel réel pour la thérapie anti cancer du colon.

ACKNOWLEDGEMENTS

First and foremost, I thank my supervisor Dr. Satya Prakash, for giving me the opportunity to conduct this research and for his continuous support over the past four years I spent in his laboratory. Dr. Prakash was always there to listen and to give advice. He taught me how to ask questions and express my ideas. He showed me different ways to approach a research problem and the need to be persistent to accomplish any goal. Without his encouragement, patience and constant guidance I could not have finished this dissertation. He was always there to meet and talk about my ideas, to proofread and mark up my papers, abstracts and chapters, and to ask me good questions to help me think through my problems (whether philosophical, analytical or computational). He also made the BTCTRL laboratory a wonderful workplace and home for the past four years by indulging my ever expanding working space and laboratory materials' needs.

Special thanks goes to my thesis committee members, Dr. Henrietta L. Galiana, Dr. Moulay Alaoui-Jamali and J. Nadeau who are responsible for helping me complete the writing of this dissertation as well as the challenging research that lies behind it. Thank you for all constructive suggestions and insightful criticism.

Besides my advisors, I would like to thank the rest of very important people who together helped me complete my thesis: Melina Narlis, who dedicated her precious time and showed her incredible skills in preparation of my histology slides, who always gave me insightful suggestions about my research and who has provided me with contacts for the very knowledgeable scientists at McGill such as Dr. Dongmei Zuo, from the McGill Molecular Oncology Group to whom I am grateful for finding time and allowing me access to the ScanScope to scan my histology slides as well as Dr. Gregory Marczynski who allowed me use his oil immersion lens and Richard Wargachuk for always letting me use his computer and explaining imaging software. I would also like to thank all the technicians at McGill's Animal Resources Centre for their help with my animal trials. Special thanks go to the Chief Animal Health Technician, Anna Jimenez for her professionalism and the remarkable care I was provided during my animal trials. Without

you Anna I would still contemplate whether that mouse is out there to bite me and if so, I could not write my thesis with my fingers chewed off.

Let me also say 'thank you' to the following people at the Biomedical Engineering Department: Pina Sorrini (Department's Secretary) for keeping great track of my records, scheduling my meetings, going "an extra mile" to get things done and always having a smile on her face; Dr. Maryam Tabrizian for allowing me to use her laboratory at any time I needed to use the space or her equipment, especially the fluorescent microscope, also for letting me use her peaceful computer space; Ross Wagner for his help with computers and Patricia Cap for her help with finances.

I would also like to express my gratitude to all my lab colleagues: Razek Coussa, Tasima Haque, Wei Ouyang, Hongmei Chen, Arghaya Paul, Safaa Sebak, Trisna Lim, Bisi Lawuyi, Fatemeh Afkhamizarreh and all the summer and exchange students: Ayla Coussa, Leonard Bienenstock, Maria Hanna, Maliha Kazmi and Sarita Singh as well as all the sudents from Dr. Tabrizian's laboratory: Christina Holmes, Jamal Daoud, Anna Hildeberg, Marc Hermann, Xuyen Hoa, Nicola Duceppe and Steven Shapka. You all contributed to achieving my degree by providing a friendly atmosphere.

In spite of all these friends, the crucial people to this PhD were my three best friends: Jasmine Bhathena, Christopher Martoni and Arun Kulamarva. Without you guys everyday life for the past four years would be plain and gray. Jasmine, thank you for listening, for helping me, for solving any "unsolvable problems", for laughing and crying together, you were great! Chris, thanks for being diplomatic and patient with me, it was fun travelling together, I know you almost always got me right! Arun, thanks for listening and giving great "male" advice, you were always very accommodating and supportive. We all had great time and our numerous photos will never let our unforgettable memories disappear!

In addition, I would like to thank my best friends Mirela and Horatiu Stanescu who looked over me and took care for the last 10 years. I will always consider myself

very lucky that I met you and am grateful for everything you've done for me. I would not be here today if it wasn't for your wake up calls and keeping me alive with your delicious home made cooking.

Above all, I am grateful to Sofiane Cherif for his constant support, for listening to my complaints and frustrations, for always motivating me and wishing the best, for being so responsible and always reminding me that my work is very important, for always being proud of me, for being more strict than my own dad, for reading my papers, working on my computer problems, for working together on my research, for his preeminent patience and for believing in me. You gave me the extra strength necessary to get things done.

Last, but not least, I thank my parents, Dr. Longina Hanczak-Urbanska and Dr. Stanislaw Urbanski, for giving me life in the first place, for bringing me up in an extraordinary way, for giving me the opportunity to travel across the world, having enough faith in me to let me live alone across the ocean, encouraging and believing in me every day knowing that despite the distance, despite the struggles, despite the time and cost it is all going to be worthwhile. It wasn't easy, hopefully now it is going to get better. My parents have always put education as a first priority in my life, and raised me to set high goals for myself. They taught me to value honesty, courage, and humility above all other virtues. I have always needed to work hard to achieve my goals in life and they have always been there for me as an unwavering support.

I feel very special and from the bottom of my heart - thank you!

I dedicate this book to the most important people in the world: my parents.

PREFACE

In accordance with the McGill University Thesis Preparation and Submission Guidelines, I have taken the option of writing the experimental section in the form of original papers either published or appropriate for publication. These papers comprise chapters 3-8 of this thesis, and are each divided into sections consisting of an abstract, introduction, material and methods, results, discussion, and conclusions. In addition, this thesis contains an overall abstract, introduction, literature review, as well as a summary of results, final conclusions, claims to original contributions, and recommendations.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS AND TERMINOLOGY

5-FU/LV	5-Fluorouracil/Leucovorin
AC	Alginate-Chitosan
ACS	American Cancer Society
APA	Alginate-Poly-L-Lysine-Algiante
APC	Adenomatous Polyposis Coli
ATP	Adenosine-5'-Triphosphate
BSA	Bovine Serum Albumin
BSH	Bile Salt Hydrolase
BV	Bacterial Vaginosis
BW	Body Weight
CA	Cholic Acid
CCAC	Canadian Council of Animal Care
CD	Crohn's Disease
CIHR	Canadian Institute of Health Research
CLMF	Cytotoxic Lymphocyte Maturation Factor
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CRC	Colorectal Cancer
CRP	C-Reactive Protein
DAC	Demethylating Agent 5-Aza-2'-Deoxycytidine
DCA	Deoxycholic Acid
DMH	1,2-Dimethylhydrazine

DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPSBO	Early Postoperative Small-Bowel Obstruction
FAP	Familial Adenomatous Polyposis
FEA	Follicle-Associated Epithelium
FOBT	Fecal Occult Blood Test
FOLFOX	Fluorouracil, Leucovorin, Oxaliplatin
FW	Formula Weight
GALT	Gut-Associated Lymphoid Tissue
GI	GastroIntestinal
GIN	Gastrointestinal Intraepithelial Neoplasia
GIT	Gastrointestinal Tract
Gr-1	Granulocyte-1
GRAS	Generally Regarded As Safe
HEMA	Hydroxyethyl Methacrylate
HMW	High Molecular Weight
Hz	Hertz
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IFN-γ	Interferon-γ
IFN-γR	IFN-γ Receptor
IL-1β	Interleukin-1β
IL-6	Interleukin-6

IL-12	Interleukin-12
ISH	In Situ Hybridization
L	Liter
LAB	Lactic Acid Bacteria
KD	KiloDalton
M	Molarity
MF	Milk Fat
MRS broth	Man, Rogossa and Sharpe broth
MUC2	Main Secreted-Colonic Mucin
MW	Molecular Weigth
MWCO	Molecular Weight CutOff
NK	Natural Killer
NKSF	NK cell Stimulatory Factor
NSAIDs	Nonsteroidal Anti-Inflammatory Drugs
NSERC	Natural Sciences and Engineering Research Council
PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffer Saline
PCNA	Proliferating Cell Nuclear Antigen
PGE_2	Prostaglanding E_2
PLL	Poly-L-Lysine
PRRs	Pattern Recognition Receptors
SEM	Standard Error Measurement
SHIME	Simulated Human Intestinal Microbial Ecosystem

SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
TxA_2	Thromboxane A_2
ThB_2	Thromboxane B_2
TXS	Thromboxane Synthase
TIR	Toll/Interleukin-1 Receptor
TLRs	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor α
UC	Ulcerative Colitis
cas-3	Caspase-3
cm	Centimeter
g	Gram
h	Hour
mg	Milligram
min	Multiple intestinal neoplasia
mL	Milliliter
mm	Millimeter
nm	Nanometer
p	Pico
rpm	Rotation per minute
v	Volume
µL	Micoliter
µm	Micrometer

CHAPTER 1

General Introduction

1.1 Overview

According to the American Cancer Society (ACS) colorectal cancer (CRC) is the second leading cause of cancer-related death in the US among men and women combined[1]. About 108,070 new cases of colon cancer (53,760 in men and 54,310 in women) and 40,740 new cases of rectal cancer (23,490 in men and 17,250 in women) will be diagnosed in 2008. If caught early (in stage I), the relative 5-year survival rate is higher than 90%, compared with only about 10% in people with advanced disease. Tumorigenesis of colorectal cancer is a multistep process which arises from preceding benign adenomas. Familial adenomatous polyposis (FAP), which is characterized by the development of hundreds to thousands of adenomatous polyps in the colon and rectum is a good model for elucidation of genetic alterations involved in colorectal tumorigenesis [2]. A mutation in the adenomatous polyposis coli (*Apc*) gene responsible for FAP is believed to contribute to molecular pathogenesis leading to progression of polyps resulting in colon cancer [3].

Risk factors for colon cancer include both hereditary and environmental factors. Dietary patterns represent controllable risk factors for the development of colon cancer [4]. A major cause associated with colorectal cancer is a diet rich in fat, refined carbohydrates and animal protein, combined with low physical activity [5].

Today's treatment of colorectal cancer includes: chemotherapy which makes use of anti-cancer drugs to kill tumor, radiation therapy which uses high-energy radiation to kill cancer cells and surgery to remove the tumor [6]. The choice of treatment depends on the individual case, tumor size, location and extent of the disease. Although frequent screening tests can detect early stage of tumor, chemotherapy and radiation do not guarantee the non-recurrence of the tumor later in life. Therefore, an alternative treatment is needed.

The oral route is considered the most convenient for administration of drugs to patients and conventional dosage forms dissolve in stomach. It is a serious drawback

where localized delivery of the drug is needed in the colon as it is not protected from hostile environment of the stomach. A well balanced intestinal flora is of utmost importance in prevention of polyps development [7]. As the gastrointestinal system is populated by potentially pathogenic bacteria that are capable of releasing toxins, an oral live probiotic bacterial cell therapy is a noninvasive way which aims to restore or improve the intestinal microbial balance. According to current definition adopted by WHO, probiotics are: "*Live microorganisms which when administered in adequate amounts confer a health benefit on the host"*[8].
Indeed a number of studies have confirmed this capacity and it has been found that oral daily intake of most commonly *Lactobacillus* and *Bifidobacteria* can lead to significant improvement of gut flora [9-11].

In addition, a study revealed that a promotion and progression of colon tumor can be inhibited by feeding with yogurt containing probiotic bacteria [12-14]. Yogurt, defined as the product of milk fermentation by *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, has a long history of beneficial impact on the well-being of humans [15]. In order for probiotics to preserve their expected health-promoting effect they need to survive during passage through the gastrointestinal tract. However, the therapeutic potential of live of bacterial cells has been hampered by inherent limitations in their use. For example, of those bacteria ingested only a small percentage survives gastric transit limiting the overall therapeutic effect [16]. Also, there are some practical concerns regarding the production, cost, and storage of such a product [17,18]. Furthermore, oral administration of live bacterial cells can cause a host immune response, and can be detrimentally retained in the intestine, replacing the normal intestinal flora [19]. Thus, concerns of safety have prevented regular use of this promising therapy in clinical practice.

Various studies show that microencapsulation in specialized ultra-thin semi-permeable polymer membranes allows oral delivery of live bacterial cells and improves their transit via gastrointestinal tract [20-22]. Furthermore, microencapsulation offers protection to bacteria from the harsh gastrointestinal environment during transit, limits stimulation of the host immune response, as well as minimizes risks of systemic infections, the replacement of the normal intestinal flora and gene transfer [23-25].

The rationale behind the study contained in this dissertation is to develop a suitable method for prevention and treatment of colon cancer. The survival, mechanical strength and overall performance of microencapsulated bacterial cells in gastrointestinal passage were evaluated in vitro. The effect of the oral delivery of microencapsulated probiotic *Lactobacillus acidophilus* bacterial cells in yogurt, as a carrier, as novel approach in amelioration of gastrointestinal health has been investigated in animal model. In addition, we evaluated various cancer markers to assess the efficacy and the immunomodulatory effect of a novel preparation. Thus this study will help develop a new nutraceutical probiotics formulation suitable in prevention of colon cancer.

1.2 Thesis research hypothesis and objectives

There is a direct relationship between diet and mortality rate in colorectal cancers. There is some evidence that delivery of probiotic bacteria reduce the risk of colon cancer. However, probiotic cultures have not been projected as therapeutics due to their low clinical efficacy and associated limitations. During this resreach a question was asked whether the microencapsulated probiotic bacterial cells exhibit sufficient colon cancer theraeutic features. Specifically, a hypothesis has been made that the delivery of microencapsulated live probiotic yogurt lactobacillus bacterial cells formulation will exibit colon cancer therapeutic effects. The main objective of this research was to create a yogurt based formulation containing live probiotic bacterial cells for use in colon cancer. Both in-vitro and in-vivo studies have been carried out.

Specific objectives are:

- To prepare and optimize microcapsule yogurt formulations by selecting suitable bacterial strain and polymeric membrane
- To determine the physical, chemical and microbial stability of these formulations in-vitro
- To evaluate the in vitro efficiency and safety of artificial cell microcapsules containing probiotic bacterial cultures

- To test oral bacterial suitability of these formulations in a computer controlled GI model simulating human intestinal microbial system
- To investigate preclinical efficacy of these formulations in colon cancer animal model
- To evaluate pharmacokinetics and toxicology of these formulations in colon cancer animal model
- To evaluate the overall anti-inflammatory effect of microencapsulated live lactobacilli cells using various biomarkers and other parameters
- To investigate long term efficacy of microencapsulated probiotic yogurt formulation in experimental animal model

1.3 Thesis Outline

This dissertation continues with a more detailed description of the material covered in this introduction. This thesis is divided into 10 chapters. Chapter 1 describes the background, research hypothesis and objectives of this thesis, followed by an extensive literature review in Chapter 2. Chapters 3-8 contain 6 original papers published or to be submitted to scientific journals. These research articles include the main studies performed to achieve this thesis` objectives. Chapter 9 summarizes the findings of this thesis work, and claims to the original contributions to knowledge. Recommendations for future research are included in Chapter 10 of this thesis.

CHAPTER 2

Literature Review

In the past decade the field of gastrointestinal (GI) related diseases has received a substantial interest. Gastrointestinal disorders fall into two categories: functional and inflammatory. Functional GI disorders, such as Irritable Bowel Syndrome (IBS), chronic diarrhea, functional dyspepsia, constipation and intestinal pain, and other chronic medical conditions like gastro-esophageal reflux disease and asthma are characterized by symptoms and not by a visible sign of disease or injury. Inflammatory GI disorders include Inflammatory Bowel Disease (IBD), Crohn's Disease (CD) and ulcerative colitis[26].

Physicians treating GI disorders had to face new challenges during recent years. By increasing the cost of medical care there has been an obvious need for more reliable treatment method of high efficacy.

Gastrointestinal research is constantly working on exploring new techniques, designing new systems and discovering new ways of delivering certain drugs of interest to the target organs. In particular, gastrointestinal cancers pose major public health problems in the developing countries, matching its effect in industrialized nations especially within older population who are often predisposed to those disorders [27].
Functional gastrointestinal disorders significantly impact the health-related quality of patients' life. Impaired health-related quality of life has been demonstrated, in particular, in patients with moderate to severe disease seen in referral settings [28].
Although the quality of life appears to improve in treatment responders, or correlates with symptom improvement, with at least some treatment modalities studied in functional gastrointestinal disorders the efficient and safe treatment has yet to arrive.
The significance of psychological factors such as early adverse life events or symptoms related to visceral perception such as pain and chronic stress further contribute to impairment of health-related quality of life in patients with functional GI disorders [29].

The presence of extra-intestinal symptoms appears to have a major if not greater impact on health care visits, excess health care costs and health-related quality of life in patients with functional gastrointestinal disorders [28].

2.1 Colon cancer

There are six main diseases associated with colon: Crohn's disease (ileitis or enteritis), diverticulitis, ulcerative colitis, irritable bowel syndrome (IBS), colonic dismotility and colon cancer. Despite considerable efforts to improve early diagnosis and treatment cancer continues to be one of the major health and socio-economic problems worldwide. Colorectal cancer is the most common cancer in Western countries and the second leading cause of cancer related deaths. Colorectal cancer is the second leading cause of cancer related deaths in the United States, accounting for nearly 60,000 deaths each year [30]. In addition, at least 100 million patients in the USA and Canada have recurrent long-term gastrointestinal (GI) problems. According to The American Cancer Society, about 50 000 Americans die from colorectal cancer each year. An estimated 106,680 cases of colon and 41,930 cases of rectal cancer are expected to occur in 2006. Sporadic lesions represent 75-80% of all colorectal cancer, whereas 20-25% are in younger individuals or in patients with a family history of cancer, suggesting a heritable susceptibility [31].

In the European Union, lung cancer is the principal cause of death in men (25% of all male cancer deaths) followed by colorectal and prostate cancers. In women, the three major causes of death are breast cancer (16% of all female deaths), colorectal (12%) and lung cancer (9%) [27].

Animal studies have provided important insights into the etiology of colon cancer; however, there are no major breakthroughs in the prevention of this disease. The lack of treatment is mostly due to no advances made in understanding the cause of this disease.

2.2 Limitations of current modalities for colon cancer treatment

Current treatment modalities of colorectal cancer involve: surgery, chemotherapy and radiation.

Surgery or colon cancer resection is the most frequently performed procedure to manage colorectal cancer; they are used to cure stage I colon cancer patients. Patients with stage II or III colon cancer can undergo a surgery for a chance to be cured and stage III patients would receive cancer-fighting drugs (chemotherapy). Treatment of stage IV cancer rarely leads to a cure [32]. Colon cancer resection requires a specialist surgeon, the use of disposable instruments, long operating hours and increased costs due to investment in the equipment. Early postoperative small-bowel obstruction (EPSBO) is a common serious complication after colectomy [33]. Multiple metastases (in various locations) ultimately limit surgical removal and the effectiveness of anti-cancer drugs.

Chemotherapy is the most widespread option of systemic treatment, because it can reach and destroy cancerous cells throughout the body, even the ones that might have already metastasized. Chemotherapy may be used alone or in combination with other forms of treatment [27].

A recent in vitro study investigated a new drug for treatment of metastatic colorectal cancer Irinotecan (CPT-11) [34]. The cytotoxic effect of the new drug was evaluated alone and in combination with another drug mitomycin C (MMC) along with three colorectal cancer cell lines: CACO-2, HT-29, and DHD/K12/TRb (PROb)[34]. It was found that the combination of CPT-11 and MMC had a large spectrum of cytotoxicity in in vitro models. This indicates that a clinical use of MMC and CPT-11 to treat colorectal peritoneal carcinomatosis of colorectal origin was designed. However, further in vivo studies need to confirm these findings.

A recent in vitro and in vivo study tested the effect of combined therapy with demethylating agent 5-aza-2'-deoxycytidine (DAC) and irinotecan (CPT-11) on the human colon cancer cell line HCT-15 [35]. In this study it was observed that DAC might increase the tumor sensitivity to chemotherapy through demethylation and restoration of gene expression. In addition it was noted that a low-dose of DAC may have the potential to be used as a "biosensitizer" of DNA-damaging agents such as CPT-11given as a pretreatment when the apoptotic pathway is inactivated as a result of aberrant promoter methylation in the cancer[35].

There is a number of studies indicating that regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) may be associated with decreased colorectal cancer risk [36-

[39]. However, the studies did not take into consideration the specific location of the tumors. In a study by Mahipal *et al.* the impact of nonsteroidal anti-inflammatory drugs (NSAIDs) on the proximal and distal colon was evaluated [40]. The study found not significant association between either colon segment and aspirin or nonaspirin NSAID use. Unfortunately, the use of NSAID drugs like aspirin, whether enteric or non-enteric coated, leads to side effects like gastrointestinal hemorrhage [41-43] and digestive system problems [44,45]. A comprehensive review on aspirin-related gastrointestinal side-effects and the mechanism by which aspirin causes gastrointestinal damage was published by Hochain *et al* [46]. Today the adjuvant therapy for colorectal cancer consists primarily of combinations of 5-fluorouracil/leucovorin (5-FU/LV) with oxaliplatin or oral capecitabine. In addition, bevacizumab (angiogenesis inhibitor) and cetuximab (epidermal growth factor receptor inhibitor) are supplemented [47].

Detailed analysis of Phase III trials of infusional 5-fluorouracil/leucovorin and oxaliplatin (FOLFOX) as a new standard of care in the palliative and adjuvant treatment of colorectal cancer have been summarized in a review by Grothey *et al.*[48].

In addition to already described pharmacologic treatments, the nonpharmacologic, complementary and alternative strategies are being tested as well and according to initial trials they result in modest alteration of sporadic adenoma recurrence rates. These include supplements in a form of calcium[49], folic acid[50], and selenium[51]. Table 2.1 summarizes various GI-related diseases along with proposed medication and their limitations.

Table 2.1: Various gastrointestinal diseases and their treatment limitations (reprinted with permission from *Biologics: Targets and Therapy*) [52].

Disease	Proposed treatment	Potential adverse symptoms/problems	References
Collagenous colitis (CC)	Prednisolone Boswellia serrata	accumulation of lymphocytes in the colonic epithelium and connective tissue thickening of the subepithelial collagen table	[53, 54]
Diarrhea	Prednisolone Omeprazole Asprin Bisphosphonates	frequent watery, loose bowel movements damage to the mucosal lining an inhibition of absorption	[55, 56, 57]
	Bismuth subsalicylates Budesonide	Profuse watery diarrhea Higher incidence of immune diseases	[58, 59, 60]

Microscopic Colitis	Prednisolone Boswellia serrata Cholestyramine 5-aminosalicylic acid		
Gastritis	Omeprazole Asprin Mistoprostol Tetracycline Metronidazole	Antibiotic resistance Gastric ulcers Gastrointestinal bleeding Recurrent abdominal pain Increased gastric cancer risk	[61] [62] [63], [64],
Inflammatory Bowel Disease (IBD)	Sulfasalazine Mesalazine Azathioprine 6-mercaptopurine Methotrexate Glucocorticosteroids Infliximab	Leukopenia Mononucleosis-like syndrome Nephrotoxicity Steroid resistance which leads to surgical interventions	[65] [66] [67], [68], [69], [70],
Colon Cancer	Irinotecan (CPT-11) Mitomycin C (MMC) 5-aza-2'-deoxycytidine (DAC) irinotecan (CPT-11) NSAID 5-fluorouracil/ leucovorin (5-FU/LV) Oxaliplatin Capecitabine	Toxicity Gastrointestinal bleeding Digestive complications Temporary effect on bone marrow Hair loss Anemia Overall fatigue May increase cardiovascular adverse events Lowered resistance to infections No data about specificity of treatments	[71] [72] [40], [73], [74], [75], [76], [47], [77],

2.3 Colon, an ideal place for action of biotherapeutics molecules – overview of gastrointestinal tract and its molecular basis

The human GI tract has the following fuctions: ingestion, digestion, absorption and defecation. It consists of the upper and lower GI tracts. The upper tract is made of mouth, pharynx, esophagus, and stomach. The lower includes the small (duodenum, jejunum and ileum) and large (cecum, colon and rectum) intestines and anus [78]. In a healthy human, the small intestine measures from 3.5 to 6.5 meters in length [79]. The large intestine, a tube-like organ is divided into six regions: cecum, ascending colon, transverse colon, descending colon, sigmoid colon, rectum and is 90-125 centimeters in

length [79]. It is derived from the embryonic hindgut, supplied by the inferior mesenteric artery and serves principally as a storage organ [79]. The colon is viewed as the preferred absorption site for oral administration of protein and peptide drugs because of the relatively low proteolytic enzyme activities in the colon [80]. Below figure depicts an illustration of the human gastrointestinal tract and figure 2.2 shows an in vitro simulation of the human GI tract using human intestinal microbial system. This dynamic system is composed of 5 vessels each representing a distinct part of the GI tract. It is being computer monitored and its contents are being kept in anaerobic conditions by flushing the headspace of each vessel with N_2 and the temperature is kept constant at 37°C [81].

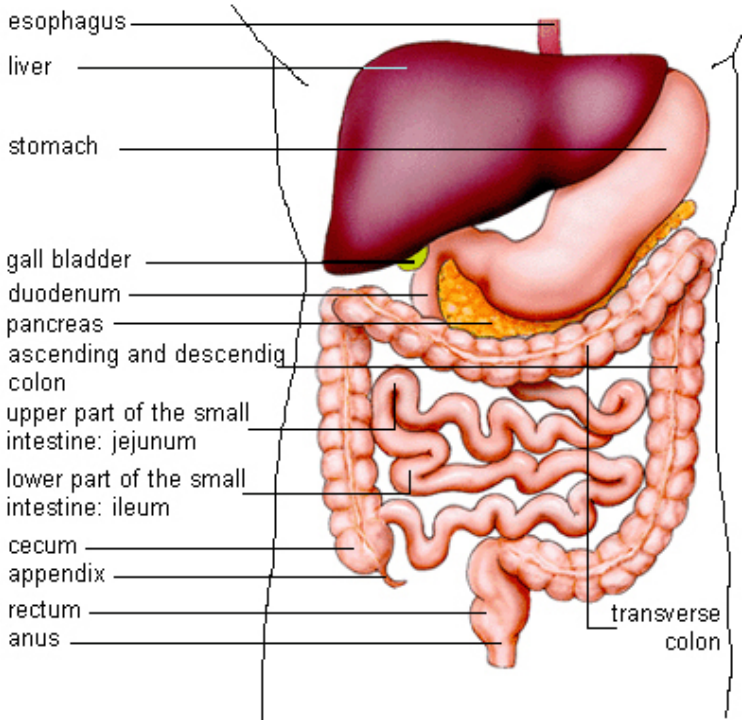

Figure 2.1: Schematic representation of the human gastrointestinal tract.

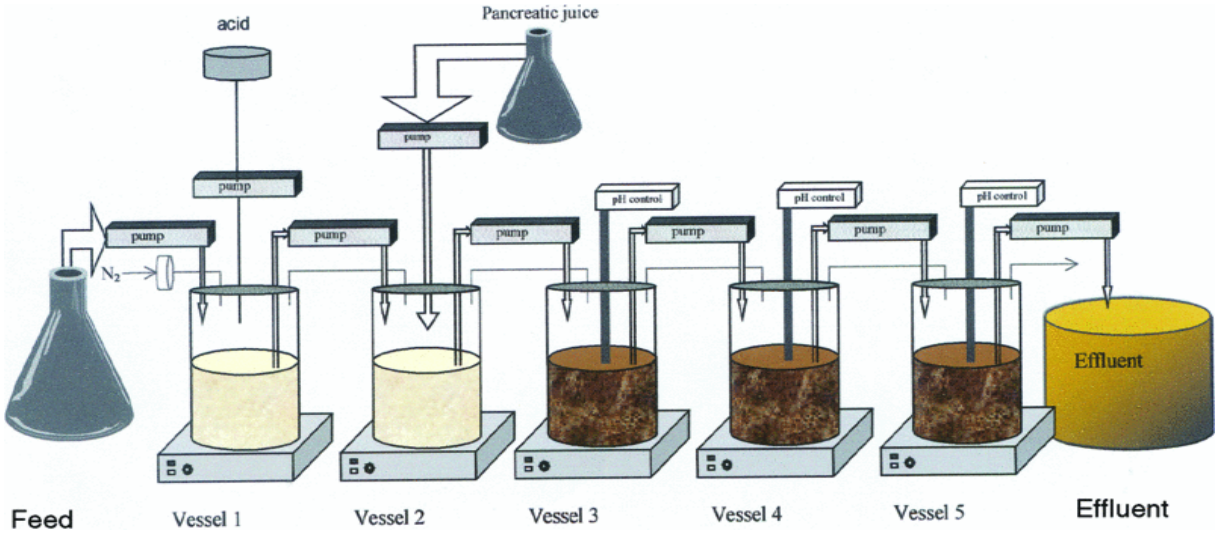

Figure 2.2: Schematic representation of the simulated human gastro-intestinal (GI) model. Vessel 1: Stomach; Vessel 2: Small intestine; Vessel 3: Ascending colon; Vessel 4: Transverse colon; and Vessel 5: Descending colon[81].

2.3.1 The microbial flora of the colon

The "microflora" or "microbiota" refer to the microbial ecosystem colonizing the gastrointestinal tract. In mammals, the GI tract is highly complex and diverse. It includes bacteria (95% are obligate anaerobes and 10% are facultative anaerobes) few fungi and protozoa [82]. It has been estimated that there are approximately 10^{12} viable bacteria per gram of large bowel content in humans (it ranges from < 10^3 colony-forming units/ mL (CFU/mL) in the stomach, to 10^{11} - 10^{12} CFU/mL within the colon, where anaerobes outnumber aerobes by a ratio of 1000 : 1) with the presence of at least 400 to 500 species [83,84]. Although substantial part of bacterial population within the human gut still needs to be identified, their relevance and impact on the host physiology and pathology are already well documented. The main functions of intestinal microflora include (1) metabolic activities translating into energy and nutrients uptake, and (2) host protection against invasion by foreign microorganisms [85]. A complex set of interactions and successful coexistance between the host and microorganisms colonizing the gut defines its normal structure and function [86]. The intestinal lumen hosts large amounts of immunogenic and pro-inflammatory molecules leaving gut mucosa in a state of restrained

immune reactivity driven by the intestinal flora. Enteric epithelial cells of the colon act as sensors, especially for the presence of pathogenic organisms, and signal the onset of mucosal inflammation. A constant bacterial stimulus is needed for the induction and perpetuation of inflammation [87]. However, not all commensal bacteria are equal in their ability to induce inflammation as they are influenced by the host genetic background [88].

2.3.2 Balance, regulation and inflammation of the gut mucosa

There is a constant communication and, under healthy condition, balance, between lumenal contents and bacteria. Bacteria are capable of producing such products as peptidoglycans and lipopolysaccharides, in addition to other substances, which stimulate the intestinal immune system and subsequently define mucosal inflammatory pathways[89]. The straightforward definition of inflammation is "a reaction of the microcirculation characterized by movement of fluid and leukocytes from the blood into extravascular tissues" [79]. The response of endothelial cells to inflammatory conditions is marked by altered production of vasoregulatory mediators, such as PGI_2, endothelium-derived relaxing factor (EDRF) and endothelin, or the modulation of platelet aggregation and thrombus formation and finally, the accumulation and recruitment of inflammatory cells [79].

2.3.3 The gut epithelium and its molecular basis of immunity

Although the macromolecular transport by the gut epithelium was established in the 1970s, we still have insufficient knowledge about the fate of the simple protein antigens during gut transport. The maintenance of immune homeostasis is essential so antigens selectively do not get degraded during transport across the epithelium [90]. In addition, an intestinal mucosa plays an important gut barrier role [91]. Its main functions include physical and epithelial barrier with intercellular tight junctions and constant cell turnover, a site for glycoproteins to compete with gut surface for bacterial or antigen binding as well as a site which favors bacterial colonization [91]. The importance of mucus as a potent anti-oxidant and inflammatory mediator is well recognized [92,93]. The mucous layer and mucin production undergo a qualitative and quantitative alteration in many situations of intestinal stress, such as the inflammatory bowel diseases (IBD) [93], ulcerative

colitis (UC) and Crohn's disease (CD) [94]. Moreover, in the active phase of the disease, UC patients exhibit reductions in the thickness of the colonic mucous layer, in the number of mucus-containing goblet cells, and in ex vivo analyzed MUC2 production (the main secreted-colonic mucin) [95].

The intricate system of digestive tract to restrict its potentially harmful contents is further made of the epithelial layer with presence of intercellular tight junctions as well as expression of adherence factors on the surface [96]. The tight junctions provide protection of the intercellular spaces and restrict the passage via the extra cellular pathway; any perturbation of the intestinal barrier may lead to promotion of bacterial adherence [96]. For instance, a study revealed a redistribution of the tight junctional transmembrane protein upon infection with *Campylobacter jejuni* which is a leading cause of human enterocolitis and is associated with postinfectious complications, including irritable bowel syndrome (IBS) and Guillain-Barre syndrome [97]. The adherence factors are expressed on the surface of epithelial cells. For example, *Helicobacter pylori* has been identified as such agent which once attached to the epithelial surface initiates infection. In addition to *H. pylori*, there are other specific adhesins identified, all possessing same role: adherence to gastric epithelial cells directly or through interaction with other adhesins [98]. The presence of adherence factors is genetically determined but the expression can be modified [99].

Pattern recognition receptors (PRRs) play a role in allowing innate immune cells to distinguish between "own" and microbial "foreign" based on the recognition of pathogen-associated molecular patterns (PAMPs) via structures such as bacterial flagella, peptidoglycans and endotoxins [100]. Toll-like receptors (TLRs) play a role in microbial recognition, induction of antimicrobial genes, and the control of adaptive immune responses and are attractive targets for designing various drugs for such disorders as inflammation, infections, autoimmunity, allergies and cancer. [101]. Figure 2.1 summarizes briefly the concept of TLRs.

TLRs are expressed in epithelial cells of the skin, respiratory, intestinal, and genitourinary tracts that form the first protective barrier to invading pathogens [102]. TLRs activate downstream effectors through adaptors that contain Toll/interleukin-1 receptor (TIR) domains, but the mechanisms accounting for diversification of TLRs effector

functions are unclear [103]. More detailed description of molecular composition and functions of TLRs is beyond the scope of this review.

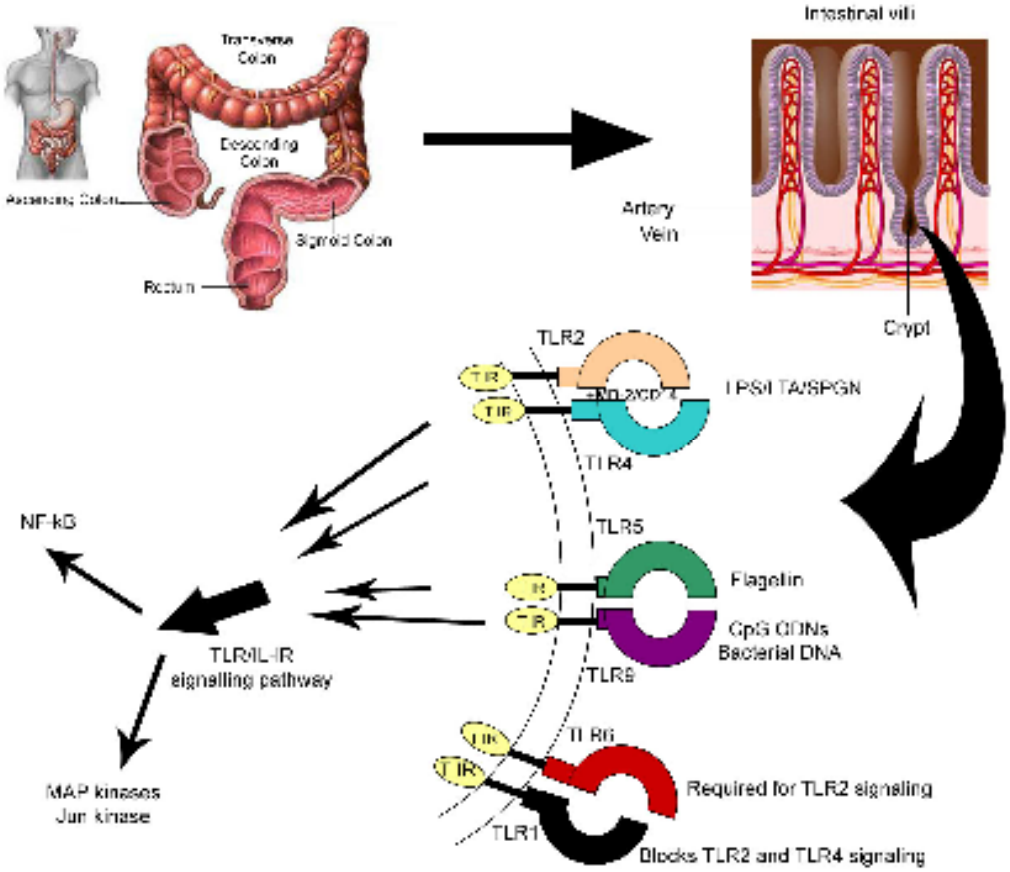

Figure 2.3: Schematic representation of molecular Toll-like receptors (TLRs) of the human gastrointestinal tract (reprinted with permission from *Biologics: Targets and Therapy*) [52].

2.5 Biomarkers and diagnostics

According to US Food and Drug Administration in *Guidance for industry-pharmacogenomic data submissions* a biomarker is defined as the one that is "measured in an analytical test system with well-established performance characteristics and for which there is an established scientific framework or body of evidence that elucidates the physiologic, toxicologic, pharmacologic, or clinical significance of the test results" [104].

The identification of new constituents as biomarkers for early diagnosis of neoplastic/benign cells and the discovery of new type of treatments with their mechanistic actions are crucial to improve cancer therapy. As the colon cancer develops through an adenoma-carcinoma sequence and its outcome is strongly correlated with poor diet (high quantities of red/fat meat and lack of fiber) its screening parameters should be able to detect the effect of dietary factors and chemotherapeutic agents contained in them on colon carcinogenesis [105]. Two of the most recommended screening tests for colorectal cancer available to patients today include a home fecal occult blood test (FOBT) or colorectal endoscopy (sigmoidoscopy or colonoscopy) [106]. Although widely available and effective in reducing mortality, the studies show that colorectal cancer screening prevalence in the United States is quite low [106]. This is due to the factors such as lack of health-care coverage as well as poor access to information allowing such screening in the first place. Today, biomarkers are used extensively in both animal studies and clinical trials to investigate the effect of various factors on colon and other cancers.

2.5.1 Feccal Occult Blood Test (FOBT)

Men and women who are genetically predisposed to colorectal cancer development and who are at average risk should be offered screening for this cancer and any presence of potential polyps at the age of 50 years old. FOBT using a guaiac-based test should be performed on yearly basis and if any positive outcome is received, they should follow with screening using colonoscopy [107]. The major disadvantage of this test is that it might fail to detect all present polyps as well as it could have result in false positive readings. In either case, if test is used regularly, it can still detect up to 92% of cancers [108]. Using this test in animal models is also considered a valid screening tool.

2.5.2 C-Reactive Protein (CRP)

C-reactive protein (CRP) is a well known acute-phase marker of inflammation in the body and it has been reported to be an independent prognostic factor of colorectal cancer [109]. CRP is produced mainly in the liver along with other acute-phase proteins in

response to cytokines, such as interleukin-6 (IL-6) released by phagocytes during infection, trauma, surgery, burns, tissue infarction, advanced cancer and chronic inflammatory conditions [110]. Several lines of evidence suggest that colorectal neoplasia may arise from colonic areas with chronic subclinical inflammation [111].
Although CRP is considered non-specific systemic marker of inflammation, many researchers have hypothesized that CRP may act as a biomarker for chronic low-grade intestinal inflammation and the subsequent development of colorectal cancer [112].

2.5.3 Interleukin -6 (IL-6)

Interleukin – 6 plays a key role in the induction and maintenance of gut inflammation during Inflammatory Bowel Disease (IBD) and it has been recently shown to influence the development and growth of colitis associated with colorectal cancer both in vitro and in vivo [113]. This immunomodulatory cytokine also plays a role in growth stimulation, metastasis, and angiogenesis in secondary tumors in a variety of malignancies [114]. It is released in large concentrations from the tumor itself as well as from tumor infiltrating leukocytes. As the levels of this cytokine increase, it increases the invasiveness of colon cancer cells and promotes secondary tumor formation through its angiogenic potency [115].

2.5.4 Interleukin-12 (IL-12)

It is known that interleukin-12 (IL-12), previously called cytotoxic lymphocyte maturation factor (CLMF) or NK cell stimulatory factor (NKSF) is a very potent antitumoral cytokine [116]. Its local and systemic administration has been extensively studied in animal models and revealed that IL-12 has powerful antitumor, anti-metastatic and anti-angiogenic activities and seems to be less toxic than other immunotherapeutics such as interferons or IL-2 [117]. It was also tested in pre-clinical tumor immunotherapy studies where adequate doses of IL-12 were delivered to patients. However, its full potential has been limited by dose-dependent toxicity [116]. In a comparative study performed by Rakhmilevich *et al.*, IL-12 was the most effective cytokine gene that could

induce eradication of experimental tumors, prevent the development of metastases, and elicit long-term antitumoral immunity [118].

2.5.5 Tumor Necrosis Factor-α (TNF-α)

TNF-α has previously been implicated in the development of colitis, and indeed pharmacologic blockade of TNF-α with monoclonal antibodies has demonstrated great efficacy in the treatment of IBD patients [119]. TNF-α has also been implicated as a positive factor in the development of some other epithelial malignancies, particularly skin cancer in mice [120] raising the possibility that TNF-α may play a similar tumor-promoting role in colorectal cancers [121]. TNF-α originating from the mucosa or possibly the epithelium itself, participates in orchestrating the activation of immune cells. Production of various proinflammatory factors by the activated immune system participates in the ensuing inflammatory response but additionally plays a role in tumor growth by providing trophic signals to the early neoplastic lesions. Loss of TNF-α signaling in immune cells, and not the mucosa, stops this cascade by aborting the mucosal inflammatory response, and this can be achieved by pharmacologic blockade of TNF-α with etanercept and possibly other agents[122].

2.5.6 Interferon – γ (INF- γ)

This pro-inflammatory cytokine, interferon-gamma, of molecular weight 34–50-kD is produced by T cells and natural killer (NK) cells as well as $CD4^+$ and $CD8^+$ T cells allowing rapid responses in the absence of specific T-cell recognition of pathogen [123]. Production of the cytokine interferon γ (IFN-γ) is pivotal in determining the effectiveness of the immune response to pathogens. Its importance is highlighted by mice and humans with defects in IFN-γ or IFN-γ receptor (IFN-γR), which result in profound deficiencies in their responses to certain intracellular pathogens, including mycobacteria and *Salmonella* [124]. Studies have shown that cytokines can effectively treat solid tumors by a direct cytotoxic effect as well as by immunomodulation [125].

2.5.7 Interleukin -1β (IL-1β)

Interleukin-1β is a crucial mediator of inflammation. It is abundant at tumor sites, being secreted by the malignant cells or microenvironmental cellular elements, in response to local inflammatory signals. IL-1 has pleiotropic effects on malignant processes, ranging from promoting invasiveness and metastasis to induction of antitumor cell immunity and inhibition of tumor growth [126]. Tumor cells secreting IL-1beta are invasive and metastatic. The production of active IL-1β is tightly controlled on several levels to avoid severe adverse effects [127]. The process of IL-1β secretion can be split into two major steps. First, gene expression and synthesis of the IL-1β precursor are induced by inflammatory signals such as PAMPs; then a second signal, namely exogenous ATP, induces inflammasome activation and secretion of mature IL-1β [128].

2.5.8 Thromboxane B2 (ThB2)

Thromboxane is one of the products obtained during the catalysis of arachidonic acid by cyclooxygenase enzyme followed by thromboxane synthase (TXS). The key regulatory step in this process is the enzymatic conversion of arachidonate to prostaglandin G_2 (PGG_2), which is then reduced to an unstable endoperoxide intermediate, prostaglandin H_2 (PGH_2). Specific prostaglandin synthases in turn metabolize PGH_2 to at least five structurally related bioactive lipid molecules, including prostaglandin E_2 (PGE_2), prostaglandin D_2 (PGD_2), prostaglandin F_2 (PGF_2), prostaglandin I_2 (PGI_2) and thromboxane A_2 (TxA_2), in a cell type-specific manner. Prostaglandins are unstable compounds that are rapidly metabolized in vivo [129]. Moreover, TXA_2 is unstable and it is rapidly degraded into an inactive form of TXB_2 in a non-enzymatic manner [130]. In immune system TXA_2 is capable of stimulating the apoptosis and DNA fragmentation of CD4+/CD8+ cells [131]. In the studies using TP-deficient mice it was found that TXA_2 induces the chemokinesis of native T cells, impairs dendritic cell–T cell adhesion, and inhibits dendritic cell-dependent proliferation of T cells, showing the modulation of acquired immunity [132].

2.5.9 Prostaglandin E_2 (PGE_2)

Prostaglandins are locally acting molecules involved in both physiological and pathological functions. PGE_2 is the most abundant prostaglandin found in human colorectal cancers, premalignant lesions, and cells derived from a number of solid malignancies [133]. Prostaglandins derived from the constitutive COX-1 isozyme are produced in many tissues of the body and thought to be responsible for physiological activities including maintenance of the gastrointestinal mucosa, kidney, and platelet functions [134]. Genetic studies using mice lacking the PGE_2 cell surface G- protein coupled receptors EP_1, EP_2, or EP_4 by which PGE_2 exerts its actions either in autocrine or in paracrine fashion, point to an important role for all three receptors in intestinal polyp formation [135]. In an experimental model for IBD, PGE_2 appears to have a dual effect. High levels of PGE_2exacerbate the inflammatory process [136]. On the other hand, PGE_2 signaling suppresses colitis symptoms and mucosal damage by protecting the integrity of the epithelial intestinal wall, presumably through the enhancement of epithelial survival and regeneration [137]. These results provide strong evidence that PGE_2 plays a pivotal role in regulating intestinal adenoma formation.

2.5.10 Cyclooxygenase – 2 (Cox-2)

Cyclooxygenase (COX)-1 and COX-2 are the two isoforms of COX enzymes that convert arachidonic acid into several eicosanoids, such as prostaglandin, the thromboxins, and prostacyclin, which participate in normal physiology and are implicated in inflammation [138]. Cox-1 is constitutively expressed in a broad range of cells and tissues and its expression remains constant under most physiological or pathological conditions. COX-1 contributes to maintenance of the gastric mucosa, regulation of renal blood flow in the afferent vessels of the kidney, and regulation of platelet aggregation [138]. On the other hand, COX-2 is an inducible, immediate-response gene which is regulated by a number of factors, including serum, growth factors, proinflammatory cytokines, hormones, oncogenes, or tumor promoters and has been associated with inflammatory diseases and cancer [139]. Therefore COX-2 is a proinflammatory mediator around which NSAID development is concentrated.

Cyclooxygenase-2 (COX-2) inhibitors are effective chemopreventive agents against colorectal cancers. Several preclinical studies have suggested that COX-2 inhibitors may enhance the effects of anticancer-agents [140,141]. Overexpression of COX-2 has also been associated with colorectal cancer. In human studies, COX-2 is increased in 80-90% of CRC tumors and in 40% of premalignant colorectal adenomas[142].
In addition to human studies, the role of COX-2 in carcinogenesis has been extensively documented, and in some tumor animal models and COX-2 overexpression has been implicated in metastasis and the development of resistance to antitumor agents [143].
Although the proposed role of COX-2 in tumor development and progression as well as the effects of COX-2 inhibitors in combination with chemotherapeutic agents such as 5FU with leucovorin against advanced cancer have not been fully elucidated, numerous studies have demonstrated that selective COX-2 inhibitors can significantly reduce polyp formation and tumor growth in vivo [144,145].

2.5.11 Immunohistochemisty

Immunohistochemisty is an integral detection technique of localizing specific cells found in tissue section using the principle that antibodies will bind to respective antigens [146]. Immunohistochemical markers are available to study intestinal cells and their malignant counterparts. Many of monoclonal antibodies against different T-cell antigens can be studied by immunohistochemistry in intestinal tissues.
Cytokine immunohistochemistry is frequently used in combination with molecular biological techniques such as in situ hybridization (ISH) and polymerase chain reaction (RT-PCR) to gain insight into the biological role of cytokines in diseased tissues [147].
There is now ample evidence that tumor growth in cancer patients and mice is associated with hematological alterations that include the accumulation of immature myeloid precursor cells in the bone marrow and spleen. In mice, these cells have the phenotype of granulocyte/macrophage precursors, and they express the granulocyte Gr-1 and macrophage Mac-1 (CD11b) markers, respectively [148].
It is considered still quite challenging to use mouse monoclonal antibodies on mouse or rat tissues due to the fact that background might develop from binding of the secondary

antibody (anti-mouse Igs) to endogenous Igs in murine tissues and false positive results might be obtained [149].

This problem has restricted or even precluded the use of immunohistochemistry with mouse monoclonal antibodies in murine tissues. Currently, numerous manufacturers have specific detection systems for mouse tissues, eliminating this problem. For instance, one solution would be the use of clocking steps before and after addition of the primary antibody. Another way of avoiding receiving false results would be to preincubate the primary antibody with biotinylated anti-mouse Fab complexes (used as secondary antibody) or blocking free binding sites in the complexed secondary antibodiess with normal mouse serum before adding the antibody mix to the tissue section [150].

2.6 Probiotics and their potentials in colon cancer treatment

For over 100 years now the beneficial effects in human health have been directly linked to ingestion of fermented milk products. Lactic acid bacteria (LAB) are the organisms most commonly used as probiotics, which are known to improve the intestinal microbial balance of the host animal [151]. Yogurt is fermented milk that contains the *Lactobacillus delbrueckii*, *Lactobacillus bulgaricus,* and *Streptococcus thermophilus* as its starter [152]. Probiotics, defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" have many attributes including the lack of adverse side effects associated with their use [153].

Probiotic bacteria play an important role in retarding colon carcinogenesis by possibly influencing metabolic, immunologic, and protective functions in the colon [154]. The use of probiotics in prevention and cancer treatment has been undergoing a recent evaluation in a number of studies. Although we should not expect miraculous outcomes in cancer treatment following probiotics administration, their immunomodulatory properties have been tested and need to be brought to publics' attention.

Table 2.2 summarizes various diseases and lists probiotic species used in their treatment, their dose, mode of delivery and potential site of action.

Table 2.2: Various pathological conditions treated with viable probiotic microorganisms – (reprinted with permission from *Biologics: Targets and Therapy*) [52].

Disease	Biotherapeutics used	Live Bacterial Cell Optimal Dose (d)	Mode of delivery	Potential site of action	Ref
Immunologic modulation	*Lactobacillus acidophilus* *Lactobacillus plantarii* *Bifidobacterum lactis* *Lactobacillus GG* *Lactobacillus johnsonii*	~10^{10}/d ~10^{10}/d ~10^{10}/d ~10^{10}/d ~10^{10}/d	Vaccines Capsules Foods	Small and large intestines	155-161
Rotavirus Diarrhea	*Lactobacillus GG* *Lactobacillus reuteri* *Lactobacillus acidophilus* *Lactobacillus bulgaricus* *Bifidobacterium bifidum* *Streptococcus thermophilus* *Saccharomyces boulardi* *Lactobacillus casei* *Bifidobacterum bulgaris*	~10^{10}/d ~10^{9}/d ~10^{10}/d ~10^{8}/ml ~10^{10}/d 500mg/d 500mg/d ~10^{10}/d ~10^{10}/d	Capsules Foods	Small and large intestines	162-169
Antibiotic associated Diarrhea	*Saccharomyces boulardi* *Lactobacillus GG*	500mg/d ~10^{10}/d	Capsules Foods	Small and large intestines	170,171
Radiation induced Diarrhea	*VSL#3 (Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Lactobacillus delbruekii* ssp. *bulgaricus*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium infantis* and *Streptococcus thermophilus*)	4.5x10^{9}/ 2 capsules	Capsules	Small and large intestines	135
Vaginal infections (bacterial vaginosis (BV), candidal vaginitis, pelvic	*Lactobacillus acidophilus*	~10^{10}/d	Ointment Capsules Foods	Skin, Stomach, small and large intestines	172-174

inflammatory disease)					
Helicobacter pylori colonization/ gastric ulcers	*Lactobacillus johnsonni* *Saccharomyces boulardi* *Lactobacillus acidophilus* *Lactobacillus gasseri*	~10^{10}/d 500mg/d ~10^{10}/d ~10^{8}/d	Capsules	Stomach, duodenum, small and large intestines	175-179
IBD CUC, Crohn's Pouchitis	*Escherichia coli Nissle* *Saccharomyces boulardi* *VSL#3* *Lactobacillus GG*	500mg/d ~10^{10}/d	Capsules	Small intestines	180
Ulcerative Colitis	*Escherichia coli Nissle*		Capsules	Small and large intestines	181-183
Irritable Bowel Syndrome	*Lactobacillus plantarii* *Bifidobacterium infantis*	~10^{10}/d ~10^{10}/d	Capsules	Small and large intestines	184-189
Collagenous Colitis	*Lactobacillus acidophilus* *Bifidobacterium animalis* subsp. *Lactis*	~10^{10}/d ~10^{10}/d	Capsules Injections	Large intestines	190,191

It is important to note that the desired effects are strain and dose specific and therefore more clinical studies are needed to screen each strain and corresponding disorder. In animals, LAB ingestion was shown to prevent carcinogen-induced preneoplastic lesions and tumors [192,193]. In the study by McIntosh *et al. Lactobacillus acidophilus* (Delvo Pro LA-1), *Lactobacillus rhamnosus* (GG), *Bifidobacterium animalis* (CSCC1941), and *Streptococcus thermophilus* (DD145) strains were examined for their influence on 1,2-dimethylhydrazine (DMH)-induced intestinal tumors in 100 male Sprague-Dawley rats when added as freeze-dried bacteria[194]. This study concluded that the strain of *L. acidophilus* supplied as freeze-dried bacteria in the diet was protective because it significantly inhibited tumors within the rat colon. There is a substantial amount of studies done by Perdignon *et al*. dealing with anti-inflammatory properties of probiotic bacteria [12,14,195]. In the study by Galdeano *et al.* the probiotic bacterium *Lactobacillus casei* was screened for its influence on the expression of receptors involved in the innate immune response in colorectal cancer BALB/c model mice [151].

Further, a complex nature of kefir was studied in BALB/c mice. Kefir is fermented milk produced by the action of lactic acid bacteria, yeasts and acetic acid bacteria, trapped in a complex matrix of polysaccharides and proteins. In addition, it is an

excellent source of proteins and calcium [196]. A study concluded that since LAB contained in kefir along with yeasts and acetic acid bacteria have an in vivo role as oral biotherapeutic substances capable of stimulating immune cells of the innate immune system they are able to promote cell-mediated immune responses against tumors and also against intracellular pathogenic infections[197]. In another kefir-related study by Vinderola *et al.* [198] , the immunomodulating capacity of kefir on the intestinal mucosal immune response in mice of viable or heat-inactivated bacteria at different doses was determined.

The adjuvant immunomodulatory effect of kefir was tested in rats, young and old[199]. It was found that only young rats had an enhanced in vitro antibody secretion by cultured lymphocytes isolated from the Peyer's patches and the intestinal lamina propria, thus only they benefited from intestinal mucosal immune response caused by kefir ingestion. In the study testing the effects of lactulose and lactitol on colonic microflora and enzymatic activity, a reduced activity of pro-carcinogenic enzymes in humans was obtained as a consequence of prebiotic intake [200]. However, in humans, there is no evidence available on whether probiotics can prevent the initiation of colon cancer. Epidemiologic studies are contradictory; some studies could not find an association between the consumption of fermented-milk products and the risk of colon cancer [201,202] whereas other studies showed a lower incidence of colon cancer in persons consuming fermented-milk products or yogurt [203-205].

In table 2.3 the protective mechanism of action of several probiotic bacteria is summarized. An important consideration expressed in the probiotic definition is the one concerned with the viability of the micro-organisms[206]. Although Ouwehand A.C. *et al*.[207] received interesting results while working on treatment for acute diarrhea suggesting that in some cases nonviable bacteria are able to produce effects similar to those obtained with viable bacteria, these findings need to be further explored and proven effective in treatment of other disorders.

Table 2.3: Postulated protectibve mechanisms of probiotics and prebiotics in the development of colon tumors[208].

Postulated protective mechanisms of probiotics and prebiotics in the development of colon tumors		
Ingestion or investigation of	Protective mechanisms	References
Lactobacillus casei, omniflora, or yogurt	Mutations in the Ames test decreased	209,210
Various strains of *Lactobacillus* and *Bifidobacterium,* cellular components and metabolites of LAB	DNA damage in colon cells decreased (antigenotoxicity)	211,212
Bifidobacterium fermented milk; fermented milk with *Lactobacillus acidophilus, Bifidobacterium bifidum, Sreptococcus lactis,* and *Streptococcus cremoris*; lactulose	Procarcinogenic enzyme activity decreased: β-glucuronidase, nitroreductase, azoreductase,and detoxifying enzyme activity increased; GST	213-216
L. acidophilus, S. cremoris, cell wall of LAB	Binding of mutagens	217-219
Milk fermented with *L. acidophilus*	Excretion of mutagens decreased	220
Milk fermented with *L. acidophilus* La1 and bifidobacteria	Immune stimulation increased	221
Fermentation of prebiotics	SCFA increased, pH decreased, probiotics increased	222,223
Butyrate	Proliferation of transformed cells decreased, apoptosis of transformed cells increased	224-226

2.7 The principle of microencapsulation, its design and potential in live biotherapeutics delivery

The principle behind the cell microencapsulation concept, otherwise known as design of artificial cell, is based on a process which consists of enclosing cells, such as bacterial or mammalian live cells, cell lines and genetically engineered cells to secrete therapeutic product in a semipermeable membrane [227]. The importance in designing an adequate membrane for a specific application is crucial as it must be able to allow bidirectional diffusion of nutrients, oxygen, metabolites, and waste but prevent high molecular weight substances, such as antibodies and immunocytes, from entering the microcapsule, as it provides immune protection for the trapped cells [228]. There is a vast amount of different polymers available for membrane composition and design. An ideal microcapsule membrane would be the one composed of natural (alginate, arabinoglycan, chitosan, agarose, poly-L-lysine, xylan and collagen) or synthetic polymers (polyaminoacids, polyacrylates, chondroitin sulfate, cyclodextrin) which would be both biodegradable and biocompatible [228]. Intrestingly, there is a large selection of plant derived polysaccharides, such as amylase, inulin, pectin and guar gum which are known to remain unaffected in the gastrointestinal environment plentiful of enzymes which enable the way for the formulation of the colon targeted delivery systems [229]. Microcapsule composed of alginate poly-L-lysine alginate (APA) polymers is one of the most well-studied and established in the literature encapsulation technologies, including entrapment of cells in alginate gel beads, formation of alginate-poly-L-lysine membrane, and liquefying of the alginate gel core to leave the cell floating in the center of the microcapsule [230]. Studies show that APA encapsulation yields intact capsules that preserve their integrity and are able to retain live bacterial cells [231].

2.8 Microencapsulated live oral biotherapeutics, their potential and limitations

2.8.1 Potential

With the emerging need for alternative treatments in the field of cancers, there is a great potential for microencapsulated live biotherapeutics. Oral delivery is the preferred route of delivery for probiotic bacteria and other viable microorganisms. In action, they are commonly added into foods, especially dairy products as both active ingredients or

nonactive supplements [232]. The following are the factors which make cell microencapsulation an interesting and worth investing time and money technique: polymer membranes protect microencapsulated material from harsh environment while allowing nutrients and metabolites to pass in and out, enormous availability of polymers, biocompatibility of polymers used to prepare artificial cells, safety of clinical grade polymers, low cost.

Cell microencapsulation is one of the promising strategies for in vivo delivery of therapeutic products and so far numerous microcapsule systems have been studied [22,233-237]. It can serve as a preventative and prophylactic method in patients suffering from GI-related diseases by inhibiting the transformation of procarcinogens to active carcinogens, binding/inactivating mutagenic compounds, producing antimutagenic compounds, suppressing the growth of pro-carcinogenic bacteria, reducing the absorption of mutagens from the intestine, and enhancing immune function[238].

So far, the current data is promising and raises hopes in medicine and biotechnology. The future will see the development of new, well characterized, scientifically proven probiotic strains with specific health benefits targeting GI-related disorders. In addition, new based technologies will develop genetically modified probiotic strains with gastrointestinal targeting carrying enhanced immunomodulatory properties. Genetically engineered bacteria have already been encapsulated and used in oral therapy [239,240,241]. This process has an instant application in renal and liver failure, physiologically responsive gene therapy and somatic gene therapy [242].

2.8.2 Limitations

Success of microencapsulation depends on its ability to yield an optimum membrane system enclosing live cells for GI delivery. For instance, a microcapsule needs to be optimized to be able to encounter and not degrade during such conditions as biological enzymes, chemical reactions, heat, pH, diffusion, mechanical pressure and other related physiological and biochemical stresses [243]. When working with polymeric membranes one needs to pay special attention to such factors as membrane retention capacity, leaking, fragility, long-term stability and mechanical properties so the

membrane is suitable to be used both in vitro and in vivo applications. Unfortunately, not all available polymers and their systems are equally applicable. For instance, when microencapsulating with poly-L-lysine, some studies show that an incomplete covering with this polymer occurs by the second alginate incubation, being exposed at the surface of the microcapsule in considerate amount [244-246]. Moreover, there is inconsistency in various reports on permeability of the APA membrane ranging from molecular weight cutoff below 31 KD [247] up to 60-70 KD [247,248]. Although the APA microcapsule system is most widely used for cell encapsulation, some inflammatory problems have been recorded [249,250].

Other problems that microcapsule production containing bacterial cells may encounter include: oxygen stress due to agitation during pH control, oxygen and pressure stresses during centrifugation or filtration, membrane damages during freeze-drying due to freezing itself and due to the drying step. In addition, such situations like production of inhibitory compounds by starter cultures, heating – pasteurization, freezing, food additives (salt, spices, flavours) and drying could further alter viability of probiotic species [251]. It has also been noted that in order to conduct a successful clinical application using microencapsulation, it was difficult to obtain a membrane optimizing all the suitable parameters using the process of binary polyelectrolyte complexation [252].

To improve the quality of the microcapsules, efficient methods need to be established and standardized for the evaluation of overall applicability of microcapsules. The amount of clinical trials is still insufficient to conclude the overall safety of probiotic strains and other biotherapeutics use in therapy. Much work is still needed to exploit these benefits and to increase our understanding of their mechanisms, for instance, improvement of microcapsule membrane designs, methods for improved cell harvest, the mass production of artificial cell microcapsules and the overall cost effective storage and clinical efficacy. Nevertheless, the already available data suggests that only time and additional high quality studies will yield more promising and convincing results. Ultimately, the efficacy, long-term safety and cost effectiveness of these therapies will prove their worth.

2.9 Thesis research goal

There is a great potential of oral delivery of live cells in medicine and biotechnology. However, today's available microcapsule formulations stil lack in performance and approval to be used clinically. Thus, the need to develop new, well characterized, improved, scientifically effective system for artificial cell oral delivery applications is of utmost importance. The main goal in this thesis is to prepare and characterize a novel microencapsulated probiotic yogurt formulation and investigate this system for potential live cell oral delivery in colon cancer prevention and theraphy and other applications.

PREFACE FOR CHAPTERS 3-10

Presented in the following eight chapters are the studies performed in order to achieve the research objectives. Each chapter discusses various important aspects of the thesis research project. Chapter 3 investigates the potential of APA microcapsule system in various gastrointestinal conditions and as a carrier of probiotic bacteria in yogurt formulation. Chapter 4 describes AC membrane system preparation and its characterization in human GI model for oral artificial cell therapy. Chapter 5 presents initial findings of APA microencapsulated probiotic yogurt formulation in animal model and it compares its efficacy to APA microcapsules without yogurt as a carrying medium. The study also includes histological analyses. Chapter 6 further investigates in vivo potential of APA microcapsule system; inflammatory cytokines and immunohistology studies are included. Chapter 7 aims to provide more insightful information about the potential of APA microencapsulated probiotic yogurt formulation against plain saline suspension in *Apc*Min mice in a long term study. Chapter 8 focuses on immunohistochemical and histological evaluation of animals after administration of probiotic yogurt formulation. Chapter 9 summarizes the completed studies, draws conclusions and provides claims to original contributions. Lastly chapter 10 presents further research and recommendations.

During this thesis research period, I was able to contribute to 25 original research articles: 15 published and 10 in preparation; 18 research abstract/proceedings, 1 book chapter, 1 U.S. provisional patent, 1 international patent pertaining to the thesis research goal. Of these I have elicited to use 6 articles, where I am the first author as thesis chapters.

Original research articles (published/in press/to be submitted) presented in this thesis:

1) **A. Urbanska,** W. Ouyang, J. Bhathena, C. Martoni and S. Prakash (2006) Investigation of the viability of microencapsulated live *Lactobacillus acidophilus* cells for *their use in probiotic* yogurts; *International J. of Prebiotics and Probiotics*, Vol. 1, No. 3/4, p.225-232
2) **A. Urbanska**, J. Bhathena and S. Prakash (2007) Live encapsulated *Lactobacillus acidophilus* cells in yogurts for therapeutic oral delivery: preparation and in-vitro analysis of alginate-chitosan microcapsules; *Canadian Journal of Physiology and Pharmacology, 2007 Sep;85(9):884-93*
3) **A. Urbanska,** J. Bhathena, C. Martoni and S. Prakash (2008) Estimation of the potential antitumorous activity of microencapsulated *Lactobacillus acidophilus* yogurt formulation in the progression of colon cancer in *Apc*Min mice; *Digestive Diseases and Sciences, 2008 Jul 17. [Epub ahead of print]*.
4) **A. Urbanska**, J. Bhathena, C. Martoni and S. Prakash (2008) Estimation of the potential antitumor activity of microencapsulated *Lactobacillus acidophilus* yogurt formulation in the attenuation of tumorigenesis in *Apc*Min mice; (to be submitted to: *Molecular Nutrition and Food Research).*
5) **A. Urbanska**, A. Paul, J. Bhathena and S. Prakash (2008) Suppression of tumorigenesis in *Apc*Min mice: down-regulation of inflammatory cytokines by administration of microencapsulated probiotic yogurt formulation; (to be submitted to: *BMC Microbiology).*
6) **A. Urbanska,** S. Cherif, J. Bhathena and S. Prakash (2008) Oral administration of microencapsulated probiotic yogurt formulation ameliorates polyp incidence in *Apc*Min mice: histological and immunohistochemical analysis; (to be submitted to: *Journal of Pathological anatomy and histopathology).*

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1) **A. Urbanska** and S. Prakash (2008) Feasibility and efficacy in delivery of live probiotic bacterial cells: gastrointestinal diseases with emphasis on colonic targeting, *Biologics: Targets & Therapy,* (3):2; 355-378

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1) J. Bhathena, C. Martoni, A. Kulamarva, **A. Urbanska** and S. Prakash (2008). The hypocholesterolemic effect of dietary wheat bran and microencapsulated feruloyl esterase positive *Lactobacillus fermentum* 14932 in hamsters fed a cholesterol-enriched semipurified diet.
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5) J. Bhathena, C. Martoni, A. Kulamarva, **A. Urbanska** and S. Prakash (2008). Cholesterol-lowering and anti-atherosclerotic effects of wheat bran and microencapsulated ferulic acid esterase positive *Lactobacillus reuteri* in hypercholesterolemic hamsters.
6) J. Bhathena, C. Martoni, **A. Urbanska**, A. Kulamarva and S. Prakash (2008). Effects of wheat bran and microencapsulated ferulic acid esterase positive *Lactobacillus farciminis* on plasma, liver and fecal cholesterol levels in Golden Syrian hamsters.
7) J. Bhathena, A. Kulamarva, **A. Urbanska** and S. Prakash (2008). In-vitro assessment of the acid tolerance and feruloyl esterase activity of microencapsulated lactobacilli.
8) R. Coussa, **A Urbanska**, J. Bhathena, C. Martoni, and S. Prakash, (2008) Characterisation of Microencapsulated Live Yeast Cells in Alginate-Poly-*L*-Lysine-Alginate Microcapsules for Use in Renal Failures.

9) R. Coussa, **A Urbanska**, J. Bhathena, C. Martoni, and S. Prakash, (2008) *In vitro* Analysis of APA Microcapsules for Oral Delivery of Live *Saccaromices cerevisea.*

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Patents and copyrights:

1. S. Prakash and **A. Urbanska** (2007) Microcapsules yogurt formulations and their use for probiotic cell delivery. International Patent (filled).
2. S. Prakash and **A. Urbanska** (2006) Microcapsules yogurt formulations and their use for probiotic cell delivery. US Provisional Patent (14647-59)(filled).

Technology Transfer:

1. S. Prakash and **A. Urbanska** (2007) Microcapsules yogurt formulations and their use for probiotic cell delivery. International Patent (2008)–(This technology developed during this thesis is optionated to Micropharma).

Other refereed contributions (Research Abstracts):

1. **A. Urbanska**, and S. Prakash (2008) Euprobio, 2nd European Conference on Probiotics and their Applications, Cracow, Poland (Oct 15-17).
2. **A. Urbanska**, J. Bhathena, C. Martoni, and S. Prakash (2008) Poprad, Slovakia, (June 4-7).
3. J. Bhathena, C. Martoni, **A. Urbanska**, A. Kulamarva and S. Prakash (2008) Ferulate 08**,** An International Conference on Hydroxycinnamates and Related Plant Phenolics**,** St. Paul, USA (Aug 25-27).
4. J. Bhathena, A. Kulamarva, C. Martoni, **A. Urbanska** and S. Prakash (2008) CSPS, Banff, AB (May 23-25).

5. C. Martoni, J. Bhathena, **A. Urbanska** and S. Prakash (2008) 8th World Biomaterials Congress, Amsterdam, (May 28-June 1).
6. **A. Urbanska**, J. Bhathena, C. Martoni, A. Kulamarva and S. Prakash (2008) The 1st international conference on drug design and discovery, Dubai, UAE. (Feb 3-6)
7. J. Bhathena, A. Kulamarva, **A. Urbanska**, C. Martoni and S. Prakash (2007) Pacific Rim Summit on Industrial Biotechnology and Bioenergy, (Nov. 14-16).
8. C. Martoni, **A. Urbanska,** J. Bhathena (2007) Singapore, (Dec. 10-12).
9. **A. Urbanska**, J. Bhathena, C. Martoni, A. Kulamarva and S. Prakash (2007) Singapore, (Dec. 10-12).
10. **A. Urbanska** and S. Prakash (2007) 7th International Colorectal Symposium, Lodz, Poland (May 16-18).
11. **A. Urbanska**, J. Bhathena and S. Prakash (2007) BIO International Convention, Boston (May 6-9).
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13. **A. Urbanska**, J. Bhathena, C. Martoni, A. Kulamarva and S. Prakash (2006) Probiotics and Health: Towards evidence based health effects, Montreal, Canada.
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Book Chapter

1. S. Prakash, J. Bhathena, and **A. Urbanska** (2006) Live bacterial cells as orally delivered therapeutic; Artificial cell, cell engineering and Therapy, Woodhead Publ. Ltd, England [chapter] (published).

Invited Speaker

1. **A. Urbanska,** Institute of Biocybernetics and Biomedical Engineering, Warsaw Poland (May 2007).

CONTRIBUTIONS OF AUTHORS

In all the original research articles included in this thesis as individual chapters, I am the first author and was responsible for researching the literature about specific study, setting objectives, researching methods, designing studies, conducting experiments, collecting data, analyzing data, using equipment available in the laboratory and localizing and using equipment outside the laboratory and preparing manuscripts. Dr. S. Prakash, reported as the last author in all manuscripts, is the research advisor and also the corresponding author. Other reported co-authors had the following contributions to the following articles:

Original article 1: "Investigation of the viability of microencapsulated live *Lactobacillus acidophilus* cells for their use in probiotic yogurts"; Wei Ouyang contributed by performing experiments measuring the mechanical stability of empty APA microcapsules at various exposure times; he also provided photomicrographs in figures 3.2 and 3.3.; Jasmine Bhathena contributed by providing suggestions and assistance in performing experiments; Christopher Martoni also provided suggestions to experimental design and proofread the manuscript.

Original article 2: "Live encapsulated *Lactobacillus acidophilus* cells in yogurts for therapeutic oral delivery: preparation and in-vitro analysis of alginate-chitosan microcapsules" Jasmine Bhathena contributed by providing suggestions and assistance in performing experiments.

Original article 3: "Estimation of the potential antitumor activity of microencapsulated Lactobacillus acidophilus yogurt formulation in the attenuation of tumorigenesis in *Apc*Min mice"; Jasmine Bhathena contributed by assisting in weighing animals, labelling their tails, collecting blood samples and explaining the statistics software SAS in addition to providing suggestions; Christopher Martoni contributed by performing fecal bile acid analysis using a blood analyzer (model 911 Hitachi); he also proofread the manuscript.

Original article 4: "Probiotic microencapsulated *Lactobacillus acidophilus* formulation ameliorates inflammatory cytokines and reduces tumor incidence in *Apc*Min mice"; Jasmine Bhathena contributed by assisting in experimental design. Christopher Martoni

contributed by performing fecal bile acid analysis using a blood analyzer (model 911 Hitachi); he also proofread the manuscript.

Original article 5: "Suppression of tumorigenesis in *Apc*Min mice: down-regulation of inflammatory cytokines by administration of microencapsulated probiotic yogurt formulation"; Arghya Paul contributed by helping with Western Blot experiments, design and materials; he helped in formulating the title and assigning the most suitable journal for publication and he also proofread the article; Jasmine Bhathena contributed by helping in the analyses of Western Blot, provided suggestions in experimental design and also proofread the manuscript.

Original article 6: "Oral administration of microencapsulated probiotic yogurt formulation ameliorates polyp incidence in *Apc*Min mice: histological and immunohistochemical analysis"; Sofiane Cherif contributed to the article by custom designing a Matlab program used to quantify the immunohistochemical antibody staining of the animal samples; he provided suggestions about experimental design, helped with statistical analyses using MatLab, formatted figures and tables and proofread the manuscript. In addition, he translated the abstract of this thesis into French.

CHAPTER 3
Original Paper 1

Investigation of the viability of microencapsulated live *Lactobacillus acidophilus* cells for their use in probiotic yogurts

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Original paper published in: ***International Journal of prebiotics and probiotic;*** **2006, Vol. 1, No. 3/4, p.225-232**

Preface

This paper evaluates physical, chemical and microbial stability of APA microcapsule system enclosing live *L. acidophilus* cells in plain 2% M.F. yogurt which serves as a platform for probiotic bacterial cell oral delivery. In vitro testing includes optimized microcapsule preparation, mechanical studies of prepared microcapsules, evaluation of viability of microencapsulated live *L. acidophilus* cells in yogurt as well as bacterial survival in simulated gastric fluids, using human GI model and different pH conditions. Comparative studies between free and microencapsulated bacterial cells are presented as well. This study assesses the potential of APA microcapsule for use in further in vivo studies.

3.1 Abstract

Herein we report the potential of yogurt and microcapsules as a platform for probiotic bacterial cell oral delivery. In-vitro data suggests that capsules containing live *L. acidophilus* cells showed superior mechanical stability and demonstrated significantly higher bacterial cell survivals compared to free bacterial cells over a period of 4 weeks. Using an in-vitro simulation human stomach model, we monitored the survival rates of free and alginate-poly-L-lysine(PLL)-alginate (APA) membrane microencapsulated *L. acidophilus* cells at 37ºC over two hours, the approximate time it takes food to pass through the stomach. Results show that 7.10 log cfu/g of microencapsulated *L. acidophilus* cells were found alive compared to only 5.51 log cfu/g of free *L. acidophilus* cells in the presence of simulated gastric fluid (SGF) and 2% milk fat M.F. yogurt . In addition, data shows that only 6.66 log cfu/g of microencapsulated *L. acidophilus* cells survived in SGF fluid in the absence of yogurt. The high survival rates of encapsulated *L. acidophilus* cells strongly suggest the use of microcapsules and yogurt for probiotic bacterial cell delivery.

KEY WORDS: Probiotic, Microcapsule, Alginate, APA membrane, Oral Delivery, Immobilization, Artificial Cells

3.2 Introduction

A well balanced gut microflora is known to contribute to the maintenance of a healthy intestinal mucosa. The density of gastrointestinal (GI) microflora increases from the stomach to the large intestine reaching 10^{10} - 10^{12} cfu/g in the colon [253]. One of the most important groups of bacteria for intestinal health are lactic acid bacteria (LAB) [253,254]. LAB are considered probiotic; live microorganisms that remain in the GI tract to benefit the host [253,255,256]. Although their mechanism of action is not known, it is believed that LAB, like other probiotic microorganisms, compete and suppress the growth of undesirable microorganisms in the colon and intestines leading to the stabilization of the digestive system [257].

There are several reports that probiotic yogurt has significant clinical benefits[18]. It is estimated that a decrease of at least 60-70 percent in breast, colorectal, and prostate cancers and 40-50 percent in lung cancer would occur when a diet is complied with (according to the anti-cancer diet guidelines) which includes probiotic yogurt products. In order to be labeled probiotic, yogurt must contain a cell load of at least 10^7 cfu/g at the time of manufacture [258]. However, it has been found that this level of live bacterial cells in probiotic yogurt is not adequate to provide the maximum benefit, especially considering that many bacteria do not survive storage [18,259], [260], [261] or passage through the stomach [262]. Therefore several attempts have been made to deliver a greater number of live bacterial cells. One strategy to deliver more live bacteria to the intestines is bioencapsulation. This procedure involves immobilizing live bacteria in small droplets of an appropriate biomaterial and then applying one or more coats of additional biopolymer to obtain the desired properties. This technology has developed over the last 20 years [263], [264], [265], [266,267], [268], [269], [270], [271 272], [273], [274 275], [276]. However, the use of this technology in probiotic yogurt formulation has not yet been investigated. This study for the first time investigates the feasibility of using yogurt as delivery platform for oral delivery of live encapsulated bacterial cells for use in probiotic applications. To achieve this, first we studied APA microcapsule formulation for their suitability in in-vitro GI conditions and tested the feasibility of designing these formulations using yogurt and various experimental in-vitro GI environments.

3.3 Methods and materials

Sodium alginate (low viscosity), poly-L-lysine (MW=27,400) (lot 71K5120) and calcium chloride (desiccant, 96+%, A.C.S. reagent, FW 110.99, d 2.15, batch # 05614AC) were purchased from Sigma-Aldrich, Canada. MRS AGAR Difco ™ Lactobacilli and MRS BROTH Difco ™ Lactobacilli were purchased from Becton, Dickinson and Company Sparks, USA. Liberty plain yogurt 2% M. F. containing active *Acidophilus* and *Bifidus* cultures was procured from a local store.

3.3.1 Bacteria cultures, propagation and enumeration

L. acidophilus (ATCC 314) cells were inoculated in 100 mL of MRS broth. The bacteria were cultured in MRS Broth at 37ºC in a Professional Sanyo MCO-18M Multi-Gas Incubator. Cultures were grown for 24 hours and centrifuged at *3000x* g for 15 minutes at 37 ºC. The media was decanted; the cells were suspended in 100mL of fresh MRS media and incubated for an additional 20 hours at 37ºC. After growth was performed, the resulting cell wet weights were noted. Anaerobic jars and gas generating kits (Atmosphere Generation System AnaeroGen™; Oxoid Ltd., Hampshire, England) were used for creating anaerobic conditions. Microcapsules containing live bacteria were homogenized manually to dilution and plating. Cell count was determined by anaerobic spread plate on MRS agar after 48 hours and was kept constant at 10^{10} cfu/g throughout the experiment.

3.3.2 Preparation of APA microcapsules loaded with *L. acidophilus*

APA capsules were prepared aseptically using an Inotech Encapsulator® IER-20 (Inotech Biosystems Intl. Inc. Switzerland) with a nozzle size of 300 µm at a frequency of 1160 Hz, 26.9 syringe pump speed and a voltage of 1.000 kV using a 60 ml syringe. 60ml of 1.5% (w/v) sodium alginate (low viscosity) was mixed with 3g of harvested bacterial cells (approximate cell load 10^{10} cfu/g) by centrifuging twice at *3000x* g for 15 minutes with a single wash in 0.85% physiological solution between centrifugations. Formed microcapsules were hardened in 0.1 M calcium chloride solution for 30 minutes,

the optimal hardening time [277]. The resulting microcapsules were coated with 0.1% PLL and 0.1% alginate solution in the same manner as in preparation of APA microcapsules mentioned below. These APA microcapsules loaded with bacterial cells were washed twice with 0.85% physiological solution and stored at 4°C until further use.

3.3.3 Preparation of non-loaded APA microcapsule

APA capsules were prepared according to the standard protocol [268] with several modifications. Briefly, Ca-alginate beads were exposed to PLL solution (0.1% w/v) for 10 minutes, washed twice with physiological solution (0.85%w/v, pH 7.2); finally put in alginate solution (low viscosity, 0.1% w/v) for 10 minutes. The resulting APA microcapsules were washed twice with 0.85% physiological solution and stored at 4°C until used.

3.3.4 Microcapsule mechanical stability test

For mechanical stability evaluations, spherical (580 ±26 μm) APA membrane microcapsules were subjected to in-vitro mechanical shaking incubation (200rpm) in MRS broth for 76 hours in a Lab Line Environ Shaker at 37°C. Empty and *L. acidophilus* loaded APA microcapsules were also exposed to various test fluids: simulated gastric fluid (SGF) and simulated intestinal fluids (SIF), for 3, 12 and 24 hours at 150 rpm shaking and at 37ºC. Samples were withdrawn and visually analyzed for physical damage using an optical light microscope.

3.3.5 Evaluation of microencapsulated live *L. acidophilus* cells viability in yogurt

Over the four-week study, we tested for the survival of encapsulated *L. acidophilus* cells in yogurt. The test samples contained 10g of APA microcapsules loaded with *L. acidophilus* cells and 10g of empty APA microcapsules, each immersed in 100mL of yogurt. Two control samples were set up as follows: 1g of APA microcapsules loaded with *L. acidophilus* cells in 10mL of (0.85%, pH 7.2) physiological solution and

1g of empty APA microcapsules in 10mL of (0.85%, pH 7.2) physiological solution. The microcapsules were filled into 200mL polyethylene wide mouth dilution tubes in which the bottoms were cut out and replaced with mesh net (200 microns) and placed into 2L polyethylene containers. The microcapsules were trapped to ensure a proper separation from the bacterial cultures of *L. acidophilus* cells already present in the yogurt when purchased. Before microcapsules were analyzed for the viability of the encapsulated bacterial cells they were washed in (0.85%, pH 7.2) physiological solution 10 times to ensure complete removal of yogurt particulates. All the samples were stored at 4ºC and exposed to shaking at 100 rpm. Sampling was performed on a weekly basis and photomicrographs were taken at the same time.

3.3.6 Microcapsule leakage study

Microcapsule membrane leakage was monitored on a weekly basis by plating the 0.85% physiological solution in which the APA microcapsules loaded with *L. acidophilus* cells were stored for a period of 4 weeks at 4°C.

3.3.7 Evaluation of the survival of microencapsulated *L. acidophilus* cells in different pH environments with and without addition of yogurt

To test for survival of *L. acidophilus* cells in different GI pH environments, the following buffers were prepared: pH 2 of 0.2M KCl buffer, pH 3 of 0.1M KHP buffer and pH 4 of 1.0M KHP buffer, pH 6 of 0.1M KH_2PO_4 buffer and pH 8 of 0.1M TRIS buffer. For the experiments 400mL of each buffer was autoclaved and cooled to room temperature and 100mL of yogurt was added. The bottoms of 15mL polyethylene tubes were cut out and replaced with a 200 µm nylon mesh. These modified tubes were then filled up with 10g of *L. acidophilus* loaded APA microcapsules. Samples were stored in anaerobic conditions at 37ºC in glass bottles. Sampling under sterile conditions was performed during the following time intervals: 5, 10, 15, 30, 60, 120, 180, 360, 1080, 2520 and 4320 minutes.

In addition, a second survival test was performed in which the above mentioned buffers were loaded with the same microcapsule bacterial load but without the addition of 2% M.F. yogurt. Samples were stored in anaerobic conditions at 37ºC in glass bottles. Sampling under sterile conditions was performed in the following time intervals: 30, 120, 180, 1080, 2520 and 4320 minutes.

3.3.8 Evaluation of microencapsulated *L. acidophilus* cells survival in human GI model -bioreactor simulating the stomach

Microcapsules containing live bacterial cultures *L. acidophilus* were tested using computer controlled simulated human GI model. In the model, each of the five reactor vessels represents distinct parts of the human GI tract in the following order: the stomach, the small intestine, the ascending colon, the transverse colon and the descending colon. In this experiment, 2 hour testing was performed in the first vessel representing the stomach in which a simulated gastric fluid (SGF), a carbohydrate-based diet was composed of arabinogalactan 1.0 g/L, pectin 2.0 g/L, xylan 1.0 g/L, starch 3.0 g/L, glucose 0.4 g/L, yeast extract 3.0 g/L, peptone 1.0 g/L, mucin 4.0 g/L, cystein 0.5 g/L and pH was adjusted with 0.2N HCl was used. 1.5g of APA microcapsules loaded with *L. acidophilus* was added to 10mL of SGF fluid and 5mL of yogurt. The control sample was SGF fluid. The study compared the survival of free *L. acidophilus* in SGF fluid only and APA microcapsules loaded with *L. acidophilus* in SGF fluid but in the absence of yogurt.

3.3.9 Statistical methods

The Microsoft® Excel SP-2 software (Microsoft Corporation, USA) was used for all statistical analysis and the data are presented as mean and standard deviation.

3.4 Results

3.4.1 Formation of the APA microcapsules

In order to investigate the viability of *L. acidophilus* cells in various media, a microencapsulation procedure with specific parameters was followed which yielded

spherical APA microcapsules of narrow size distribution and a constant bacterial cell load. Figure 3.1 displays photomicrographs of freshly encapsulated empty capsules and capsules loaded with *L. acidophilus* cells. In the photomicrographs, under light microscopy, the capsules reveal their homogeneity, spherical shape and similar size. The empty APA microcapsules appear translucent and *L. acidophilus* loaded APA microcapsules are opaque owing to a dense load of *L. acidophilus* cells. Each subsequent microencapsulation yielded a similar bacterial cell load, kept constant at 10^{10}cfu/g.

3.4.2 Mechanical and GI stability of APA microcapsules

To assure viability of the *L. acidophilus* cells in the APA microcapsules, the microcapsules need to be resistant to mechanical stress. Empty and *L. acidophilus* loaded APA microcapsules were exposed to 200 rpm mechanical in vitro shaking for 76 hours in MRS broth at 37 ºC. Figure 3.2 depicts photomicrographs of freshly prepared empty APA microcapsules as well as those loaded with *L. acidophilus* cells after an incubation period of 76 hours. A study of the APA capsule morphology revealed that no structural damage was visually noticeable; and therefore they were considered suitable for further testing. An evaluation of the GI stability of APA microcapsule was carried out by exposing the APA microcapsules containing live LAB cells to simulated gastric fluid (SGF) solutions (pH 1.98) at 37ºC for 3, 12 and 24 hours with 150 rpm mechanical shaking. Microscopic assessment was performed to evaluate microcapsule integrity. Results show that APA microcapsules were sturdy after exposure and remained intact in SGF for up to 24 hours at pH 1.98 and with 150 rpm shaking (Figures 3.3a, 3.3b and 3.3c). We also evaluated the APA microcapsule stability in simulated intestinal fluid (SIF) at 37ºC and with150 rpm mechanical shaking. The APA membrane was found to have remained intact and microcapsules shown to preserve their original spherical shape after 24 hours. APA microcapsules were seen to swell after 3 hours (data not shown).

A comparative study wherein APA microcapsules were exposed to a combination of simulated fluids was also performed. Figure 3.4 shows the percentage of undamaged APA microcapsules as a function of time; 100% of the APA microcapsules were unchanged after exposure to SGF for 3 hours and SIF for 3 hours. Moreover, no damage

was found to occur to the APA microcapsules after treatment for 3 hours in SGF and 12 hours in SIF. However, up to 3% of the APA microcapsules were found to have been damaged after treatment in SGF for 3 hours and SIF for 24 hours.

3.4.3 Microencapsulated *L. acidophilus* cells survival rates in yogurt and microcapsule permeability study

Studies were designed to investigate APA encapsulated bacterial cell survival in probiotic yogurt. Figure 3.5 shows photomicrographs of APA microcapsules loaded with *L. acidophilus* cells. Pictures Y1 to Y4 were taken weekly over a period of 4 weeks and show APA microcapsules stored in 2% M.F. plain yogurt exposed to mechanical shaking at 100 rpm at 4^{o}C. Photomicrographs P1 to P4 show APA microcapsules stored in 0.85% physiological solution, over 4 weeks, stored under similar conditions of 4ºC and shaking at 100 rpm. This 4-week study revealed that APA microcapsules loaded with *L. acidophilus* cells preserve their shape and integrity over time.

The survival of encapsulated *L. acidophilus* over the 4-week study is shown in Figure 3.6(A). There was a constant drop observed in bacterial cell survival and it reached 7.53 log cfu/g of live bacterial cells after the fourth week of testing. This is however, a rather acceptable loss considering the cell count decreased from 10^{10} cfu/g to 10^{7} cfu/g, which is usually the minimum requirement for a yogurt to be labeled probiotic. In addition, the mean pH of the yogurt stored at 4ºC measured on a weekly basis was found to be 4.3 (data not shown). The capacity of APA microcapsules to retain its cell load was measured over 4 weeks. APA microcapsules loaded with *L. acidophilus* cells were stored in 0.85% physiological solution at 4ºC and the supernatant from the medium was plated weekly. Figure 3.6(B) shows the percentage survival of live *L. acidophilus* cells in 0.85% physiological solution over time. A steady increase in the bacterial count was found over 4-weeks. After the fourth week, it was found that 2.21 log cfu/g of *L. acidophilus* cells had seeped from the APA microcapsules into the storage medium.

3.4.5 Survival of microencapsulated *L. acidophilus* cells in different pH environments with and without supplementation with yogurt

The survival of APA microcapsules loaded with *L. acidophilus* cells, in various environments, was estimated using a series of different buffers. The viability of encapsulated *L. acidophilus* cells in the presence of 2% M. F. yogurt in a buffer was tested over 72 hours (Figure 3.7). Crucial time points at 120 minutes – the stomach's approximate retention time, and at 360 minutes – the small intestine's retention time showed 6.67 log cfu/g survival at pH 2 and 9.18 log cfu/g viability at pH 6, respectively. As expected, the lowest survival rates were found at the most acidic pH of 2 (5.38 log cfu/g) and at pH 3 (5.52 log cfu/g) after 72 hours. At pH 6 (representative of the small intestine) the viability was seen to be 8.43 log cfu/g after 72 hours, and 6.41 log cfu/g at pH 4. While there was a steep drop in the total bacterial count during the first 3 hours, a slower decline was observed from the 6^{th} hour onward until the 72^{nd} hour.
While the *L. acidophilus* bacterial cultures survived at pH 6 and pH 8, there were no viable cells present at lower pH values beyond the 30 minute sampling time interval (data not shown).

Using a computed controlled simulated model of the human GI tract, a study of the survival of encapsulated bacteria under gastric conditions of pH 1.98 was carried out. Three samples were used; the first, encapsulated *L. acidophilus* cells in SGF; second, encapsulated *L. acidophilus* cells in SGF and 2% M. F. yogurt and the third, 2% M. F. yogurt containing free bacterial cultures in SGF. An SGF sample served as a control. During the stomach's 2-hour retention time, the anaerobic survival of *L. acidophilus* cells at 37 ºC was determined. As shown in Figure 3.8, the lowest bacterial count was obtained in the sample containing 2% M. F. yogurt with free bacterial cells in SGF. The highest survival (7.10 log cfu/g) was observed in the sample containing encapsulated *L. acidophilus* cells in presence of 2% M. F. yogurt and SGF. A slightly lesser survival (6.66 log cfu/g) was determined in the sample containing encapsulated *L. acidophilus* cells in SGF.

3.5 Discussion

A novel yogurt formulation for oral bacterial delivery using microencapsulation technology was devised. The probiotic bacterium *L. acidophilus* was encapsulated within

APA microcapsule. Any matrix for cell immobilization ideally should provide physical support and uniform distribution of immobilized cells where the transport gradient of nutrients toward and waste products away is balanced and necrosis is prevented [278]. In past studies, it has been reported that the most common type of membrane used for cell therapy is the single alginate based polymer membrane. Various other substances are also being used for encapsulation such as various proteins, polyhemoglobin, and lipids [279], [280]. From a variety of naturally derived membrane materials (e.g. pectin, chitosan, hydroxyethyl methacrylate (HEMA), agarose and lipid complexes), the alginate and poly-L-lysine capsule was selected because alginate is an accepted, generally regarded as safe (GRAS) non-toxic food additive and poly-L-lysine is a natural, safe poly-aminoacid. Calcium ions provide cross-linking with sodium alginate through ionotropic gelation. The PLL coating is shown to provide immunoisolation [281]. The outer alginate layer coating the microcapsules provides better acid stability and improved mechanical strength. In doing so, the biocompatibility of the multilayer structure is optimized. The molecular weight cut off (MWCO) of the resultant APA membrane was determined to be 60-70 KD (data not shown), which provides a useful selectivity limit. This would allow the polymer membrane to protect encapsulated materials from harsh external environments, while at the same time allowing for the metabolism of selected solutes capable of passing in and out of the microcapsule [280].

The microencapsulation technique used yields spherical alginate microcapsules that have a narrow size distribution and retain *L. acidophilus* bacterial cultures (Figure 3.1). To be suitable for oral delivery, microcapsules must demonstrate good mechanical resistance and results show that the APA microcapsules maintain their integrity even after prolonged mechanical agitation (Figure 3.2). In addition, the APA microcapsules demonstrated excellent resistance to simulated intestinal and gastric fluids and only underwent a slight swelling when exposed to SGF for 3 hours and SIF for 24 hours at 37ºC with agitation at 150rpm (Figure 3.3). As shown in Figure 3.4, 97% of the microcapsules remain intact after being exposed for 3 hours to SGF and 24 hours to SIF at 150rpm and 37ºC. Microencapsulated *L. acidophilus* cells were later stored in 2% M.F. yogurt and physiological solution (0.85%, pH 7.2) over 4 weeks. The viability of live *L. acidophilus* in microcapsules and their morphology was monitored. From the

photomicrographs, taken weekly, it is seen that the shape of the microcapsules is well preserved and when compared to microcapsules stored in physiological solution, neither the 2% M.F. yogurt nor shaking at 100 rpm alters their integrity or appearance (Figure 3.5). Both media, differing significantly in their viscosities (2% M. F. yogurt and 0.85% physiological solution) serve equally well as storage media for APA microcapsules. This implies superior resistance to mechanical shear and a tolerance to the various components of the simulated GI fluids.

An initial cell load of 10^7 cfu/g is recommended by National Yogurt Association for yogurt to be called a probiotic. These high numbers have been suggested to compensate for the possible loss in the numbers of probiotic organisms during passage through the stomach and intestine. In our studies, a cell load of 10^{10} (cfu/g) was used. Higher initial load was selected to ensure delivery of a greater number of live bacteria to target sites.

Over 4 weeks storage, 7.53 log (cfu/g) of the encapsulated bacteria remained alive with 100 rpm shaking at 4°C (Figure 3.6(A)). This duration was chosen as it approximates the length of time yogurt can be stored in a refrigerator after purchase. The microcapsule permeability study performed over 4 weeks shows a steady release of the bacteria into the physiological storage solution (0.85% NaCl, pH 7.2). The cumulative count after 4 weeks was found to be approximately 2.21 log (cfu/g) of the encapsulated live bacteria (Figure 3.6(B)). Thus the microcapsules seem to retain bacteria adequately but there is a room for further improvement in the design of the capsule to prevent cell loss. Bacterial cells encounter a variety of pH's during the period of their GI transit. The ability to resist/ adapt to these changes is a desirable property in a probiotic. Buffers of various pH values were prepared to mimic the conditions microencapsulated *L. acidophilus* cells might encounter during passage in the GI tract. A comparative study was also performed in presence and in absence of 2% M.F. yogurt. Figure 3.7 shows the survival rates of microencapsulated *L. acidophilus* at different pH's in presence of 2% M.F. yogurt. The survival rates of microencapsulated *L. acidophilus* in the same conditions without the addition of yogurt, were much lower (data not shown). As expected, after a 72-hour period, the survival at pH 2 was 5.38 log (cfu/g), at pH 3 was 5.52 log (cfu/g), at pH 4 was 6.41 log (cfu/g), at pH 6 was 8.44 log (cfu/g) and at pH 8

was 8.55 log (cfu/g). This shows a lower tolerance and consequently higher sensitivity to extremely acidic environments. As prior results have shown, gastric fluids are detrimental to probiotic cell counts [282,283]. It seems however, that encapsulated cells survive gastric conditions significantly better when stored in yogurt as opposed to storage in media sans yogurt.

The survival of free and encapsulated *L. acidophilus* cells in SGF and in the presence and absence of 2% M.F. yogurt was estimated. Figure 3.8 shows the survival of encapsulated and free bacteria using a model of a human stomach at 37 ºC over two hours, the time it takes food to pass through the stomach. After two hours, 7.10 log (cfu/g) of microencapsulated *L. acidophilus* cells in the presence of SGF and 2% M.F. yogurt were still alive, while only 5.51 log (cfu/g) of free *L. acidophilus* cells were found to be viable in presence of SGF. In addition, 6.66 log (cfu/g) of microencapsulated *L. acidophilus* cells in SGF fluid without yogurt were reported alive. The difference in the survival of encapsulated and free bacterial cells could thus, as predicted, be attributed to the protective effect of the APA membrane in the presence of SGF. Moreover, the addition of 2% M. F. yogurt indicates that yogurt might posses some additional protective properties. The protective effect of yogurt on bacterial cells has been attributed to several factors. These include the strains of inherent probiotic bacteria, pH, hydrogen peroxide, storage atmosphere, concentration of metabolites such as lactic acid and acetic acids, dissolved oxygen, and buffers such as whey proteins [284-286].

The difference in the survival of microencapsulated *L. acidophilus* cells in the presence and absence of 2% M.F. yogurt indicates that yogurt may further help protect microencapsulated *L. acidophilus* cells. Currently, however, there are conflicting reports on the survival of probiotic bacteria in yogurts during storage. Some market surveys on commercial yogurts have found counts far below the recommended 10^7 (cfu/g), of *L. acidophilus* and bifidobacteria at the expiry date of the yogurt [287]. Other surveys have reported satisfactory viability of probiotic bacteria throughout the shelf-life of yogurts [288,289]. Future studies could investigate what components of the yogurt have a protective effect on bacterial cell survival. In addition, further examination of the survival of bacterial cells in the remaining compartments of simulated GI model could be explored

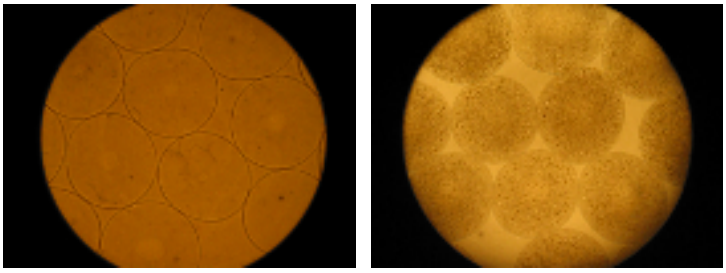
as well as whether a different microcapsule membrane could show improved cell survival rates.

3.6 Conclusion

Results show that APA microcapsules display good mechanical stability in storage solutions. This study also demonstrates the protective properties of the APA membrane in low pH conditions, and in simulated gastric fluid. This indicates that ingested microcapsules may be capable of surviving the passage through the stomach and reaching the target sites further in the GI tract with an adequate cell load which can be further enhanced by using yogurt. In-vitro result suggests that yogurt containing APA microencapsulated *L. acidophilus* may represent a significant improvement over ordinary yogurt in the delivery of probiotic bacterial cells for possible treatment of GI tract related diseases such as in colon cancer. Further studies, however, are required to substantiate this hypothesis, in particular in vivo confirmation of their effectiveness in experimental animal models.

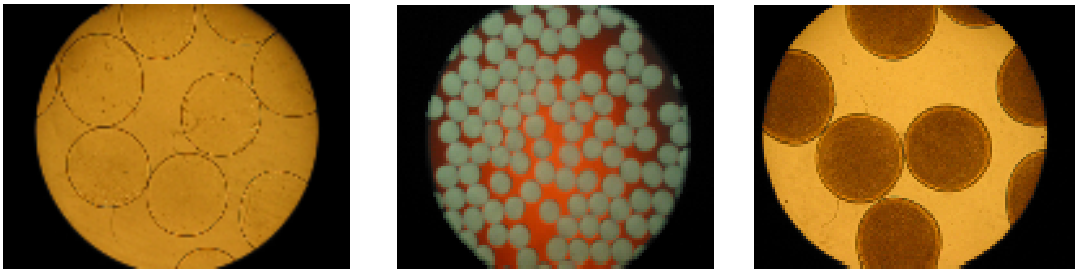
3.7 Acknowledgements

This work was supported by the research grant from Dairy Farmers of Canada (DFC) to Dr. S. Prakash.



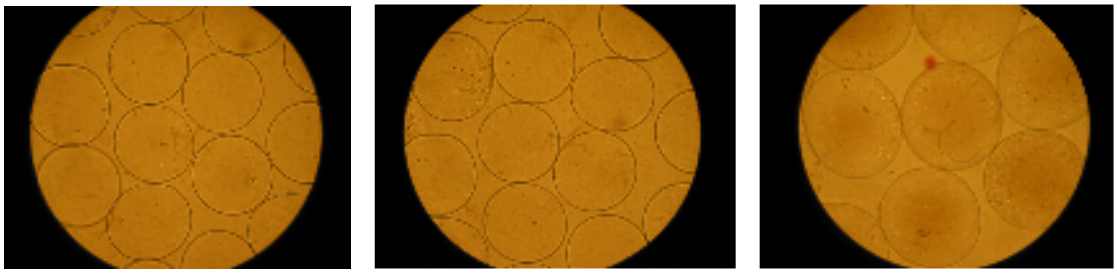
(A) **(B)**

Figure 3.1: (A)Photomicrograph of freshly prepared empty APA microcapsules (size 580 ±26 μm, magnification: 6.3*X*). (B) Photomicrograph of freshly prepared APA microcapsules loaded with *L. acidophilus* cells.



(A) **(B)** **(C)**

Figure 3.2: (A) Photomicrograph of freshly prepared empty APA microcapsules. (magnification 6.3*X*). (B) Photomicrograph of freshly prepared APA microcapsules loaded with *L. acidophilus.* (Magnification: 2.5*X*). (C) Photomicrograph of APA microcapsules loaded with *L. acidophilus* cells after 76 hours of incubation in MRS broth and 150 rpm *in-vitro* shaking at 37ºC (Magnification: 6.3*X*).



(A) **(B)** **(C)**

Figure 3.3: Photomicrographs of empty APA microcapsules exposed to shaking at 150 rpm at 37ºC: (A) in SGF (pH 1.98) for 3 hrs.(B) in SGF (pH 1.98) for 12 hrs. (C) in SGF (pH 1.98) for 3 hrs and in SIF (pH 6.5) for 24 hrs. (Magnification: 6.3*X*).

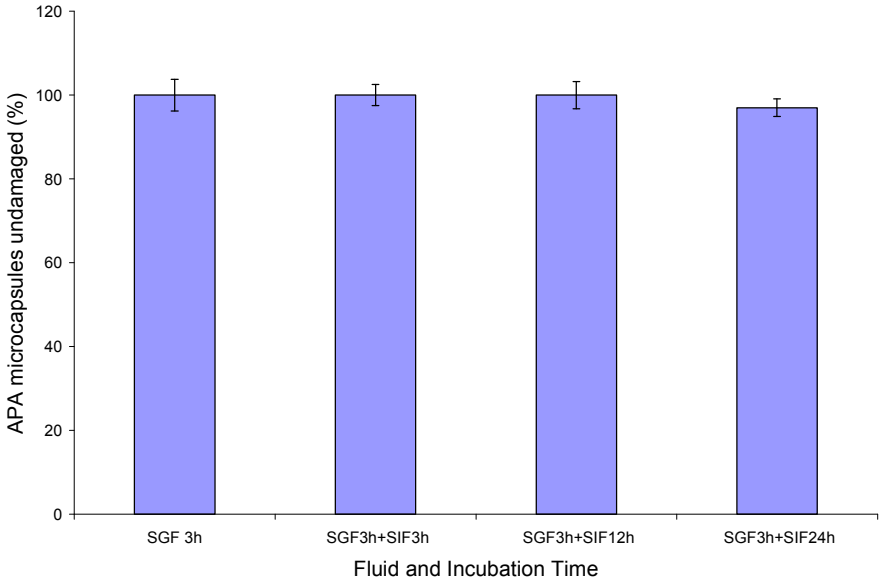


Figure 3.4: Mechanical stability of empty APA microcapsules at various exposure times in simulated gastric fluid (SGF) (pH 1.98) and simulated intestinal fluid (SIF) (pH 6.5) after shaking at 150 rpm at 37ºC.

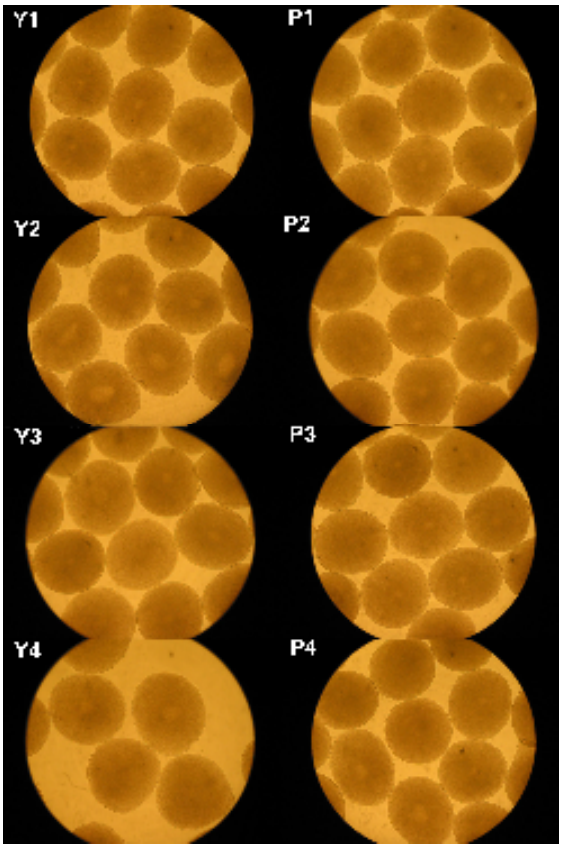
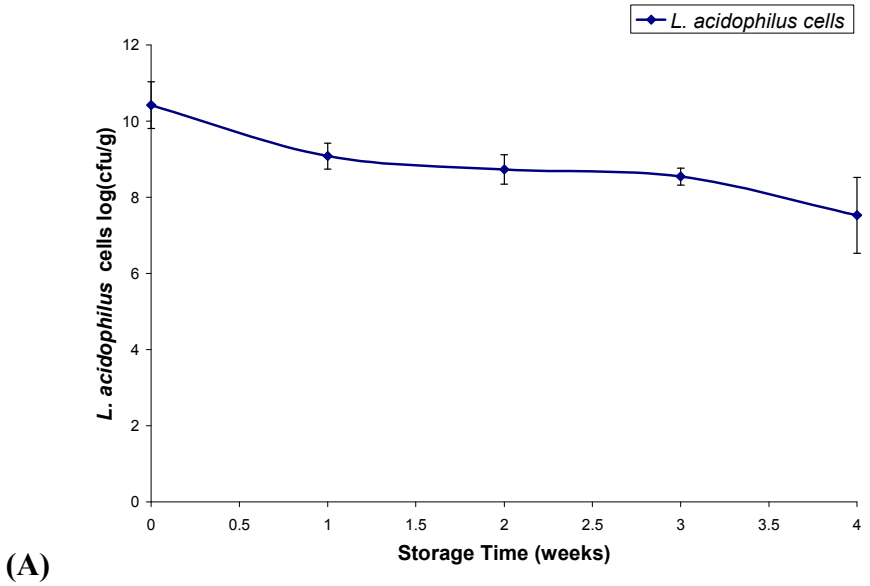
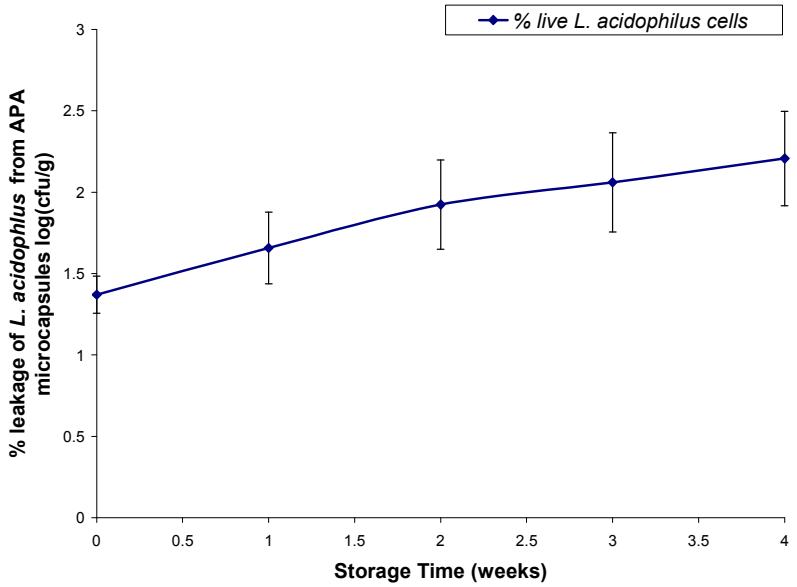


Figure 3.5: Photomicrographs of APA microcapsules loaded with *L. acidophilus* cells exposed to mechanical shaking of 100 rpm at 4 ºC; Y1) storage in 2% M.F. yogurt for 1 week, P1) storage in 0.85% physiological solution for 1 week, Y2) storage in 2% M.F. yogurt for 2 weeks, P2) storage in 0.85% physiological solution for 2 weeks, Y3) storage in 2% M.F. yogurt for 3 weeks, P3) storage in 0.85% physiological solution for 3 weeks, Y4) storage in 2% M.F. yogurt for 4 weeks, P4) storage in 0.85% physiological solution for 4 weeks. (magnification 6.3*X*).



(A)



(B)

Figure 3.6: (A)Viability of live *L. acidophilus* cells in 2% M.F. yogurt during 4 weeks of mechanical shaking at 100 rpm at 4ºC. (B) Retention capacity of APA microcapsules. The number of viable *L. acidophilus* bacteria in the supernatant of storage media gives an indication of how many *L. acidophilus* bacteria have leaked from the microcapsules. The APA microcapsules loaded with *L. acidophilus* cells were stored in 0.85% physiological solution for 4 weeks at 4ºC. No mechanical stress was applied.

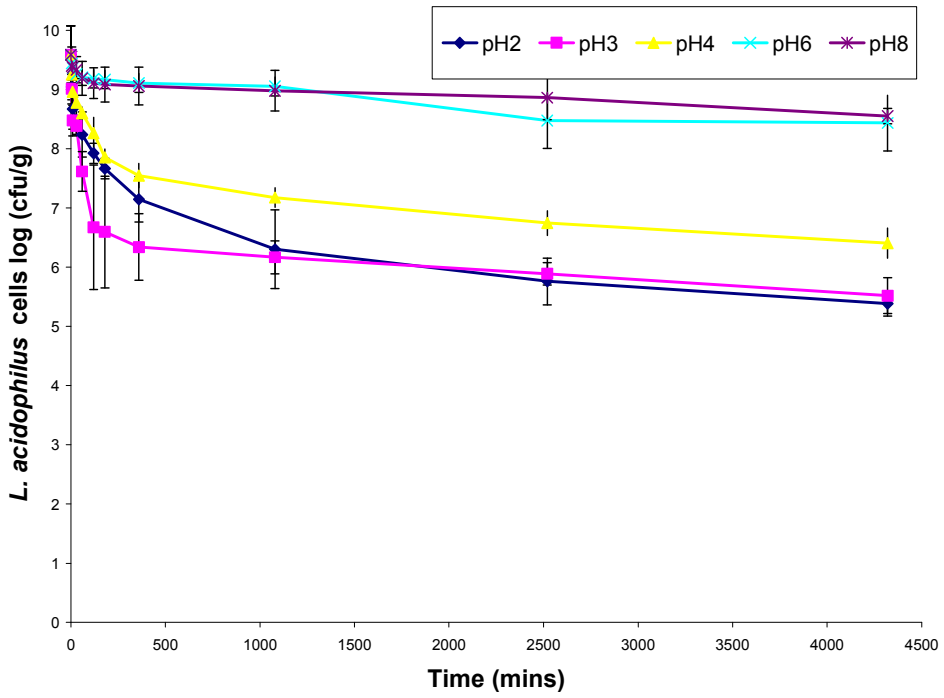


Figure 3.7: Evaluation of the survival of APA encapsulated *L. acidophilus* cells in pH 2, 3, 4, 6 and 8 in presence of 2% M. F. yogurt at 37ºC.

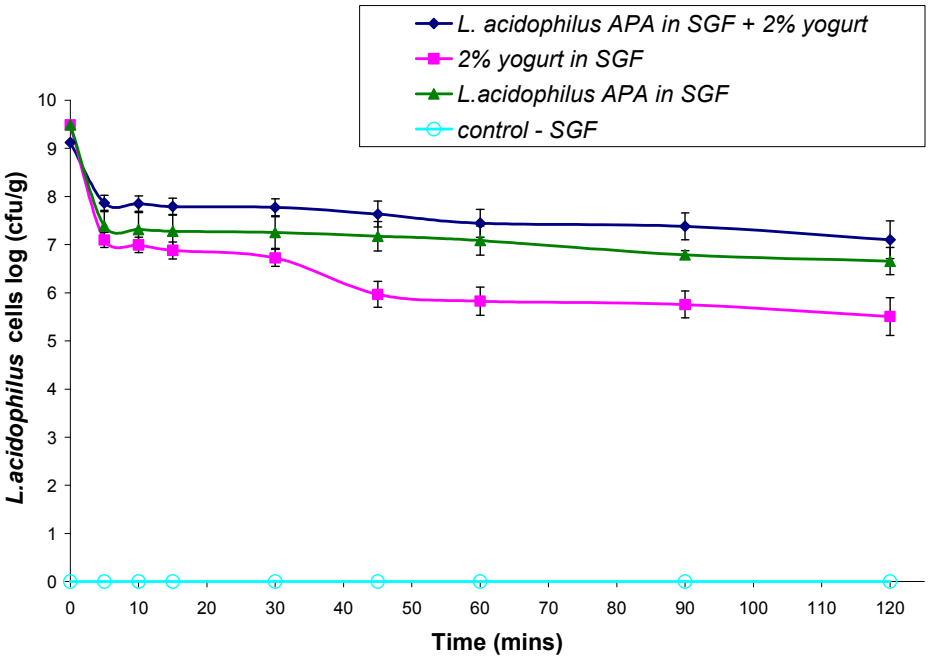


Figure 3.8: Comparison of the survival of APA encapsulated and free *L. acidophilus* cells in conditions simulating the stomach supplemented with 2% M.F. yogurt at 37ºC.

CHAPTER 4
Original Paper 2

Live encapsulated *Lactobacillus acidophilus* cells in yogurts for therapeutic oral delivery: preparation and in-vitro analysis of alginate-chitosan microcapsules

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Original article published in: ***Canadian Journal of physiology and pharmacology, (*2007), Sep;85(9):884-893**

Preface

This paper describes in vitro studies involving AC microcapsule system enclosing live *L. acidophilus* cells in plain yogurt as a platform for probiotic bacterial cell oral delivery. Mechanical and physical properties, such as stability and integrity of the AC microcapsules in MRS broth subjected to shaking over 76 hours were tested. In addition, AC microcapsules were exposed for various periods of time to simulated gastric and intestinal fluids. Morover, AC microcapsules empty and loaded with bacterial cells were characterized in presence of yogurt after being subjected to various pH environments for 76 hours. Survival and resistance of bacterial strain in AC microcapsule was evaluated in simulated human GI model. The use of 10, 50, 100 and 500 chitosan polymers derivatives and chitosan concentrations, namely 0.5%, 0.25% and 0.1% is being investigated. This study analyzes the potential of AC microcapsule as a suitable membrane for delivery of live bacterial cells. A set of similar evaluation tests is being conducted as for APA microcapsule.

4.1 Abstract

There is a strong potential in using microencapsulated targeted delivery of live bacterial cells for application in various diseases including diarrhea, kidney failure, liver failure, lowering cholesterol and others. This study investigates the potential of microcapsules composed of natural polymers, alginate and chitosan (AC) novel membrane, and the use of them in yogurt for delivery of probiotic *Lactobacillus acidophilus* bacterial live cells. Results show that AC microcapsule integrity was preserved after 76 hours of mechanical shaking in MRS broth and in simulated gastric and intestinal fluids after 12 hours and 24 hours. Using an in-vitro computer controlled simulated human gastrointestinal model, we found 8.37 log (cfu/mL) of viable cells were present after 120-minute gastric exposure and 7.96 log (cfu/mL) after 360-minute intestinal exposure.

In addition, AC microcapsules composed of chitosan 10 and 100 at various concentrations were subjected to 4-week storage in 2 % M.F. yogurt and 0.85 % physiological solution. It was found that 9.37 log (cfu/mL) and 8.24 log (cfu/mL) of cells

encapsulated with chitosan 10 and 100 and stored in 2 % M.F. yogurt were alive, respectively. The most suitable AC capsule for this purpose was composed of 0.5 % chitosan 10 providing highest bacterial survival of 9.11 log (cfu/mL) after 4 weeks. Finally, an investigation of bacterial viability over 72 hours in different pH buffers yielded highest survival of 6.34 log (cfu/mL) and 10.34 log (cfu/mL) at pH 8 for free and AC encapsulated cells, respectively.

We conclude from these findings that encapsulation allows accomplishing a higher bacterial delivery to desired targets of GI tract and that microcapsules containing bacterial cells are very good candidates for oral artificial cell therapy.

KEY WORDS: Bacteria, Probiotic, Microcapsule, Alginate, Chitosan, Oral Delivery, Artificial Cells, Biotherapy.

4.2 Introduction

Over the last 20 years, there has been a great interest in probiotic microorganisms. Various excellent studies are available for using bacterial cells for therapy. Moreover, great efforts have been made in the field of incorporation of these bacterial cells into dairy products as food supplements for health benefits. Probiotic bacteria, derived from such foods as yogurt, can help maintain a balanced microflora in the intestine which contributes to a healthy gut barrier. According to the International Dairy Federation the recommended amount of active and abundant bacteria for therapeutic effects should be 10^7 CFU/g. However, various studies show that probiotic bacteria may not survive in sufficient numbers when incorporated into dairy products because of low pH and during their passage through the gastro-intestinal (GI) tract [290].

The use of lyophilized culture, immobilization and other methods has been proposed with little success[291]. Recently, it has been suggested the use of microencapsulation technologies, whereby the probiotic is separated from its environment by a protective coating which can retain high cell concentrations and protect live bacterial cells during storage and gastric transit [292]. Therefore, microencapsulation of probiotic bacteria is currently being investigated. Arrays of polymers, such as agarose, alginate, chitosan, cellulose, gellan and kappa-carrageenan have been chosen as the support material for the embedding matrix. Among these membranes, alginate-poly-L-lysine-alginate (APA) membrane has shown great potential [280]. However, there are studies available suggesting that suitability of APA microcapsule may not be feasible for oral administrations of live bacterial cells [293]. Recently it has been shown that chitosan based microcapsule can be used for live cell encapsulation and targeted deliveries.

Chitosan (poly b-(1-4)N-acetyl-d-glucosamine), a deacetylated form of chitin, is a natural antimicrobial compound. It can be either obtained from crustacean shells (crabs, shrimp and crayfishes) either by chemical or microbiological processes and or it can be produced by some fungi (*Aspergillus niger, Mucor rouxii, Penecillium notatum*) [294, 295,296]. Chitin and chitosan are non-toxic, biocompatible, and biodegradable high molecular weight polymers. Earlier chitosan polymer has been projected as a promising candidate for gene delivery, cell culture, and tissue engineering [297] as well as in

agriculture, medicine, biotechnology and pharmaceutical industries [298]. The development of applications for chitosan in drug delivery has expanded rapidly in recent years owing to its interesting biological, physiological, and pharmacological properties [299], [300], [301].

The objective of this study is to design a new enhanced yogurt formulation incorporated with an alginate-chitosan encapsulated probiotic bacteria - *Lactobacillus acidophilus*. We investigate suitability of a novel AC microcapsule yogurt formulation for therapeutic oral delivery of live bacterial cells in-vitro using flask and computer controlled in-vitro GI models.

4.3 Materials and methods

4.3.1 Materials

Sodium alginate (low viscosity), poly-l-lysine (MW = 27,400) (lot 71K5120) and calcium chloride (desiccant, 96+%, A.C.S. reagent, FW 110.99, d 2.15, batch # 05614AC) were purchased from Sigma-Aldrich, Canada. MRS AGAR Difco ™ Lactobacilli and MRS BROTH Difco ™ Lactobacilli were purchased from Becton, Dickinson and Company Sparks, USA. Commercial-grade chitosan 10, 50, 100 and 500 were from Wako Chemicals, Japan. Liberty plain yogurt 2% M. F. containing active *L. Acidophilus* and *L. Bifidus* cultures was procured from a local store.

4.3.2 Computer Controlled Simulated Human Gastrointestinal Model

The reactor set-up was adapted from the Simulated Human Intestinal Microbial Ecosystem (SHIME), representing the gastrointestinal (GI) tract of an adult human, as described by Molly *et al*. [302]. Briefly, this GI model consists of a succession of 5 reactors representing the different parts of the human gastrointestinal tract, i.e., the stomach, small intestine, ascending colon compartment (proximal colon compartment), and transverse and descending colon compartment (distal colon compartments). In order to simulate gastric conditions a carbohydrate-based diet was prepared which was composed of arabinogalactan 1.0 g/L, pectin 2.0 g/L, xylan 1.0 g/L, starch 3.0 g/L, glucose 0.4 g/L, yeast extract 3.0 g/L, peptone 1.0 g/L, mucin 4.0 g/L, cysteine 0.5 g/L and pH was adjusted with 0.2N HCl. Pancreatic fluid was composed of 12 g/L $NaHCO_3$, 6 g/L oxgall

and 0.9 g/L pancreatin. For this experiment, all physiological and biochemical parameters of the GI model, including transfer of contents from one vessel to another were computer controlled using LabView 6i software. All compartments were continuously stirred reactors with a constant volume and pH control [303]. Retention time, pH, and temperature settings were described previously by Possemiers *et al.* [304]. A schematic representation of the SHIME was presented by De Boever *et al.*[305]. As mentioned above this dynamic *in vitro* GI tract model mimics the various stages and conditions of the human intestinal tract.

4.3.3 Bacteria cultures, propagation and enumeration

L. acidophilus (ATCC 314) cells were inoculated in 100 mL of MRS broth. The bacteria were cultured in MRS Broth at 37 ºC in a Professional Sanyo MCO-18M Multi-Gas Incubator. Cultures were grown for 24 hours and centrifuged at *3000x* g for 15 minutes at 37 ºC. The media was decanted; the cells were suspended in 100 mL of fresh MRS media and incubated for an additional 20 hours at 37 ºC. After growth was performed, the resulting cell wet weights were noted. Anaerobic jars and gas generating kits (Atmosphere Generation System AnaeroGen^{TM}; Oxoid Ltd., Hampshire, England) were used for creating anaerobic conditions. Microcapsules containing live bacteria were homogenized manually to dilution and plating. Cell count was determined by anaerobic spread plate on MRS agar after 48 hours and was kept constant at 10^{10} cfu/mL throughout the experiment.

4.3.4 Preparation of AC microcapsules loaded with *L. acidophilus*

Alginate-Chitosan (AC) microcapsules were prepared aseptically using an Inotech Encapsulator® IER-20 (Inotech Biosystems Intl. Inc. Switzerland) in a Microzone Biological Containment Hood (Microzone Corporation ON, Canada). The following parameters for microencapsulation were used: a nozzle size of 300 µm at a frequency of 918 Hz, 24 syringe pump speed and a voltage of >1.000 kV using a 60 mL syringe. All membrane components were filter sterilized through a 0.22 µm Sterivex-GS filter prior to

use. The pellet of wet cells was centrifuged twice at *3000x* g for 10 minutes, weighted and kept constant at 1.7 g, suspended in 0.85 % saline, pooled and slowly added to a gently stirred 60 mL sterile 1.5 % (w/v) sodium alginate (low viscosity) solution. The approximate cell load was kept constant at 10^{10} cfu/mL. Formed microcapsules were hardened in 0.1 M calcium chloride solution for 30 minutes, the optimal hardening time [306]. The resulting microcapsules were coated with 0.5 %, 0.25 % and 0.1 % chitosan 10 or 100 solution dissolved in dilute acetic acid at a pH of 5.3 for 30 min. These AC microcapsules loaded with bacterial cells were washed twice with 0.85 % physiological solution and stored at 4 °C until further use.

4.3.5 Preparation of non-loaded AC microcapsule

AC capsules were prepared according to the standard protocol [307] with several modifications. Briefly, Ca-alginate beads were exposed to chitosan solution (0.5 % w/v) for 30 minutes, washed twice with physiological solution (0.85 % w/v, pH 7.2). The resulting AC microcapsules were washed twice with 0.85 % physiological solution and stored at 4 °C until used.

4.3.6 Microcapsule mechanical stability test

For mechanical stability evaluations, spherical (550 ± 26 μm) AC coated microcapsules were subjected to in-vitro mechanical shaking incubation (150 rpm) in MRS broth for 76 hours in a Lab Line Environ Shaker at 37 °C. Empty and *L. acidophilus* loaded AC microcapsules were also exposed to various test fluids: simulated gastric fluid (SGF) and simulated intestinal fluids (SIF), for 3, 12 and 24 hours at 150 rpm shaking and at 37 ºC. Samples were withdrawn and visually analyzed for physical damage using an optical light microscope.

4.3.7 Evaluation of microencapsulated *L. acidophilus* cells viability in yogurt

Over the four-week study, we tested for the survival of encapsulated *L. acidophilus* cells in yogurt. The test samples contained 10 g of AC microcapsules loaded with *L. acidophilus* cells and 10 g of empty AC microcapsules, each immersed in 100 mL of yogurt. Two control samples were set up as follows: 1g of AC microcapsules loaded with *L. acidophilus* cells in 10mL of (0.85 %, pH 7.2) physiological solution and 1g of empty AC microcapsules in 10mL of (0.85 %, pH 7.2) physiological solution. The microcapsules were filled into 10 mL polyethylene dilution tubes in which the bottoms were cut out and replaced with mesh net (200 microns) and placed into 250 mL glass bottles. The microcapsules were trapped to ensure a proper separation from the bacterial cultures of *L. acidophilus* cells already present in the yogurt when purchased. Before microcapsules were analyzed for the viability of the encapsulated bacterial cells they were washed in (0.85 %, pH 7.2) physiological solution 10 times to ensure complete removal of yogurt particulates. All the samples were stored at 4 ºC and exposed to shaking at 100 rpm. Sampling was performed on a weekly basis and photomicrographs were taken at the same time.

4.3.8 Evaluation of the survival of microencapsulated L. acidophilus cells in different pH buffers with and without addition of yogurt

To test for survival of *L. acidophilus* cells in different GI pH environments, the following buffers were prepared: pH 2 of 0.2M KCl buffer, pH 3 of 0.1M KHP buffer and pH 4 of 1.0M KHP buffer, pH 6 of 0.1M KH_2PO_4 buffer and pH 8 of 0.1M TRIS buffer. For the experiments 400 mL of each buffer was autoclaved and cooled to room temperature and 100 mL of yogurt was added. The modified polyethylene 15mL tubes were filled up with 10 g of *L. acidophilus* loaded AC microcapsules. Samples were stored in anaerobic conditions at 37 ºC in glass bottles. Sampling under sterile conditions was performed during the following time intervals: 5, 10, 15, 30, 60, 120, 180, 360, 1080, 2520 and 4320 minutes.

4.3.9 Statistical methods

The Microsoft® Excel SP-2 software (Microsoft Corporation, USA) run on a personal computer, was used for all statistical analysis. All the experiments were performed at least in triplicate and the data presented as mean and standard deviation.

4.4 Results

We studied the feasibility of delivering the encapsulated live bacterial cells to various compartments of Simulated Human GI Model. For this, spherical AC microcapsules of narrow size distribution and a constant bacterial cell load were prepared. Figure 4.1 displays photomicrographs of freshly encapsulated empty capsules and capsules loaded with *L. acidophilus* cells of 550±26 μm in size and magnification of 2.5x using light microscopy. Microcapsules exhibit homogeneous spherical shape. Empty capsules are transparent and capsules loaded with bacterial cells are opaque due to high concentration. Each subsequent microencapsulation yielded a fixed bacterial cell load, kept constant in a range of 10^{10} cfu/mL.

In order to test microcapsules mechanical stability freshly prepared capsules were subjected to mechanical stress. Figure 4.2 shows three comparative photomicrographs of freshly prepared microcapsules. Fig (4.2a) displays empty AC microcapsules, fig (4.2b) - loaded capsules with *L. acidophilus* and fig. (4.2c), same capsules after 76 hours of incubation in MRS broth exposed to mechanical shaking of 150 rpm at 37 ºC. It can be observed that the physical morphology of the capsules after being subjected to an intense mechanical stress does not impact capsules integrity or their shape. Upon close examination no damage was noted. Therefore, the capsules preserve their robustness while being exposed to harsh GI conditions.

Having obtained promising results from physical testing, microcapsules containing bacterial cells were subjected to various fluids found in GI model. Figure 4.3 displays three photomicrographs of AC microcapsules exposed to simulated gastrointestinal fluid (SGF) (pH 1.98) for 3 hours (4.3a), to SGF for 12 hours (4.3b) and to simulated intestinal fluid (SIF) (pH 6.5) for 24 hours. In addition, all the samples were exposed to mechanical shaking of 150 rpm at 37 ºC. All the photomicrographs were taken using magnification of 6.3*X*. Upon close examination no physical damage was observed and the capsules remained intact.

Next, we further wanted to verify that the mechanical stability of the microcapsules is sufficient to be applied in oral formulation of encapsulated bacteria. For this microcapsules were exposed to a combination of simulated fluids and mechanical shaking of 150 rpm at 37 º. In figure 4 results represent 97.42 %, 91.11 %, 88.43 % and 84.19 % integrity of AC capsules treated in SGF for 3 hours, SGF and SIF for 3 hours, SGF 3 hours followed by 12 hours in SIF and SGF 3 hours followed by 24 hours in SIF, respectively. As in previous studies, the microcapsules were exposed to mechanical shaking of 150 rpm at 37 ºC.

Studies were designed to investigate AC encapsulated bacterial cell survival in probiotic yogurt. (Figure 4.5) For the experiment survival of encapsulated bacterial cells in SGF with and without addition of 2 % M.F. yogurt as well as the survival of free bacteria contained in the yogurt was investigated. Results show significantly better survival of yogurt fortified encapsulated cells. Fig 4.5 shows the highest survival rate of AC encapsulated bacterial cells in presence of yogurt, which after 120 mins yielded 8.37 log (cfu/mL). After 120 min of exposure to SGF, 7.20 log (cfu/mL) of viable cells was found of encapsulated *L. acidophilus* and 7.00 log (cfu/mL) of free bacterial cells in yogurt.

Figure 4.6 displays survival of AC encapsulated and free bacterial cells in yogurt obtained by exposure to simulated intestinal fluid conditions. The viability of encapsulated *L. acidophilus* and free cells in the presence and absence of 2% M. F. yogurt was tested over 6 hours. Crucial time points at 120 minutes – the stomach's approximate retention time, and at 360 minutes – the small intestine's retention time showed 8.05, 7.47, 6.54 and 7.96, 7.09 and 6.24 log (cfu/mL), respectively.

As there are many chitosan polymers derivatives available, we further investigated various chitosan types namely chitosan 10, 50, 100 and 500. Results show chitosan 50 and 500 were not suitable for microencapsulation of bacterial cells and therefore excluded from future studies. Microcapsules prepared using chitosan 10 and 100 were further investigated and called as in AC 10 and AC 100, respectively. A comparative study measuring the performance of AC microcapsules in yogurt, using both chitosan 10 and 100, was performed over 4 weeks. For this microcapsules were loaded with *L. acidophilus* cells were stored in 2% M.F. yogurt and 0.85 % physiological

solution at 4 ºC and exposed to mechanical shaking of 100 rpm. Results in figure 4.7 show the survival of live *L. acidophilus* cells over the 4 - week study. There was a constant drop observed in bacterial cell survival and it reached 9.37 log (cfu/mL) of cells encapsulated with chitosan 10 and stored in yogurt, 8.24 log (cfu/mL) of cells encapsulated with chitosan 100 and stored in yogurt, 8.04 log (cfu/mL) of cells encapsulated with chitosan 10 and stored in physiological solution and 6.39 log (cfu/mL) of cells encapsulated with chitosan 100 and stored in physiological solution at the fourth week of testing. All the samples were exposed to mechanical shaking of 100 rpm and stored at 4 ºC. In addition, free bacterial cells contained in the yogurt decreased their bacterial cell counts to 7.65 log (cfu/mL). However, greater bacterial counts of 8.26 log (cfu/mL) were observed when yogurt was stored at 4 ºC. (Fig. 4.7)

We further investigated effect of using various chitosan concentrations. Figure 4.8 depicts a study performed during 4 weeks where different chitosan 10 and 100 concentrations were used, namely 0.5 %, 0.25 % and 0.1 %. For the experiments microcapsules coated with these polymers were stored in 0.85 % physiological solution (PS) and kept at 4 ºC. Free *L. acidophilus* cells in 0.85 % physiological solution were used as a control at 4 ºC. A constant drop of bacterial survival was observed over the 4-week study. However, the highest survival rate was noticed for chitosan 10 at 0.5 % concentration, 9.11 log (cfu/mL) and the lowest for chitosan 10 at 0.1 % concentration – 8.56 log (cfu/mL). Free bacterial cells have reached complete downfall at the second week. (Fig. 4.8)

Another set of experiments was designed to compare the viability of free *L. acidophilus* cells in 2 % M.F. plain yogurt in various buffers during 72 - hour study (Fig. 4.9) and compared with AC 10 encapsulated cells (Fig. 4.10). In figure 4.9, the highest survival of 6.34 log (cfu/mL) was observed for free *L. acidophilus* cells at pH 8, followed by 2.14 log (cfu/mL) and 1.44 log (cfu/mL) at pH 6 and 4, respectively. However, when the cells were exposed to pH 2 and 3, they entirely died after 180 and 360 min of exposure, respectively. However, a gradual decrease in bacterial cell number was observed when AC microcapsules were exposed to the same conditions (Fig. 4.10). In addition, results in figure 4.10 show the survival of AC 10 encapsulated live *L. acidophilus* cells in buffers of pH 2, 3, 4, 6 and 8 supplemented with 2 % M.F. yogurt. In

this study, cells exhibited the highest survival at pH 8, 10.34 log (cfu/mL), and lowest at pH2 of 7.48 log (cfu/mL). Moreover, at pH 6 cells reached 10.07 log (cfu/mL), at pH 4, 7.56 log (cfu/mL) and 7.82 log (cfu/mL) at pH 3.

4.5 Discussion

It has been noted before that for a membrane to perform best in in vivo conditions it must exhibit sufficient strength [308]. Microcapsules composed of alginate cross linked with chitosan have undergone various testing in this study. They have exhibited a sufficient mechanical strength when exposed to harsh conditions and preserved their structural stability well. In vitro studies have showed that AC microcapsules maintain their structural integrity after 76 hours of incubation and mechanical stress. This indicates that they can be used successfully in oral delivery without any breaking of the capsules which is an important consideration for the protection of the encapsulated live bacterial cells through the GI tract. These studies showed that the AC capsules maintain their structure after treatment with simulated gastric fluid showing that they can successfully pass though the stomach without any destruction. Similarly, after 24 hours in simulated intestinal fluid the microcapsules remain wholesome and intact. In contrast to previous studies with APA capsules [309], AC capsules show less mechanical strength when passaged thought the simulated gastric and intestinal conditions. AC capsules seem to degenerate faster in simulated conditions, 16 % capsules of the test sample burst after 3 hour treatment in SGF followed by 24-hour treatment in SIF. Comparatively only 3% of APA capsules were found to have deteriorated after the same treatment.

Yogurts contain lactic acid bacteria which contribute to beneficial effects of the yogurt. In fact guidelines are available for the recommended concentrations of bacteria in probiotic products. For example, the Japanese Fermented Milks and Lactic Acid Bacteria Beverages Association [310] and the International Dairy Federation [290] require that a minimum of 10^7 viable microorganisms per gram or milliliter should be present in a food product in order for it to be considered ‘probiotic’. However, survival of these bacteria through the GI tract is not sufficient to maintain the minimum therapeutic dose required to show a beneficial effect and the bacterial cultures suffer from exposure to gastric acidic conditions [311]. We aim to supplement yogurt with probiotic bacteria encapsulated

in a protective membrane to enhance the delivery of these bacteria to the GI tract. *Lactobacillus acidophilus* cells were encapsulated in AC membranes and incorporated into 2 % M.F. yogurt and subjected to SGF treatment for 2 hours, which is the average time these capsules are expected to spend in the stomach on ingestion. It was found that in comparison to AC encapsulated *L. acidophilus* cells in SGF and 2% M.F. yogurt in SGF, the AC encapsulated *L. acidophilus* cells in 2 % M.F. yogurt showed better survival.

After gastric transit, the capsules would continue into the intestines where they would spend about 6 hours before excretion. AC encapsulated *L. acidophilus* cells in 2 % M.F. yogurt, AC encapsulated *L. acidophilus* cells and 2% M.F. yogurt were subjected to 6 hours of SIF treatment. The *L. acidophilus* cells encapsulated in AC membrane and suspended in 2 % M.F. yogurt retained their viability best. On the other hand, bacteria inherent in 2 % M.F. yogurt showed a loss in their viability.

In both SGF and SIF conditions, the bacteria are postulated to survive better than non-encapsulated cells or yogurt inherent cells because of the protective effects of the AC membrane. In addition the 2 % M.F. yogurt may afford extra stability and shielding to the encapsulated bacteria.

Haque *et al.* [312] demonstrated that AC capsules made with chitosan 10 were robust and maintained the cell viability under adverse conditions. We investigated further by using chitosan 50, 100 and 500 for use in the oral delivery of live bacterial cells. As mentioned previously, encapsulation of *L. acidophilus* cells with chitosan 50 and 500 was shown to be inefficient due to excessive viscosity of the alginate-chitosan solution. (data not shown)

L. acidophilus cells encapsulated in AC using chitosan 10 (AC 10) and 100 (AC 100) were subjected to external rotational force at 100 rpm and 4 ºC. The bacterial viability was evaluated in 2 % M.F. yogurt as well as in 0.85 % physiological solution. AC 10 in 2 % M.F. yogurt maintained its viability without excessive loss of active bacterial cells over 4 weeks. In contrast, AC 100 in yogurt showed a greater decrease in their viable count demonstrating the superior protection offered by alginate-chitosan 10 capsules. AC capsules in 0.85 % physiological solution drastically decreased in their viable cell load attesting to the protective effect of the 2 % M.F. yogurt.

Chitosan 10 capsules are shown to have a superior protection and mechanical strength [312]. Various percentile of chitosan 10 (0.1, 0.25 and 0.5 %) were used to formulate AC capsules to determine the optimal concentration for use in yogurt. The capsules were incubated in 0.85 % physiological solution at 4 ºC over 4 weeks. AC capsules in which *L. acidophilus* cells were encapsulated using 0.5 % chitosan 10 were superior to AC capsules made with 0.1 % and 0.25 % chitosan 10 in terms of retaining cell viability. Free *L. acidophilus* cells in 0.85 % physiological solution were in contrast found to have minimum survival after only 2 weeks.

The viability of free *L. acidophilus* cells in 2 % M.F. yogurt was assessed at different pH. As expected, free *L. acidophilus* cells at pH 2 and pH 3 showed a dramatic decline in cell viability within 180 and 360 minutes, respectively. On the other hand, free cells seem to survive better with increasing pH, from pH 4 to pH 8. Even so at pH 8 cells decreased from 10.78 to 6.34 log (cfu/mL) over 72 hours. This proves the necessity of encapsulating live bacteria in membranes such as AC 10 to improve survival and thus shelf life of probiotic yogurts. Finally, the survival of *L. acidophilus* cells was estimated when encapsulated in AC 10 and suspended in 2 % M.F. yogurt. As seen with free cells the viability of these cells at pH 2, 3 and 4 considerably decreased over 72 hours, whereas, at pH 6 and 8 the cells showed even better survival with minimum losses. It is seen that encapsulation of live bacterial cells therefore allows sufficient survival so that these cells may be maintained above the minimum effective dose required [310] at 4ºC in probiotic yogurts.

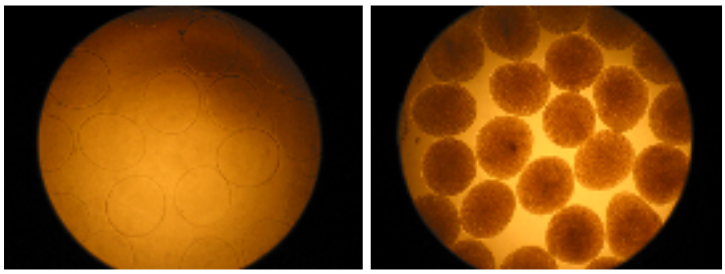
4.6 Conclusion

In conclusion, this study shows that alginate-chitosan microcapsules can serve as suitable vehicles for oral delivery of live bacterial cells to the intestines. AC 10 membrane provides sufficient mechanical stability and resistance to GI tract interactions. It is also suitable for safe and targeted oral delivery of probiotics. However, further research is required. By encapsulating *L. acidophilus* cells and using them in yogurt as a delivery system, artificial cell technology may provide a formulation which could serve as an alternative potential colon cancer treatment. In order to test the full potential of this

application, however, further detailed studies on the biocompatibility and cell delivery features of the membrane in vivo remain to be tested.

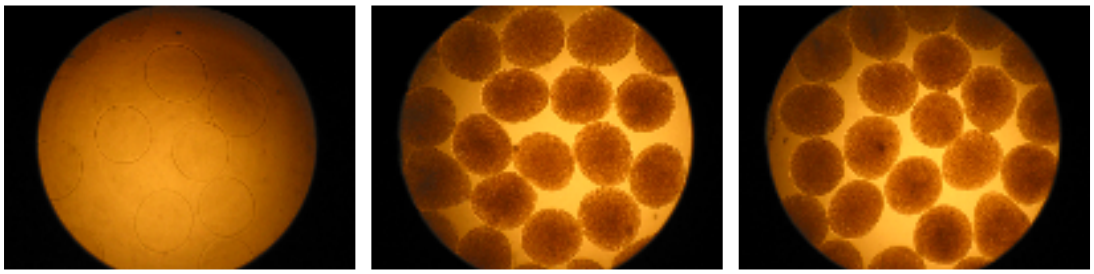
4.7 Acknowledgements

This work was supported by research grants (to S.P.) from the Canadian Institute of Health Research (CIHR). A Faculty of Medicine Internal Postgraduate Scholarship to A. Urbanska is acknowledged.



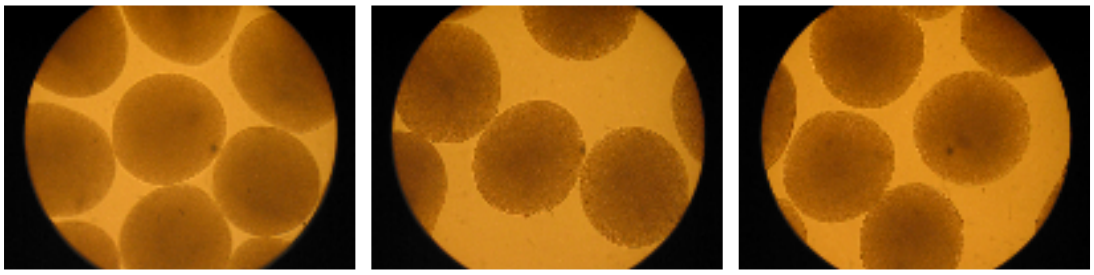
(A) (B)

Figure 4.1: (A) Photomicrograph of freshly prepared empty AC 10 microcapsules (size 550±26 µm, magnification: 2.5*X*). (B) Photomicrograph of freshly prepared AC 10 microcapsules loaded with *L. acidophilus* cells.



(A) **(B)** **(C)**

Figure 4.2: (A) Photomicrograph of freshly prepared empty AC 10 microcapsules. (B) Photomicrograph of freshly prepared AC 10 microcapsules loaded with *L. acidophilus*. (C) Photomicrograph of AC 10 microcapsules loaded with *L. acidophilus* cells after 76 hours of incubation in MRS broth and 150 rpm in-vitro shaking at 37ºC (Magnification: 2.5*X*).



(A) **(B)** **(C)**

Figure 4.3: Photomicrographs of AC 10 microcapsules loaded with *L. acidophilus* cells exposed to shaking at 150 rpm at 37ºC: (A) in SGF (pH 1.98) for 3 hrs. (B) in SGF (pH 1.98) for 12 hrs. (C) in SGF (pH 1.98) for 3 hrs and in SIF (pH 6.5) for 24 hrs. (Magnification: 6.3*X*).

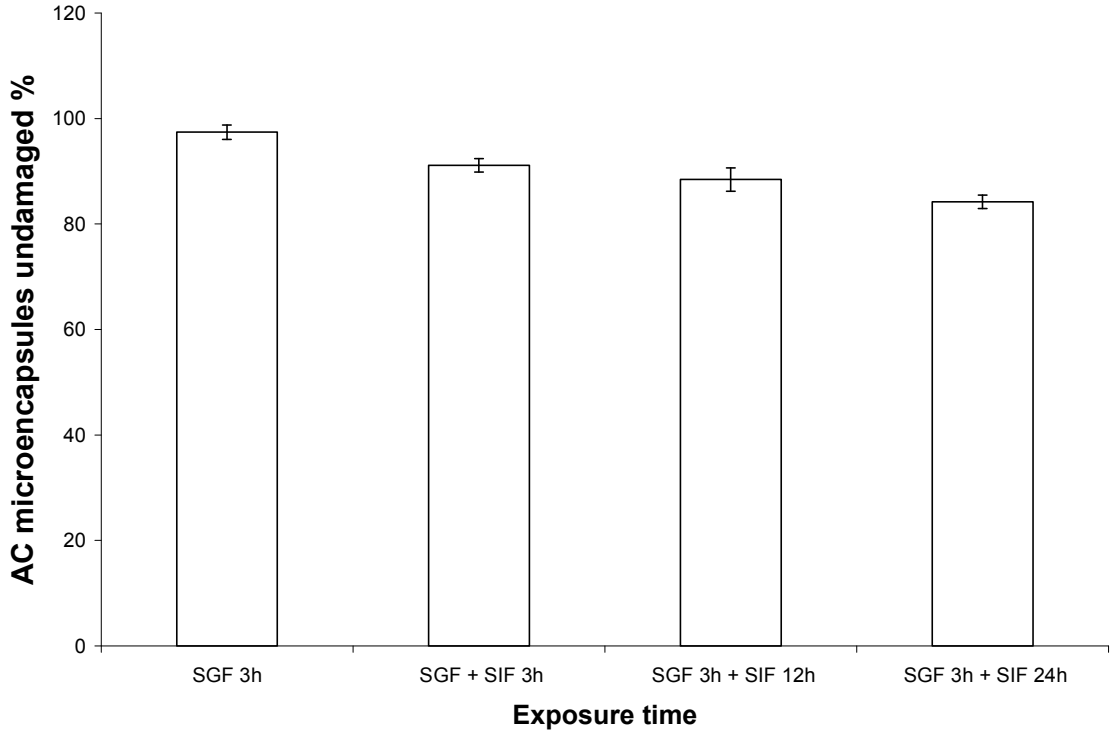


Figure 4.4: Mechanical stability of AC 10 microcapsules loaded with *L. acidophilus* cells at various exposure times in simulated gastric fluid (SGF) (pH 1.98) and simulated intestinal fluid (SIF) (pH 6.5) after shaking at 150 rpm at 37ºC.

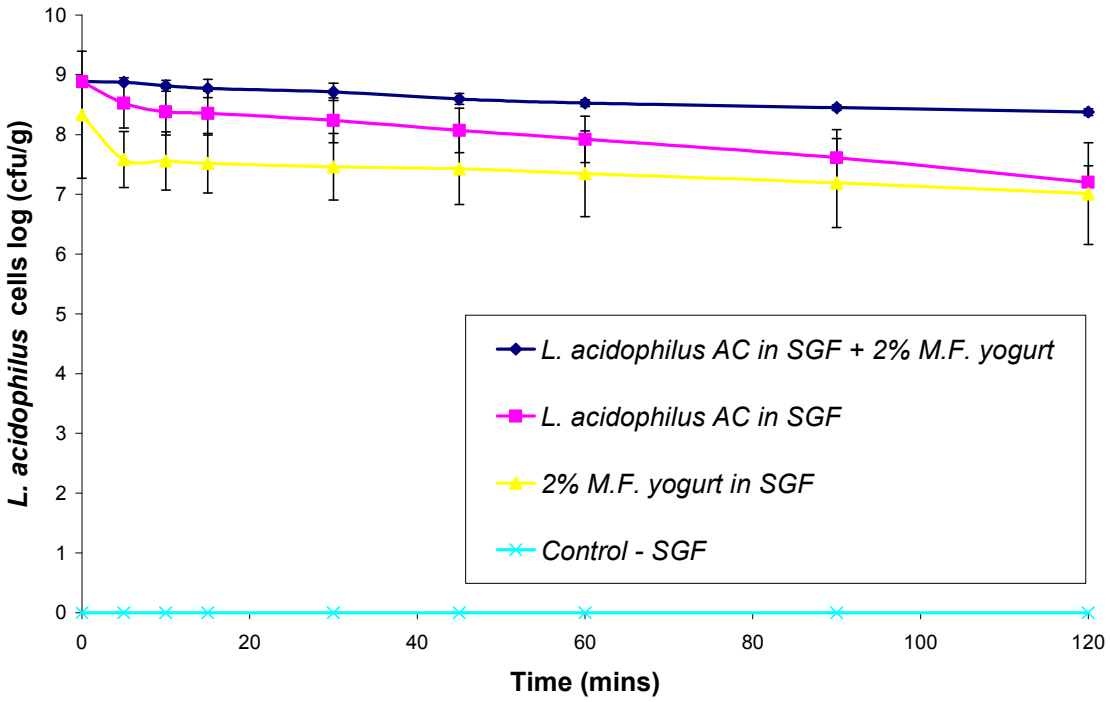


Figure 4.5: Comparison of the survival of AC 10 encapsulated and free *L. acidophilus* cells using computer controlled human GI model - conditions simulating the stomach supplemented with 2% M.F. yogurt at 37ºC.

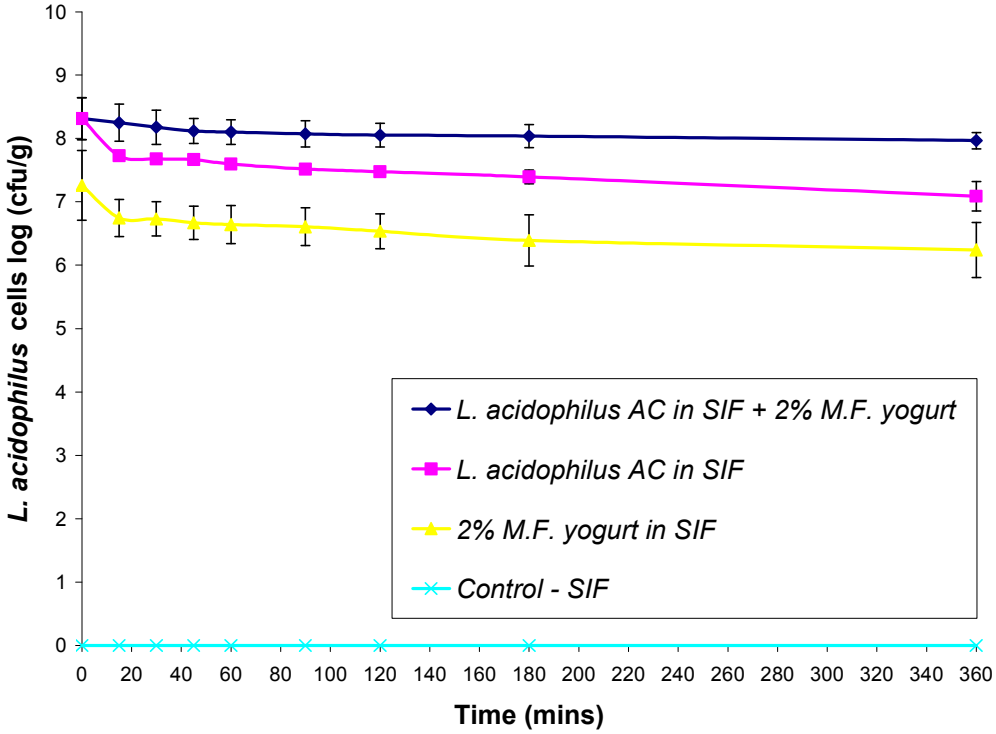


Figure 4.6: Comparison of the survival of AC 10 encapsulated and free *L. acidophilus* cells using computer controlled human GI model – conditions simulating the intestines supplemented with 2% M.F. yogurt at 37ºC.

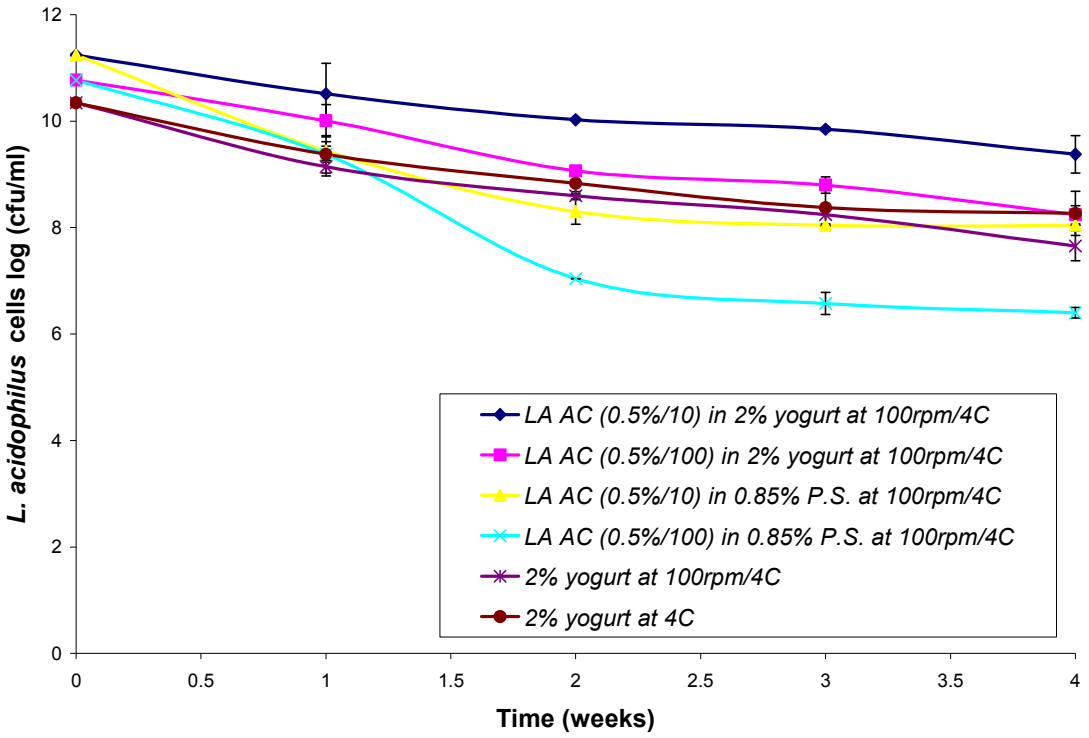


Figure 4.7: Comparative study – survival of AC 10 and AC 100 encapsulated *L. acidophilus* in presence and of 2% M.F. yogurt at 4ºC and mechanical shaking of 100 rpm.

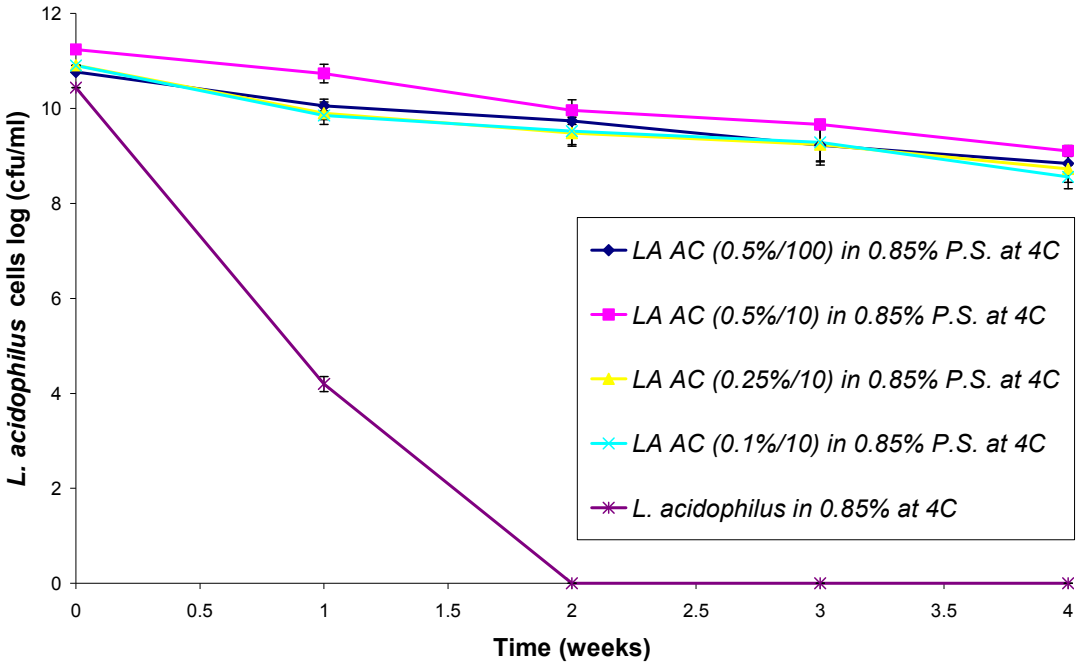


Figure 4.8: Comparative study of microencapsulated *L. acidophilus* bacterial cells viability in various chitosan concentrations and polymers (0.5%/100, 0.5%/10, 0.25%/10, 0.1%/10) in 2% M.F. yogurt with free *L. acidophilus* bacterial cells in 0.85% saline during 4 weeks of mechanical shaking at 100 rpm at 4ºC.

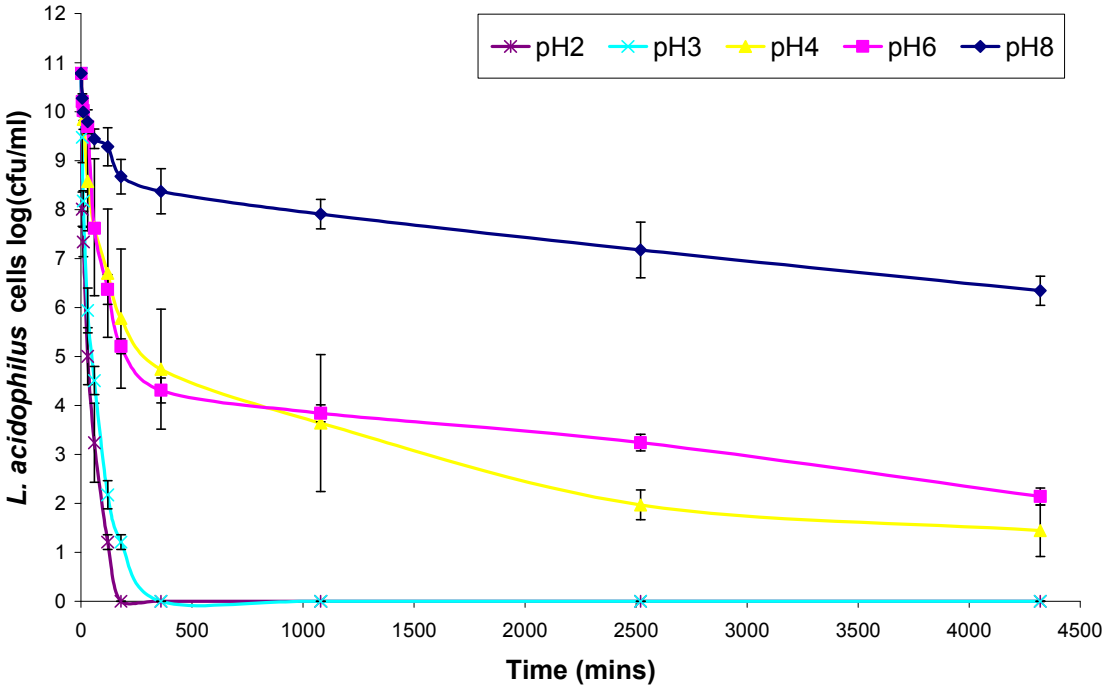


Figure 4.9: Viability of free *L. acidophilus* cells in 2%M.F. plain yogurt in buffers: pH2, pH3, pH4, pH6 and pH8.

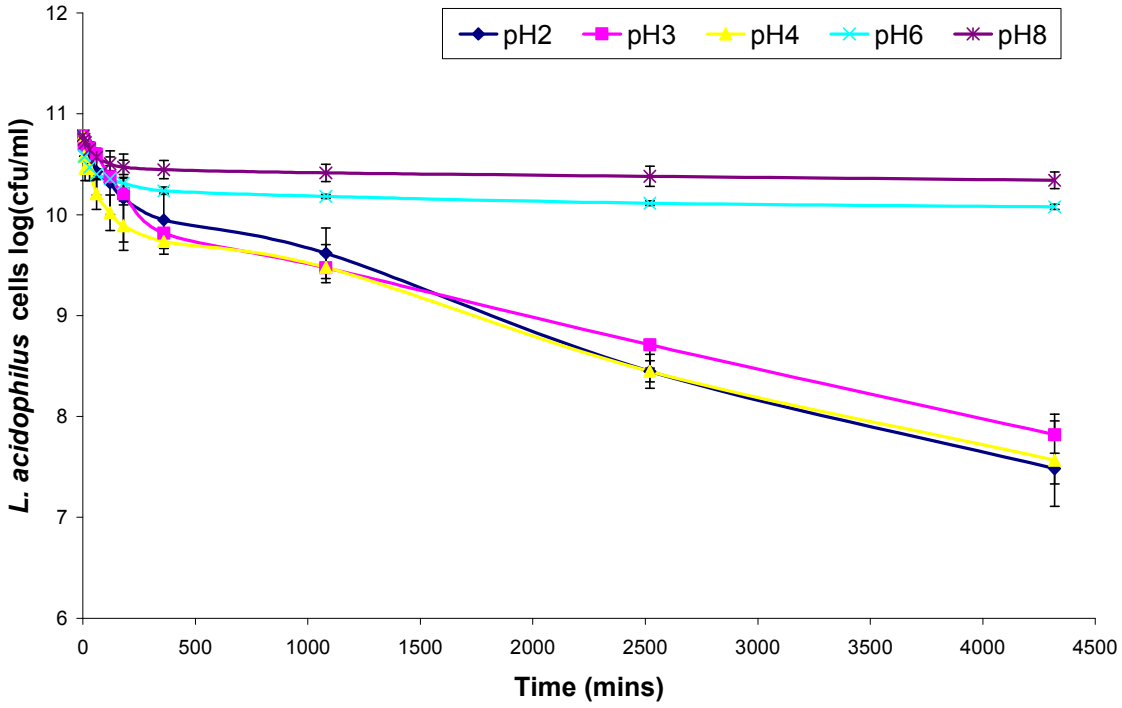


Figure 4.10: Survival of AC 10 encapsulated live *L. acidophilus* cells in buffers: pH2, pH3, pH4, pH6 and pH8 supplemented with 2%M.F. plain yogurt.

CHAPTER 5
Original paper 3

Estimation of the potential antitumor activity of microencapsulated *Lactobacillus acidophilus* yogurt formulation in the attenuation of tumorigenesis in *Apc*Min mice

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Original paper published in: ***Digestive Diseases and Sciences; 2008 Jul 17. [Epub ahead of print].***

Preface

This paper presents first in vivo study and it evaluates two treatment formulations: APA microencapsulated bacterial cells in probiotic 2% M.F. yogurt and APA microencapsulated bacterial cells in saline solution. As APA microcapsules resulted in being more robust, had a higher retention capacity for bacterial cells, preserved better bacterial viability and had a greater resistance to GI environment, they were selected to be used in in vivo studies. Multiple intestinal neoplasia (Min) male mice with a mutation at codon 850 of the *Apc* gene were used as it is a well-established animal model for studies on human colorectal cancer.

The APA microcapsules' efficacy after oral administration to *Apc*Min mice is being evaluated using biomarkers such as IL-6 and bile acid test against animals receiving empty APA microcapsules suspended in saline. Histological analysis including adenomas enumeration in small and large intestines and their classification is also included.

5.1 Abstract

There is a strong correlation between orally administered probiotics and suppression of the low-grade inflammation which can lead to restoration of normal local immune functions. We studied the potential immunomodulatory and antitumorogenic properties of microencapsulated probiotic bacterial cells in a yogurt formulation in Min mice carrying a germline *Apc* mutation. Daily oral administration of microencapsulated *Lactobacillus acidophilus* bacterial cells in the yogurt formulation mice resulted in significant suppression of colon tumor incidence, tumor multiplicity and reduced tumor size. Results show that oral administration of microencapsulated *L. acidophilus* contributed to stabilization of animal body weight and decreased release of bile acids. Histopathological analyses revealed fewer adenomas in treated vs. untreated animals. Furthermore, treated animals exhibited fewer gastrointestinal intraepithelial neoplasias with a lower grade of dysplasia in detected tumors. Results suggest that oral administration of microencapsulated probiotic *L. acidophilus* exerts anti-tumorous activity which consequently leads to reduced tumor outcome.

Key words: Microencapsulation, Probiotic Bacteria, Colon Cancer, Fecal Bile Acids, Oral Delivery.

5.2 Introduction

Colorectal cancer is the second most common cause of cancer death in men and women. Although 70% of tumors are resectable when detected early, 25% of patients will have recurrent disease[313]. The current available treatments include chemotherapy and surgery but they contribute vastly to a loss in the quality of life. Therefore, there is an immense need for an alternative preventative treatment.

Lactic acid bacteria (LAB) are commonly used probiotics ubiquitously found, for instance, in yogurts and other functional foods. The immunomodulating and immunostimulating properties of yogurt and fermented milks have been well documented [12-14]. Recent *in vitro* and *in vivo* studies have shown that the growth of transplantable and chemically induced tumors was inhibited by yogurt and other lactic acid bacteria [4,195,314]. Several researchers have attempted to elucidate the inhibitory effect of yogurt on colon tumors[152,195,315,316]. Moreover, the probiotic effect on various forms of intestinal inflammations has been evaluated in mice[317-320]. Probiotics like lactobacilli and bifidobacteria have been claimed to deconjugate and absorb bile acids. In doing so, they contribute to the overall reduction of the colonic mucosal secretion of mucin and fluids which may lead to changes in colonic bacterial flora[321]. Bile acids have been reported to cause oxidative stress, DNA damage, and mitochondrial membrane instability in intestinal lining leading to formation of cancerous cells[322,323]. IL-6 is an important regulatory cytokine with multiple actions on immune functions such as being involved in the differentiation of cytotoxic T lymphocytes and regulations of T-cell proliferation[324].

Microencapsulation in specialized ultra-thin semi-permeable polymer membranes has been successfully shown to protect live bacterial cells in oral and other delivery applications. Various methods of microencapsulation of probiotics as therapeutics in the prevention and treatment of various disorders, including GI diseases, have been thoroughly described in a review by Bhathena *et al*[325]. Whilst the numerous studies and methods described were very promising, the overall therapeutic effect was diminished by

the fact that only 1% of free bacteria ingested survive GI transit[291]. Lim and Sun [326] first proposed the alginate-poly-L-lysine-alginate (APA) microcapsule membrane in 1980 and since then, microencapsulation has proven to be an effective strategy for cell implantation and cell-based gene therapy for the treatment of diabetes, metabolic or neurologic disorders, and cancer[327-330].

This study investigates a novel approach for the oral delivery of microencapsulated probiotic bacteria *Lactobacillus acidophilus,* in yogurt, as a potential carrier and investigates its potential performance in the prevention of colon cancer and in the amelioration of gastrointestinal health.

5.3 Materials and Methods

5.3.1 Chemicals

Sodium alginate (low viscosity), poly-L-lysine (MW=27,400) (lot 71K5120) and calcium chloride (desiccant, 96+%, A.C.S. reagent, FW 110.99, d 2.15, batch # 05614AC) were purchased from Sigma-Aldrich, Canada. MRS AGAR Difco ™ Lactobacilli and MRS BROTH Difco ™ Lactobacilli were purchased from Becton, Dickinson and Company Sparks, USA. Liberty plain yogurt 2% M. F. containing active *Acidophilus* and *Bifidus* cultures was procured from a local grocery store.

5.3.2 Bacteria and Culture Conditions

Lactobacillus acidophilus (ATCC 314) cells were cultivated and serially propagated three times in the MRS medium before experimental use. Incubations were performed at 37°C in a Professional Sanyo MCO-18M Multi-Gas Incubator in anaerobic conditions (1-2% CO_2, Atmosphere Generation System AnaeroGen™; Oxoid Ltd., Hampshire, England). Bacteria to be encapsulated were isolated after 20 hours of the 3rd passage. Microcapsules containing live bacteria were homogenized manually and re-suspended in 0.9 mL of saline. Samples were serially 10-fold diluted in diluent (saline). Duplicate plates were inoculated with 0.4 mL samples from the appropriate dilutions and incubated

under 5% CO_2 at 37°C for 48 h before counting. Cell count was kept constant on average 10^{10} cfu/mL throughout the experiment.

5.3.3 Microencapsulation method

The bacterial strains were microencapsulated into Alginate-Poly-L-Lysine-Alginate (APA) membranes. All membrane components were filter-sterilized through a 0.22 μm Sterivex-GS filter prior to use. Grown cultures were centrifuged at *3000 x*g for 15 minutes at 25ºC and the supernatant broth was decanted. The pellet of wet cells was weighted and suspended in 0.85% saline, pooled and slowly added to a gently stirred sterile 3.3% sodium alginate solution (diluted 50% with 0.85% saline). The entire procedure was performed under sterile conditions in a Microzone Biological Containment Hood (Microzone Corporation ON, Canada) and all solutions were autoclaved with the exception poly-L-lysine which was 0.22 μm sterile-filtered prior to usage. APA microcapsules were prepared aseptically using an Inotech Encapsulator® IER-20 (Inotech Biosystems Intl. Inc. Switzerland). Freshly prepared microcapsules were washed twice with 0.85% saline and stored at 4°C. Parameters for microencapsulation were as follows: gelation time in $CaCl_2$ – 30 minutes, coating time – 10 minutes, nozzle diameter - 300 μm, vibrational frequency – 918 Hz, voltage > 1.00kV and current 2 amp.

5.3.4 Treatment formulation preparation

APA microcapsules loaded with *L. acidophilus* bacterial cells were blended with Liberty plain yogurt 2% M.F. and 0.85% saline in the proportions of 3:1, respectively. Empty APA microcapsules were suspended in 0.85% saline using the same formulation. Alginic acid and its salts are considered to be Generally Recognized as Safe (GRAS) according to the Food Additive Status List [331] and have been used in the food industry as thickening-agent, preservative, antioxidant, flavoring agents as well as an encapsulant material because it has the benefit of being nontoxic [332]. Treatment group consisting of animals being fed yogurt only was not included in this study as previous reports tested its potential in colorectal cancers [333] as well as our focus was to test efficacy of APA microencapsulated probiotic bacteria.

5.3.5 The mouse colorectal cancer model

Multiple intestinal neoplasia (Min) mice are heterozygous for *Apc*Min, a germ-line truncating mutation at codon 850 of the *Apc* gene and spontaneously develop pretumoric numerous intestinal neoplasms [334]. The *Apc*Min mouse is a popular animal model for studies on human colorectal cancer [335]. It is used to study the effects of genetics, diet, or chemical compounds on the incidence and development of intestinal precancerous lesions, the adenomas [336]. The germ-line mutations in the *Apc* gene lead to FAP, but inactivation of *Apc* is also found in 80% of sporadic colorectal cancers [337].

Male heterozygous C57BL/6J-*Apc*$^{Min/+}$ mice [338], weighing 20-25 g, were obtained from The Jackson Laboratory (Bar Harbor, ME). The animals kept in the Duff Medical Building Animal Care Facility on a 12-hour light-dark cycle and controlled humidity and temperature. Mice were maintained in a barrier facility. They were allowed sterile water and the laboratory rodent diet 5001 from Purina Land O'Lakes *ad libitum*. Animals overall health was monitored daily.

The protocol was approved by the Animal Care Committee of McGill University and animals were cared for in accord with the Canadian Council on Animal Care (CCAC) guidelines.

5.3.6 Animal Protocol

Mice 7 or 8 weeks old were used. The life span of these mice is 119 ± 31 days [339]. The mice were separated into three experimental groups: 1) Control - animals were gavaged empty APA microcapsules suspended in 0.85% saline, 2) Treatment 1 – animals were gavaged APA microencapsulated *L. acidophilus* bacterial cells blended in 2% M.F. yogurt and 3) Treatment 2 – gavaged APA microencapsulated *L. acidophilus* bacterial cells suspended in 0.85% saline. Upon arrival, animals were randomly placed in the cages and allowed one week of acclimatization period. Based on initial serum IL-6 values the animals were ranked and randomly block assigned to the aforementioned groups. There were 12 animals per group. Animals were weighed individually every week; the

saphenous vein was bled every 4 weeks and fecal samples were collected at specific intervals throughout the experiment. There were 3 end points at weeks 8, 10 and 12 of treatment at which 9, 9 and 15 animals were sacrificed, respectively.

5.3.7 Analytical techniques

5.3.7.1 Interleukin- 6 Determination

Interleukin-6 (IL-6) is a cytokine secreted by diverse cell types under homeostatic and inflammatory conditions [340]. Interleukin (IL)-6 mRNA expression in general is low in normal, adenomatous and cancerous human colon mucosa; except in rather undifferentiated lesions, in which IL-6 is over expressed. IL-6 has been shown to be associated with cancer development. However, its role in gastric cancer has never been investigated. For this test, blood samples were collected every 4 weeks into heparinized tubes, which after blood collection were centrifuged at 5000 *x*g for 20 minutes to yield plasma which was used in further testing. The release of IL-6 from plasma samples into the culture medium was quantified by enzyme-linked mouse immunosorbent assay (ELISA, Biosource, Invitrogen, USA) according to manufacturer's instructions. Briefly, 50 µl plasma plus 50 µL standard diluent buffer were added to each well and incubated for 3 hours and 30 minutes at room temperature. Upon completion of the assay procedure, the plate was read at 450 nm wavelength using a Perkin Elmer Victor microtiter plate reader.

5.3.7.2 The Hemoccult SENSA test

Used according to Beckman Coulter instructions. Briefly, using applicator provided small fecal sample were collected, thin smear was applied covering Box A. Second applicator was used to obtain second sample from a different part of feces, covering Box B. Three days later, samples were developed by applying one drop of Hemoccult SENSA Developer between the positive and negative Performance Monitor areas. Results were read within 10 seconds.

5.3.7.3 Fecal Bile Acids Determination

Feces were collected at specific intervals throughout the experiment and the analysis was performed per group per cage. Total fecal bile acids were determined as previously described [341], [342] with the following modifications. 25 uL of sample were used to determine total bile acid concentration enzymatically as previously described [343] using a commercially available kit (Sigma Diagnostic Bile Acids 450A, Sigma Diagnostics, St. Louis, Missouri, USA) Calibration was carried out according to bile acid internal standard included in the same kit.

5.3.7.4 Adenomas Enumeration, Classification and Histopathology

Mice were euthanized by CO_2 asphyxiation, and the small, large intestine and cecum were excised. Upon removal, the intestines were infused with 10% Phosphate Buffered Formalin (PBF) after which the Swiss Roll was performed by which they were placed in cassettes and immersed in 10% PBF as a fixative. Five-µm paraffin-embedded sections were stained with H&E for histological evaluation.

Polyp scoring was performed by a blinded veterinary pathologist to the treatment. The lesions observed were divided into two categories mostly based on the size of the lesion: gastrointestinal intraepithelial neoplasia (GIN)(<1mm) and adenomas (>1mm). The standards for the histological assessment were established from the MMHCC-sponsored symposium and are detailed on the MMHC web site: (http://emice.nci.nih.gov/emice/mouse_models/organ_models/gastro_models/murine_intestinal_neoplasia/mo dels_colorectal_cancer).

5.3.7.5 Statistical Analyses

The Statistical Analysis System (SAS Enterprise Guide 4.1 (4.1.0.471) by SAS Institute Inc., Cary, NC, USA) was used to analyze the data. Data were expressed as means ± SEM. Differences in body weight, IL-6 concentration, adenomas and gastrointestinal intraepithelial neoplasia number between the groups were analyzed statistically by ANOVA Mixed Models. Data were considered significant at $p \leq 0.05$.

5.4 Results

5.4.1 Microencapsulation and impact of treatment on *Apc*Min mice body weight

The microencapsulation technique used yielded spherical alginate microcapsules with a narrow size distribution of 433±67 μm. Figure 5.1 displays photomicrographs of fresh microcapsules loaded with *L. acidophilus* bacterial cells under light microscopy (77*X* (a) and 112*X* magnification (b)). Using optimal settings microencapsulation yielded a consistent bacterial cell load of approximately 10^{10} cfu/mL in all batches throughout the experiment.

After an acclimatization period of one week, the mice were randomly assigned using a block design into 3 groups, each consisting of 11 animals. Body weights were recorded on weekly basis (Figure 5.2a). Results show a steady drop in the body weight of control group animals (24.6±0.48 to 22.0±1.47 grams over 12 weeks). However, a stable body weight was observed in animals receiving treatment; 24.2±0.47 to 24.0±1.32 grams (treatment 1) and 24.3± 0.36 to 24.0±0.71 grams (treatment 2). After 17 weeks, rapid decline in body weight was observed in control group animals as compared to treated animals.

5.4.2 Interleukin- 6 levels in experimental *Apc*Min mice

Results show the average levels of anti-inflammatory interleukin-6 (IL-6) were 11.17±1.59 for treatment 1 group, 17.45±2.74 for treatment 2 group and 18.33±1.46 pg/mL for the control group at the time of sacrifice (Figure 5.2b). The expression levels increased steadily in animals of the control group and decreased after the 16^{th} week in animals of treatment group 1 and remained stable in the animals of treatment group 2.

5.4.3 Detection of fecal blood in *Apc*Min mice (Hemoccult SENSA test)

Abnormal bleeding is associated with gastrointestinal disorders; the fecal samples from individual cages were collected at the beginning and end of the treated period and analyzed for fecal blood using the Hemoccult SENSA test. The Hemoccult SENSA is a qualitative test with a higher sensitivity than standard guaiac tests. The occult blood test

was performed in triplicate. All tests were positive and the coloration intensities were qualitatively scored by three blinded observers, + being the least intense to +++ being the most intense. The results are displayed in Table 5.1a. Results show that all animals had presence of blood in their fecal samples on arrival. After the experimental period, the control animal group displayed higher blood content in their fecal samples as compared to both treatment groups. Furthermore, treatment 2 was more effective in controlling fecal blood than treatment 1.

5.4.4 Fecal Bile Acid levels in experimental animal model

To determine the effect of microencapsulated probiotic bacteria on luminal bile acids, the levels of bile acids in fecal samples from individual cages of each group were measured. Using bile acid standard with a concentration of 100-umol/L, the correlation of the determinant factors (R^2) of 0.9955 was obtained and used in this experiment. There was a constant drop in fecal bile acid observed in all groups (Figure 5.3) with the greatest reduction observed in the treatment group 2 where animals were gavaged *L. acidophilus* bacterial cells in APA microcapsules suspended in saline (448±2.82 to 105±21.36 (nmol/g fecal sample/100g BW)). A decrease in luminal bile acids (442±4.87 to 210±3.66 (nmol/g fecal sample/100g BW)) was also observed in the treatment group 1 receiving microencapsulated *L. acidophilus* bacterial cells in 2% M.F. yogurt. Total fecal bile acid values averaged over time are presented in Table 5.1b. Results show an average decrease of 310.25±75.22 (nmol/g fecal sample/100g BW) in treatment group 1 and 229.15±101.95 (nmol/g fecal sample/100g BW) in treatment group 2 compared to control animals 358.44±53.93 (nmol/g fecal sample/100g BW) receiving no treatment.

5.4.5 Adenomas reduction in the treated animals: Classification and Histopathology

The number of adenomas, low grade dysplasia, high grade dysplasia, and gastrointestinal intraepithelial neoplasias (GIN) were scored for each treated and non treated animal group in both the small and large intestine. The numbers were averaged per animal in a given group. In the large intestine, 0.8 adenomas were found in the

control group as compared to 0.4 and 0.7 in treatment 1 and 2 groups, respectively (Figure 5.4a).

In the small intestine, 28 adenomas were found in the control group versus 13 and 18 in treatment groups 1 and 2, respectively (Figure 5.4c). In the large intestines, there were 0.3 GIN found in control group versus 0.2 and 0.1 in treatment 1 and treatment 2 groups, respectively (Figure 5.4b). In the small intestine, there were 8 GIN found in control group versus 4 and 6 in treatment 1 and 2 groups, respectively (Figure 5.4d). Control group animals had a statistically higher number of adenomas and GIN in the small intestine than treatment 1 group mice. Although there were no statistically significant changes in numbers of adenomas or GIN in the large and small intestine between treatment groups; there was a trend toward fewer polyps compared to controls.

To evaluate the overall impact of the treatment procedure, after 12 weeks of treatment, histological analysis of colon lesions was analyzed and compared with control animals (Fig. 5.5). Results show that the tumors were mostly well differentiated pedunculated adenomas with high grade of dysplasia in a representative animal of the control group (Fig. 5.5a) compared to treatment groups (Fig. 5.5 b, c and d; arrows indicate areas affected most). A clear trend in controlling colon lesions was observed in the treatment groups. Histological analysis of treatment group 2 (Fig. 5.5 c and d) indicates that the animals had tumors in a more advance stage characterized by low grade of dysplasia (Fig. 5.5c) and broad-based adenomas (Fig. 5.5d). In contrast, the animal in treatment 1 group had mostly microadenomas (Fig. 5.5b).

5.5 Discussion

The development of colorectal cancer, one of the most frequent cancers, is influenced by the inflammatory state with changes in mucosal function and structure as well as changes in the colonic bacterial flora [321]. Studies have shown that yogurt and other probiotic foods reduce the incidence of colorectal cancer and other gastrointestinal diseases [344-347]. The main objective of this study was to show the efficacy of microencapsulated probiotic bacteria.

There is a strong correlation between diet and disease occurrence. Furthermore, there is a relationship between food intake and age. As mentioned before, the life span of

*Apc*Min mice is 119 ± 31 days. After 119 days (17 weeks), no weight gain was observed due to a pronounced decline in food intake. The decline in food intake at advanced ages is usually due to social factors such as depression and social isolation; physical factors such as changes in taste and smell, and medical conditions including gastrointestinal disease, malabsorption syndromes, acute and chronic infections [348]. In our case older mice had a greater degree of intestinal obstruction in the form of polyps and were therefore less likely to maintain food consumption levels due to an inability to properly excrete.

Secretion of IL-6 is strongly associated with the pathogenesis of Inflammatory Bowel Diseases (IBD) [349], [350] and overproduction of IL-6 by intestinal epithelial cells is thought to play a part in the pathogenesis of IBD [351]. IL-6 can initiate the innate immune response by inducing the acute phase of inflammation [352,353] and appears to be involved in malignant transformation, tumor progression, and tumor-associated cachexia as reported in studies on Kaposi's sarcoma [354], multiple myeloma [355], renal cell carcinoma [356], prostate cancer [357], ovarian cancer [358] and breast cancer [359]. Results in the present study indicate an overall decreasing IL-6 trend for both treatment groups compared to control (*p* values: T1/T2= 0.55, C/T2= 0.99 and C/T1= 0.57). This indicates that an anti-inflammatory state correlates with the beneficial effect of the probiotic bacteria on the involved immunomodulatory mechanisms. The differences in inflammation levels among the treatment groups could be the result of prevention of pathogenic bacterial growth, binding to or penetration of pathogens to mucosal surfaces, stimulation of mucosal barrier function, or altering immunoregulation (decreasing proinflammatory and promoting protective molecules).

Fecal occult blood testing (FOBT) is a screening method for colorectal cancer that has the most evidence for efficacy and is also the cheapest approach [360]. It helps reducing mortality by detecting early signs that may lead to the formation or presence of polyps in the colon or rectum. Rectal bleeding was observed in animals on arrival and the test was repeated at the end of experiment to verify whether the treatment has an effect in decreasing the bleeding from the GI tract. This qualitative test detected the presence of blood in the feces in all animal cages at the end of the treatment, but did not reveal any significant differences among groups. Although this test is more sensitive than other

occult blood tests, it is considered to be a qualitative screening rather than decisive test; therefore it cannot provide conclusive evidence of the presence or absence of gastrointestinal bleeding or pathology.

Bile acids contribute to colonic carcinogenesis by disturbing the fine balance between proliferation, differentiation, and apoptosis in colonic epithelial cells [361-363]. Secondary bile acids have been implicated as an important etiological factor in colorectal cancer. In addition, the bile acids in the feces act as a promoter of colon cancer, in particular deoxycholic acid (DCA), a secondary bile acid. The ratio of DCA/cholic acid (CA) in feces is also said to have a diagnostic significance in colon cancer [364]. It has been established previously that lactobacilli are unable to bind the major conjugated bile acid, glycocholic acid (GCA) [365]. Further, it is also known that the colonic microflora probably has bile salt hydrolase (BSH) activity which causes the breakdown of conjugated bile acids to the secondary bile acids, most notably DCA [321]. Testing in the lab revealed that *L. acidophilus* used in this study had no significant BSH activity (data not shown). Owing to the fact that DCA is the primary bile acid measured in feces using the total bile analysis kit, the overall decreasing trend may indicate that a minor amount of primary bile acids were deconjugated to secondary bile acids. Also, we postulate that this trend could result in the replacement of BSH positive colon flora with one that exhibits lesser BSH activity, over the course of the experiment. Although more studies are needed, our results indicate that microencapsulated bacterial cells may have an influence in tumorogenesis.

Histological examinations are the most reliable methods for evaluation of the tumor status and therapy efficacy. The number of adenomas found in each of the treatment groups in the colon and in the small intestine indicates that treatment 1 had a greater impact than treatment 2. This may be explained in two ways; firstly, the synergistic effect between bacterial cultures and yogurt along with its nutrients (i.e. calcium, vitamins A and D) might have a superior effect over bacterial cells suspended in saline solution; secondly, the protective effect of 2% fat contained in the yogurt could have effectively shielded microcapsules and encased bacteria from environmental stresses, therefore enhancing overall efficacy. In addition, the number of gastrointestinal intraepithelial neoplasias (GIN) found in the colon were fewer than in the small intestine.

Although there were no statistically significant differences between groups in the large intestines, there was a statistical difference between control and treatment 1 group in the small intestine in number of adenomas and GIN (Fig 5.4c, 5.4d).

Among the organs examined in this study, there was only one malignant tumor (adenocarcinoma) found in the small intestine of a control animal. The nuclei were enlarged and pleomorphic with variable loss of their polarity. The glandular structure was distorted and resembled that seen in overt colonic carcinoma. The highest tissue damage was observed in the colon of control group animals under the same conditions as applied to the other tissues. This is probably due to the fact that the colonic wall, including the mucosa and the submucosa, is much thinner than that of the other organs.

Most of the adenomas found were sessile/broad-based and were composed of papillary projections of lamina propria covered by an epithelium. There were no lesions or adenomas found in the ceca. The greatest loss in mucin secretion was displayed in severely dysplastic glands of control group animals sacrificed at 12 week of the experiment. The glands were closely packed and a structural atypia, e.g., "back to back" arrangement was more prominent. Nuclei were plump but still uniform and smaller than those in carcinomatous glands. Cytological abnormalities detected included cellular and nuclear pleiomorphism and loss of polarity. Architectural abnormalities included the presence of intraglandular papillary projections and of cribriform and solid epithelial areas. There were, however, no major differences in animal tissues collected from animals sacrificed at different time periods. The tumors found in both treatment groups showed some features of papillary carcinoma-grooved nuclei and papillary architecture, but these were not consistent (Fig 5.5). Future work could incorporate additional cellular markers to reveal immunocytochemical indices of tumour growth dynamics such as proliferation-associated antigens (e.g. Ki-67, PCNA) and to examine reciprocal correlations between the intensity of apoptosis and the expression of such pro- and anti-apoptotic cell markers as caspase-3 (cas-3), MT and Ki-67 antigen.

The present study therefore demonstrates that microencapsulated probiotic bacteria in yogurt exert beneficial action by maintaining the constant body weight, minimizing intestinal inflammation and delaying overall polyp progression in

experimental *Apc*Min mice. This study will have implication in colon cancer, IBD, and other GI diseases.

5.6 Acknowledgements

We acknowledge the Canadian Institute of Health Research (CIHR) grant to S. Prakash. We also acknowledge support for A.M. Urbanska by a PGS-D scholarship from the Natural Sciences and Engineering Research Council (NSERC) of Canada and a McGill Faculty of Medicine Internal postgraduate scholarship. J. Bhathena acknowledges the Canadian Liver Foundation for a Graduate Studentship Award. C. Martoni and A. Kulamarva acknowledge graduate scholarships (doctoral) from NSERC. We acknowledge the aid of Dr. Marilene Paquet, Veterinary Pathologist McGill Cancer Center in performing the blinded scoring for the histological experiments as well as Arun Kulamarva for help with fecal samples collection.

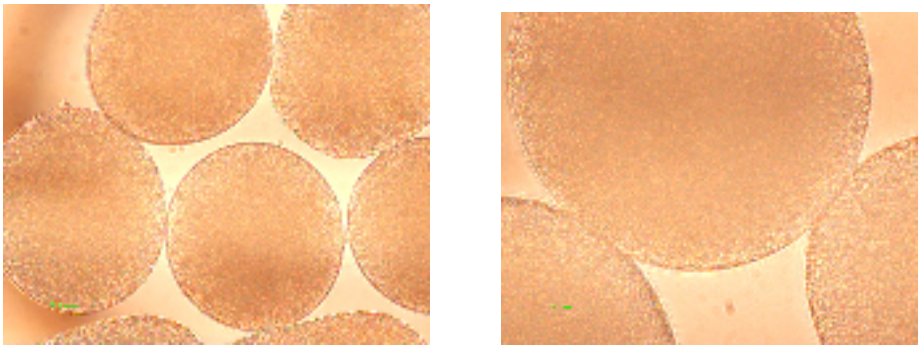
	Animal age – 8 weeks			Animal age – 20 weeks		
	Cage 1-3	**Cage 4-6**	**Cage 7-9**	**Cage 1-3**	**Cage 4-6**	**Cage 7-9**
Control (C)	+	+	++	+++	+++	+++
Treatment 1 (T1)	+	++	+	++	+++	++
Treatment 2 (T2)	++	+	+	++	++	++

(A)

	Average Total Fecal Bile acid (nmol/g fecal sample/100g BW)	**Repeated measures ANOVA using Mixed Models Analysis *p* values**
Control (C)	358.44±53.93	0.0296 (T1+C)
Treatment 1 (T1)	310.25±75.22	0.0187 (T1+T2)
Treatment 2 (T2)	229.15±101.95	0.0037 (T2+C)

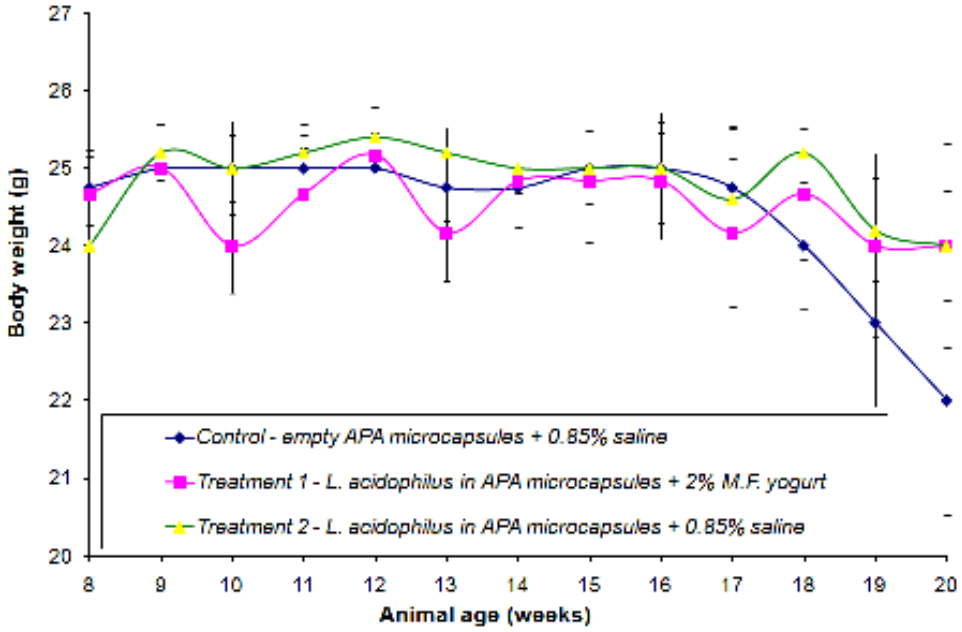
(B)

Table 5.1: Detection of fecal blood using The Hemoccult SENSA test (A) and comparison of average total fecal bile acid per group and their *p* values (B).

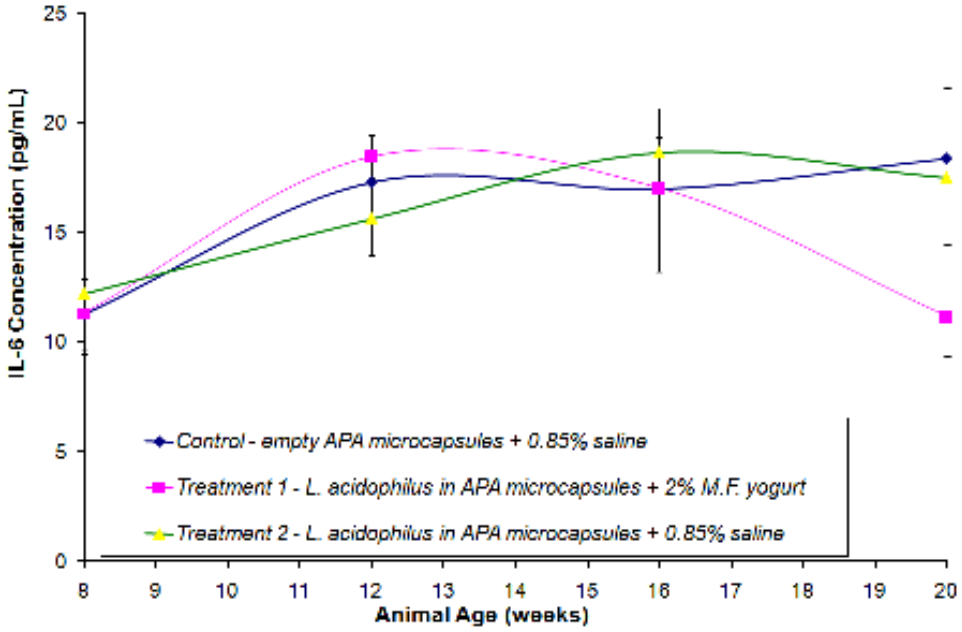


(A) **(B)**

Figure 5.1: Photomicrograph of APA microcapsules loaded with *Lactobacillus acidophilus* bacterial cells at (a) 77*X* magnification and (b) at 112*X* magnification. (size 433±67 µm)



(A)



(B)

Figure 5.2: The effect of treatment in the C57BL/6J-*Apc*$^{Min/+}$ mice examined at different time intervals on (A) animal body weights and (B) on the concentration levels of anti-inflammatory interleukin-6; *n=12*. Data represent the mean ± SEM of concentration levels per group.

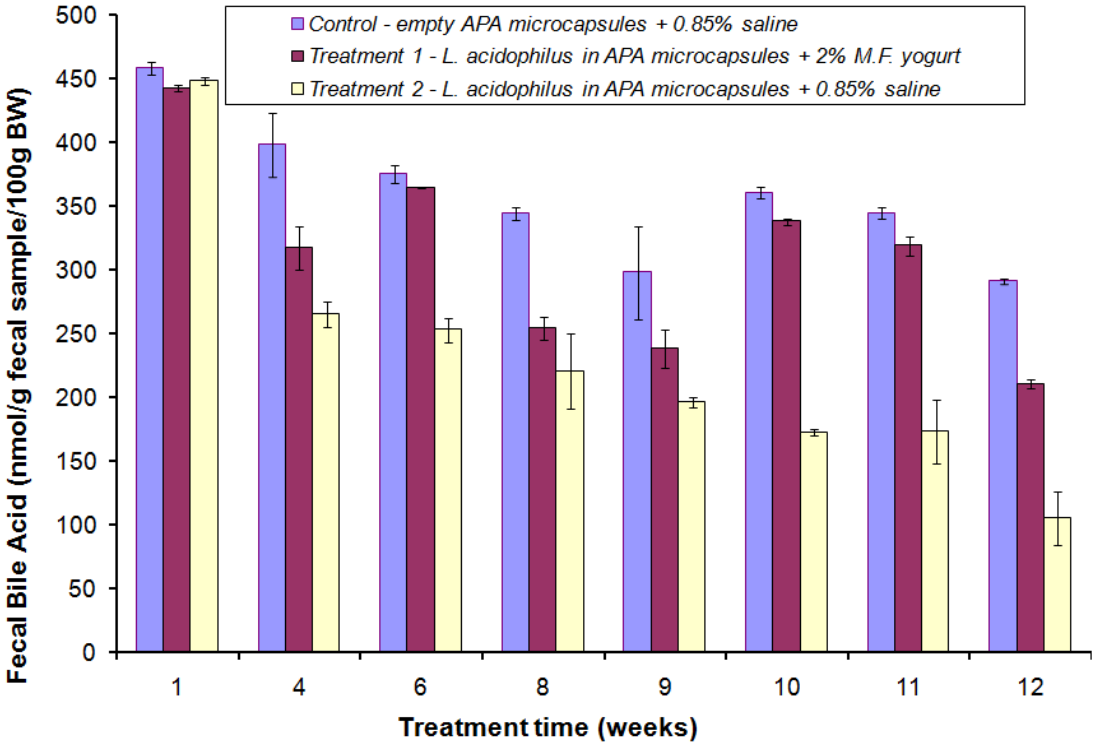


Figure 5.3: Effect of treatment on total fecal bile acid levels. Data represent the mean ± SEM of concentration levels per group.

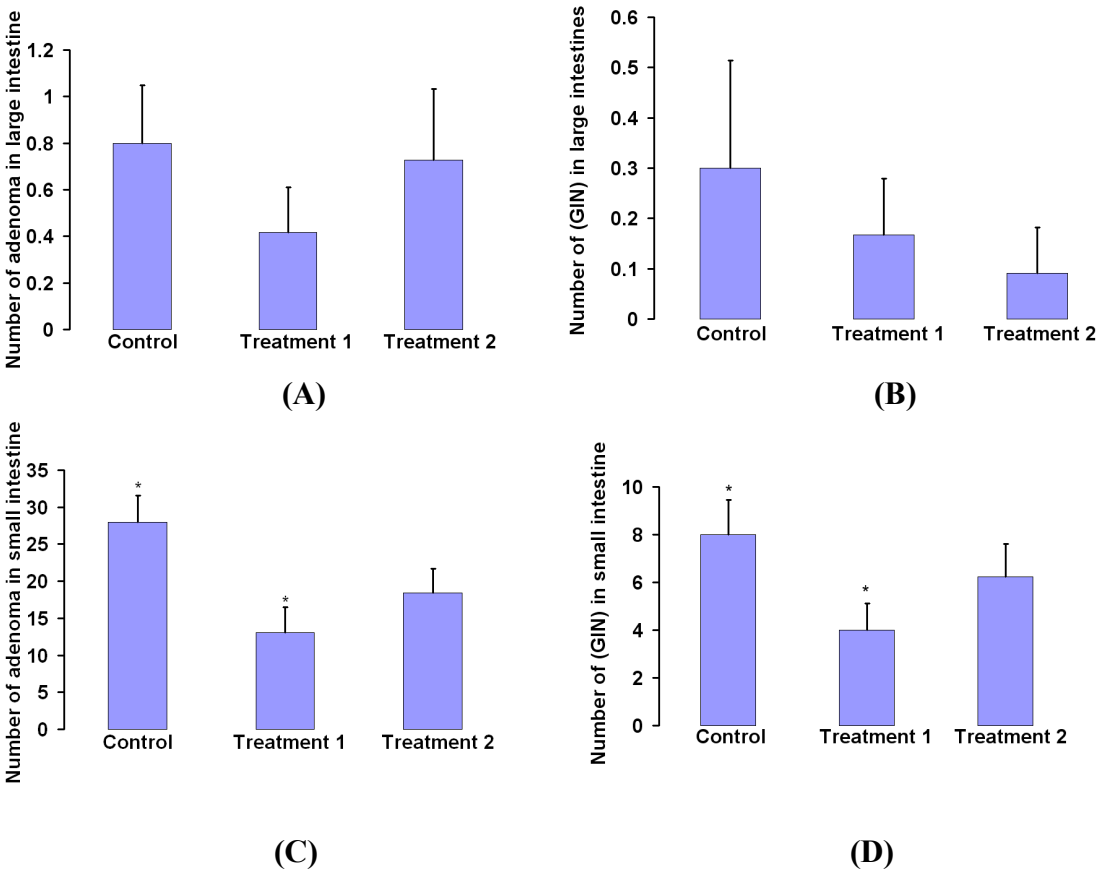
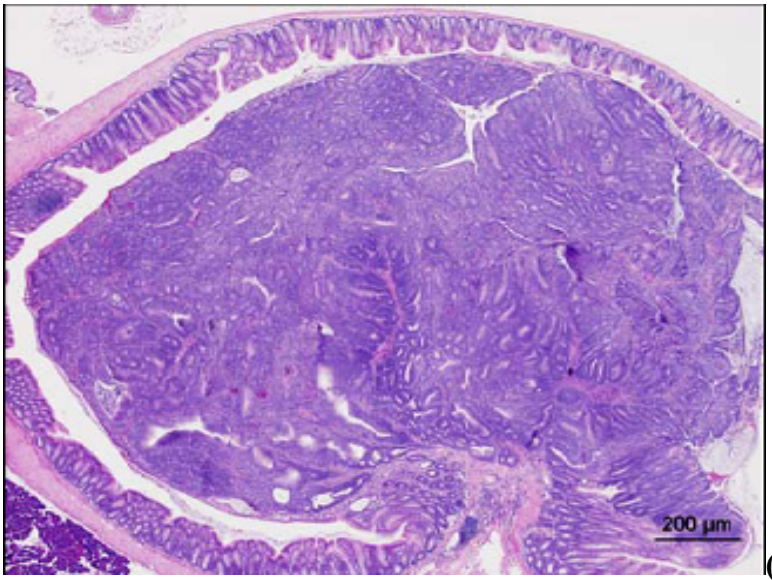
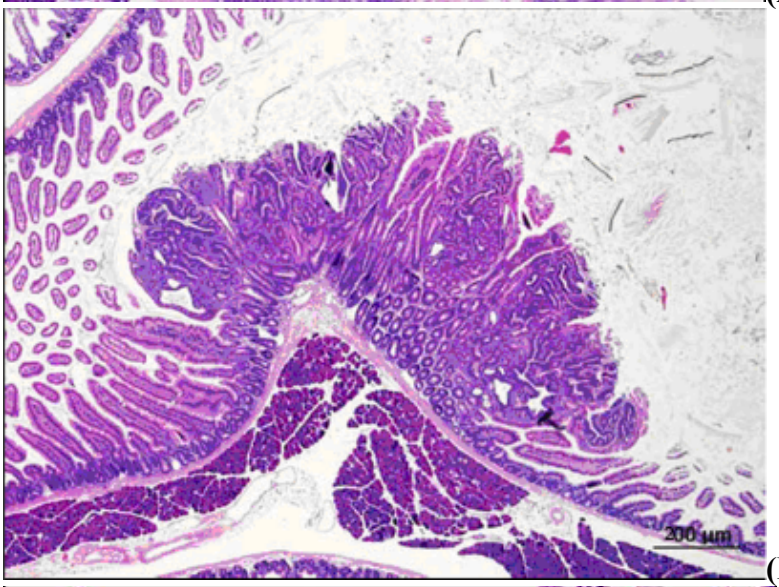


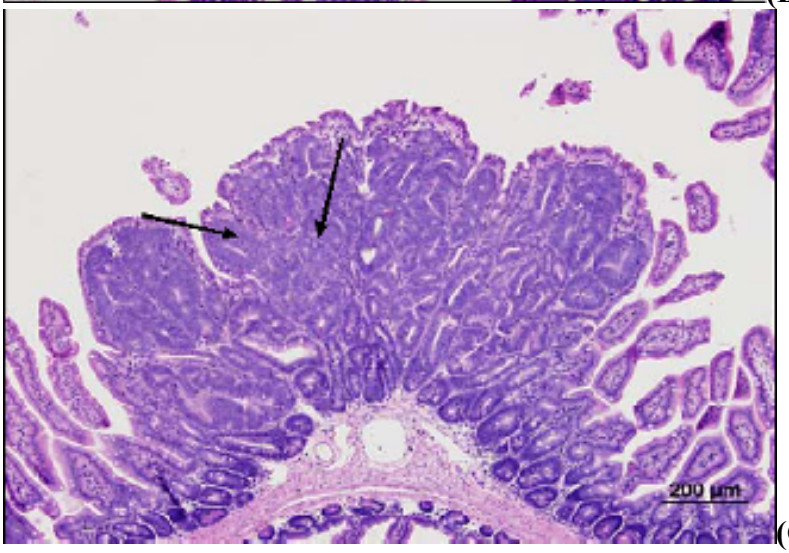
Figure 5.4: The number of adenomas (A), (C) and Gastrointestinal Intraepithelial Neoplasias (GIN) (B), (d) for three groups: Control (empty APA microcapsules + 0.85% saline), Treatment - 1(*L. acidophilus* bacterial cells in APA microcapsules + 2% M.F. yogurt) and Treatment - 2 (*L. acidophilus* bacterial cells in APA microcapsules + 0.85% saline found in large) (A), (B) and small intestines (C), (D). Data represent the mean ± SEM per group. Asterisks, Statistical differences ($p < 0.05$).



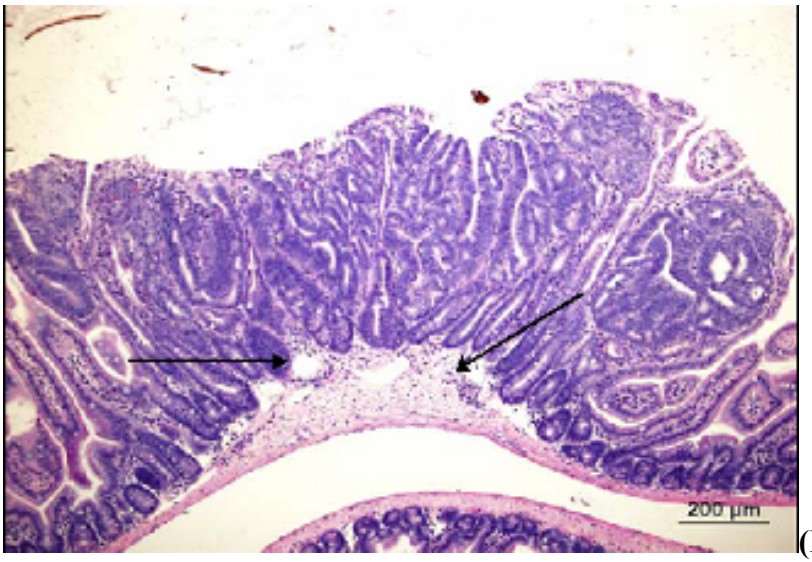
(A)



(B)



(C)



(D)

Figure 5.5: Histological sections showing intestinal changes in C57BL/6J-*Apc*$^{Min/+}$ mice. (A) A representative tumor of the colon found in a control untreated mouse shows pedunculated (polypoid) adenomas with high grade of dysplasia. Original magnification 40*X*. (B) Gastrointestinal intraepithelial neoplasia (microadenomas) of small intestine found in a treatment 1 mouse gavaged with *L. acidophilus* bacterial cells in APA microcapsules + 2% M.F. yogurt. Note the increased Nuclear / Cytoplasmic ratio, the nuclear crowding at the lamina propria/crypt. Original magnification 100*X*.(C) Papillary Adenoma in small intestine, sessile with low grade of dysplasia (arrows) (Sessile adenomatous polyp) found in a treatment 2 mouse gavaged with *L. acidophilus* bacterial cells in APA microcapsules + 2% M.F. yogurt 0.85% saline. Original magnification 100*X*. (D) Broad-based adenoma of small intestine found in a treatment 2 mouse gavaged with *L. acidophilus* bacterial cells in APA microcapsules + 0.85% saline. Original magnification 100*X*. All tissues were stained with hematoxylin eosin.

CHAPTER 6
Original paper 4

Probiotic microencapsulated *Lactobacillus acidophilus* formulation ameliorates inflammatory cytokines and reduces tumor incidence in mice

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Original paper to be submitted to: ***Molecular Nutrition and Food Research***

Preface

The following paper further evaluates the potential of APA microencapsulated probiotic yogurt formulation in *Apc*Min mice. The oral administration of microencapsulated *L. acidophilus* bacterial cells suspended in saline is being conducted for duration of 15 weeks. Control animals are being gavaged with empty APA microcapsules also in saline solution. Biomarkers such as IL-6 and IL-12 are being evaluated in the blood collected from animals on montly basis. From the fecal collection, an analysis of animals' fecal bile acid concentrations is being calculated. The levels of IL-12, IgA, TNF- α and IFN- γ are tested at the time of sacrifice using luminal digesta obtained from the small intestine. In addition, parameters such as animals' overall health, their body weights and presence of fecal blood are being monitored during the length of the experiment. Moreover, histological analysis including adenomas enumeration in small and large intestines and their classification is also included. Staining of frozen-OCT cross sections with CD8+ T cells as well as Mac-1 cells further scrutinizes the impact of treatment on animal polyp presence and progression. Immunohistochemical analysis on intestinal samples is performed. To further investigate the treatment effect on recruitment of T cells in animal tissues, the TUNEL method is being conducted to show cell proliferation and apoptosis.

6.1 Abstract

*Apc*Min mice spontaneously develop pretumoric adenomas in the intestinal mucosa and therefore are considered an important animal model to study mechanisms leading to both carcinogenesis and cancer prevention. We studied the relationship between the level of inflammation and adenoma formation in *Apc*Min mice gavaged daily with APA microencapsulated *Lactobacillus acidophilus* bacterial cells and empty APA microcapsules for 15 weeks. The interleukin-6 and interleukin-12 plasma concentrations as well as the secretory levels of immunoglobulin-A, tumor necrosis factor-α, interferon-γ in ileal contents were analyzed by ELISA. At the time of sacrifice the treatment group had a lower concentration of IL-6 and IL-12 compared to control, namely 13.81±1.16 and 709.6±255.24, respectively. Moreover, IgA concentration was comparably similar in the duodenum, jejunum, proximal and distal segments of the small

intestine in both groups. In contrast, TNF-α and IFN-γ concentrations were lower in the entire small intestine in animals receiving treatment. Histology studies showed a significantly smaller amount of adenomas in treatment animals as compared to control. The number of CD8+ T lymphocytes and tissue macrophages (Mac-1+ cells) per villus in ileal mucosa were determined by immunohistochemistry. Treatment with the microencapsulated probiotic formulation produces significant reductions in intestinal inflammation. Results suggest that daily oral delivery of the microencapsulated probiotic formulation exerts its beneficial effect by maintaining the constant animal body weight, minimizing intestinal inflammation and delaying overall polyp progression in experimental Min mice. This has implications in managing potential risks of unbalanced microflora of the gut due to poor diet, age and other clinical situations.

Key words: Microencapsulation, Probiotic, Lactobacillus, Colon Cancer, Adenoma.

6.2 Introduction

Epidemiological investigations show that colorectal cancer (CRC) is one of the three most frequent malignancies in Western industrial nations. Although the 5-year survival rate for patients with early stage and local CRC approaches nearly 90%, survival is dramatically decreased by local recurrence and the development of distant metastases that primarily affect the liver, which are the predominant cause of CRC-related mortality. According to Dana-Farber Cancer Institute the "western" diet which is composed primarily of high amounts of red meat, fatty products, refined grains, and desserts has been closely related to a higher incidence of colonic cancer. It has been further established that certain components of such diet are responsible for altering intestinal flora in a way leading to production of carcinogens [366,367]. Although the overall data for red meat and colon cancer are strongly suggestive of an important relation, they are not conclusive. In a study by Hill *et al.* it was noted that no carcinogen could be responsible in a cancer-diet link [368]. The capability of the intestinal environment to modulate anticancer immunity not only at the mucosal but also at the systemic level is still an open

question. It is known that the immune responses to mucosal microbiota require a precise regulatory control; therefore the unlimited immune activation in response to signals from commensal bacteria could pose the risk of inflammation [369]. The same study makes a note that the regulation of microflora composition (e.g. by probiotics and prebiotics) offers the possibility to influence the development of mucosal and systemic immunity and it also can play a role also in the prevention and treatment of some diseases.

Probiotics are defined by the World Health Organization (WHO) as "live microorganisms which when administered in adequate amounts confer a health benefit to the host"[370] . Their numerous beneficial contributions to health problems have been reported including diarrhea, gastroenteritis, irritable bowel syndrome, inflammatory bowel disease (Crohn's disease and ulcerative colitis), cancer, improved immune function, lactase digestion, hyperlipidemia, hepatic diseases, *Helicobacter pylori* infections, and genitourinary tract infections. They are also postulated to be able to deconjugate and absorb bile acids. In doing so, they contribute to the overall reduction of the colonic mucosal secretion of mucin and fluids which may lead to changes in colonic bacterial flora [321].

The interest in dairy products enriched with probiotic microorganisms has never been so great, and current investigations involve mainly lactic acid bacteria with specific characteristics for specific purposes. Lactic acid bacteria (LAB), like other probiotic microorganisms, are believed to compete and suppress the growth of undesirable microorganisms in the colon and intestines leading to the stabilization of the digestive system [371]. Although many attempts of delivering live bacterial cells to the intestines have been attempted, their outcomes have been largely unsuccessful due to poor bacterial survival in acidic stomach conditions [372] storage conditions[18] and other limitations.

Microencapsulation involves immobilizing live cells such as bacteria in small droplets of an appropriate biomaterial and then applying one or more coats of additional biopolymer to obtain the desired properties. Therefore via microencapsulation we assure the proper and sufficient delivery of desired microorganisms to targeted sites.

In the present study we investigated the effect of the daily oral delivery of APA microencapsulated probiotic *Lactobacillus acidophilus* bacterial cells suspended in saline solution and compared their effect with empty APA microcapsules receiving control

mice. In addition, we studied the relationship of intestinal immune responses and tumorigenesis in the *Apc*Min mouse model using various biomarkers. The abundance of ileal tissue macrophages (Mac-1 cells) and CD8+ T lymphocytes were immunostained and the levels of secretory IL-6, IL-12, IgA, IFN-gamma and TNF-alpha in ileal contents were investigated. Interleukin-6 (IL-6) is a cytokine secreted by diverse cell types under homeostatic and inflammatory conditions [373]. Numerous studies found increased IL-6 immunoreactivity in cancerous lesions when compared to normal colon mucosa and that IL-6 concentrations correlated with tumor tissue concentrations and proliferative activity [374-376]. In several comparative studies, IL-12 was the most effective cytokine gene that could induce eradication of experimental tumors, prevent the development of metastases, and elicit long-term antitumoral immunity [377]. Additional histology sections of small intestines were performed to study the development of adenomas in response to oral delivery of microencapsulated bacterial cells.

6.3 Materials and Methods

6.3.1 Materials

6.3.1.1 Chemicals

Sodium alginate (low viscosity), poly-L-lysine hydrobromide (MW=27,400) (lot 71K5120) and calcium chloride (desiccant, 96+%, A.C.S. reagent, FW 110.99, batch # 05614AC) were purchased from Sigma-Aldrich, (Oakville, ON, Canada). MRS AGAR Difco ™ Lactobacilli and MRS BROTH Difco ™ Lactobacilli were purchased from Becton, Dickinson and Company (Sparks, USA).

6.3.1.2 Bacteria and Culture Conditions

Lactobacillus acidophilus cells (ATCC 314) were cultivated and serially propagated three times in the MRS medium before experimental use. Incubations were performed at 37°C in a Professional Sanyo MCO-18M Multi-Gas Incubator in anaerobic conditions (1-2% CO_2, Atmosphere Generation System AnaeroGen™; Oxoid,

Hampshire, England). Bacteria to be encapsulated were isolated after 20 hours of the 3rd passage.

6.3.1.3 Microencapsulation Method

The bacterial strains were microencapsulated into alginate-poly-L-lysine-alginate (APA) membranes. All membrane components were filter sterilized through a 0.22 μm Sterivex-GS filter prior to use. Grown cultures were centrifuged at 3,000 *x*g for 15 minutes at 25ºC and the supernatant broth was decanted. The pellet of wet cells was weighted and suspended in 0.85% saline, pooled and slowly added to a gently stirred sterile 3.3% sodium alginate solution (adjusted to 1.65% with 0.85% saline). The entire procedure was performed under sterile conditions in a Microzone Biological Containment Hood (Microzone Corporation ON, Canada) and all solutions were autoclaved with the exception of poly-L-lysine, which was 0.22 μm sterile-filtered prior to usage. APA microcapsules were prepared aseptically using an Inotech Encapsulator® IER-20 (Inotech Biosystems Intl. Inc. Switzerland). Freshly prepared microcapsules were washed twice with 0.85% saline and stored at 4°C. Parameters for microencapsulation were as follows: gelation time in $CaCl_2$ – 30 minutes, coating time – 10 minutes, nozzle diameter - 300 μm, vibrational frequency – 918 Hz, voltage > 1.00kV and current 2 amp.

6.3.1.4 Treatment Formulation Preparation

APA microcapsules loaded with *L. acidophilus* bacterial cells were suspended in 0.85% saline in the proportions of 1:3, respectively. Empty APA microcapsules were suspended in 0.85% saline using the same formulation.

6.3.2 Animals

6.3.2.1 The Mouse Colorectal Cancer Model

The mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The animals were kept in the McIntyre Medical Sciences Building Animal Care Facility in the

room with a 12-hour light-dark cycle and controlled humidity and temperature. Mice were maintained in a barrier facility. They were allowed sterile water and the laboratory rodent diet 5001 from Purina Land O'Lakes *ad libitum*. Animals overall health was monitored daily. The animal use protocol was approved by the Animal Care Committee of McGill University and animals were cared for in accord with the Canadian Council on Animal Care (CCAC) guidelines.

6.3.2.2 Animal protocol

Male C57BL/6J-*Apc*$^{Min/+}$ mice[338] 5 or 6 weeks old were used. The mice were separated into two experimental groups: Control - animals gavaged with empty APA microcapsules suspended in 0.85% saline and Treatment – animals gavaged with APA microencapsulated *L. acidophilus* bacterial cells suspended in 0.85% saline. Upon arrival, animals were randomly placed in the cages and allowed one week of acclimatization period. Based on initial serum IL-6 values the animals were ranked and randomly block assigned to the aforementioned groups. There were 7 animals per group. Animals were weighed individually every week; the bleed from saphenous vein was performed every 4 weeks and feces samples were collected weekly throughout the experiment.

6.3.3 Analytical Techniques

6.3.3.1 Interleukin- 6 and Interleukin-12(*p*40) Determination

To determine the concentration levels of IL-6 and IL-12, blood was collected from animals' saphenous vein every 4 weeks into heparinized tubes. The release of IL-6 and IL-12(p40) from plasma samples into the culture medium was quantified by enzyme-linked immunosorbent assay (ELISA, Biosource, Invitrogen, USA) according to manufacturer's instructions. Briefly, 50 µl plasma plus 50 µL standard diluent buffer were added to each well and incubated for 3 hours and 30 minutes at room temperature. Upon completion of the assay procedure, the plate was read at 450 nm wavelength using a Perkin Elmer Victor microtiter plate reader.

6.3.3.2 Fecal Bile Acids Determination

Feces were collected weekly throughout the experiment and the analysis was performed per group per cage. Total fecal bile acids were determined as previously described [378,379] with the following modifications. 25 μL of sample were used to determine total bile acid concentration enzymatically as previously described [380] using a commercially available kit (Sigma Diagnostic Bile Acids 450A, Sigma Diagnostics, St. Louis, Missouri, USA).

6.3.4 Obtaining Luminal Digesta

For all samples including IgA, TNF- α, IFN- γ and IL-12 the luminal digesta was obtained in a similar fashion. At the time of sacrifice, the small intestine of each animal was measured and cut into 4 equal segments, approximately representing duodenum, jejunum as well as proximal and distal ileum. Each segment was gently flushed with cold PBS buffer and the contents were collected and frozen at -85°C until next use. Before the analyses, the digesta samples were treated with 1.0% BSA-50 mM Tris buffer (pH 7.5) for 60 min at room temperature to separate the food matrix and cellular material. The samples were then centrifuged at 50,000 x*g* for 15 min. The supernatants were stored at -85°C and used later for experiments.

6.3.4.1 Gastric Mucosal Luminal IgA Concentrations Determination

The luminal concentration of IgA was measured using a competitive enzyme immunoassay (Bethyl Laboratories Inc., Montgomery, TX) according to manufacturer's instructions. Briefly, 96-well plates were coated with goat anti-Mouse IgA-affinity purified capture antibody and incubated for 60 minutes. Samples were washed 3 times with wash buffer. 200 μL of blocking solution was loaded into the wells and let to incubate for 30 minutes. Samples were washed 3 times with wash buffer. 100 μL of standards and samples were added to specific wells and left to incubate for 60 minutes. Samples were washed 5 times with wash buffer. 100 μL of Goat anti-Mouse IgA-HRP

conjugate antibody was diluted to1:50,000 added to the wells and incubated for 60 minutes. 100 µL of TMB (TMB/H_2O_2, 50-76-00, Kirkegaard and Perry, Gaithersburg, MD; 1% Bovine Serum Albumin in Tris Buffered Saline, T-6789, Sigma Chemical) substrate solution was added and left for 15 minutes following addition of 100 µL of 2 M H_2SO_4 to stop the reaction. OD at 450 nm was determined using a Perkin Elmer Victor microtiter plate reader. The concentrations were counted per digesta fresh weight.

6.3.4.2 Gastric Mucosal Luminal TNF-α Concentrations Determination

TNF- α was measured using a competitive enzyme immunoassay (ELISA, Biosource, Invitrogen, USA) according to manufacturer's instructions. Briefly, 100 µL of standards were added to appropriate wells. 50 µL of incubation buffer was loaded to each well followed by 50 µL of samples and 50 µL of anti-TNF-alpha (Biotin Conjugate) solution. The plate was incubated for 90 minutes at room temperature. The samples were thoroughly aspirated and washed 4 times with buffer. The wells were loaded with 100 µL Streptavidin-HRP working solution and incubated for 30 minutes. Upon completion, samples were aspirated and washed 4 times with buffer. Addition of 100 µL of stabilized chromogen followed and the final incubation lasted 30 minutes. Before the plate was read at 450 nm, 100 µL of stop solution was added. A Perkin Elmer Victor microtiter plate reader was used to obtain readings.

6.3.4.3 Gastric Mucosal Luminal IFN-γ Concentrations Determination

Biotin gamma rabbit anti-mouse interferon-γ was purchased from Cedarlane, (Hornby, ON, Canada) and reconstituted from sterile form to 50ug/mL with PBS solution containing 0.1% BSA. Murine IFN-γ ELISA kit was purchased from Peprotech (Rocky Hill, NJ), and used as recommended by the manufacturer. Briefly, 100 µL of capture antibody was added to each well and incubated overnight. Wells were aspirated and washed with wash buffer 3 times. 300 µL of blocking buffer was loaded and incubated for 1 hour. 100uL of standard and samples were added to appropriate wells and incubated for 2 hours. Detection antibody was diluted to a concentration of 0.5 µg/mL and 100 µL

was added per well. 2 hours of incubation followed. Avidin-HRP conjugate was diluted to 1:2000 in diluent for a total volume of 11 mL. 100 µL of it was added per well and incubated 30 minutes. All incubations were performed at room temperature. Plates were washed 4 times in between each step. ABTS liquid substrate solution containing 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) in an acidic buffer (Sigma-Aldrich, Inc., St. Louis, MO) was added to each well for color development. OD at 405 nm was determined using a Perkin Elmer Victor microtiter plate reader.

6.3.5 Histology

A tissue sample was cut from the distal ileum section of each animal. The tissue sample was thus obtained from the middle of the area from which the digesta sample was collected. The sample was placed in Tissue-Tek OCT compound (Sakura Finetek, Zoeterwoude, the Netherlands), frozen in liquid nitrogen, and stored at -85°C. The tissue samples were cut with a cryostat microtome at 4 µm, and the slices were placed on SuperFrost microscope slides (Menzel-Gläser, Braunschweig, Germany), air-dried, and stored at -85°C.

6.3.5.1 Tissue Sampling

The mice were euthanized by CO_2 asphyxiation. The small intestines were excised and separated from the stomach, cecum and large intestine. The length of the small intestine was measured and 4 equal sections were cut. The portion representing the distal ileum was flushed with PBS buffer and it was Swiss rolled into a cassette and placed in 10% Phosphate Buffered Formalin (PBF) as a fixative.

6.3.5.2 Preparation of Microscopic Sections

In order to preserve the cell and tissue structure, specimens were fixed with formaldehyde. A formaldehyde solution (final concentration, 10% [*wt/vol*] paraformaldehyde) in phosphate-buffered saline (130 mM sodium chloride, 10 mM

sodium phosphate buffer, pH 7.2) was used. The material was then incubated overnight in the refrigerator (4°C) for fixation. After fixation, the tissues were immersed in 15% sucrose/PBS (~x10 volume) at 4°C with gentle rocking for 4 hours. Overnight incubation in 30% sucrose/PBS (~x10 volume) at 4°C with gentle rocking followed. The usual protocol for preparing cryosections of tissue material includes embedding the material in OCT tissue-freezing medium (OCT Tissue-Tex [Miles, Elkhart, Ind.]. A Cryotome cryostat (ThermoElectron Corporation) was used to prepare the cryosection.
The samples were flash frozen with ice acetone after being transferred from OCT. Sections with a thickness of 6 μm were cut in the cryostat with a manual cryotome. Sections were directly picked up onto an adhesive, electrostatically charged microscope slides (Fisherbrand Superfrost Plus Microscope Slides, USA). This was done immediately after the microscope slides were introduced into the cryostat. It was important that the microscope slides be at room temperature during this procedure in order to allow adhesion and smoothing out of the thawing microtomic sections. Subsequently, the mounted tissue holder was incubated at −22°C for 1 h (precooling) in the cryostat. Slides were then stored at −20°C prior to immunostaining.

6.3.5.3 Polyp Scoring

Polyp scoring was performed by a person blinded to the treatment. The lesions observed were divided into two categories mostly based on the size of the lesion: gastrointestinal intraepithelial neoplasia (GIN)(<1mm) and adenoma (>1mm). The standards for the histological assessment were established from the MMHCC-sponsored symposium and are detailed on the MMHC web site: (http://emice.nci.nih.gov/emice/mouse_models/organ_models/gastro_models/murine_intestinal_neoplasia/mo dels_colorectal_cancer).

6.3.6 Immunohistochemistry

6.3.6.1 CD8, Mac-1 Staining

Immunostaining was performed by the avidin-biotin complex technique using the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, California, USA). Briefly, 4 μm thick cryostat sections were fixed in ice cold acetone and rehydrated in 10 mM sodium phosphate, pH 7.5, 0.9% saline (PBS). Non-specific binding sites were blocked with normal serum from the kit for 10 minutes. 3% hydrogen peroxidase treatment was applied to samples for 2 minutes. Samples were rinsed with PBS buffer and incubated with 2% normal serum in PBS to avoid background staining. The slices were incubated with the biotinylated antimouse CD8a monoclonal antibody or the biotinylated antimouse Mac-1 monoclonal antibody, both from Cedarlane (Hornby, Ontario, Canada). The slices were washed in TBS and incubated with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). The slides were again washed in TBS, and the staining was developed with 3,3'-diaminobenzidine peroxidase (DAB) substrate kits of Vector Laboratories or Zymed Laboratories (South San Francisco, CA) for CD8 and Mac-1 analysis, respectively. All of the kits were used according to the manufacturers' instructions. The slides were counterstained with hematoxylin and methyl green for CD8 and Mac-1 analysis, respectively. They were mounted with VectaMount AQ mounting medium and VectaMount mounting medium (VectaMount AQ, Vector Laboratories, Inc.) for CD8 and Mac-1 analysis, respectively. Negative control samples were produced by incubating the samples in TBS plus 1% BSA without either of the biotinylated primary antibodies.

6.3.6.2 In Situ Apoptotic Cell Labeling Using the Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Method

Deparafinization of tissues was performed using xylene for 10 minutes (x3 changes), and was followed by rehydration in ethanol baths of descending ethanol concentrations; 100% EtOH (5 mins), 90% EtOH (2mins), 80% EtOH (2mins), 70% EtOH (2mins) and 30% (2 mins) followed by $_{d}H2O$. Next, the slides were suspended in PBS buffer for 30 mins at RT. Subsequently, tissues were digested for 30 minutes with Proteinase K (Roche). The TUNEL method was carried out according to manufacturer's instructions (Roche). Briefly, slides were rinsed with PBS twice. 450 μL of label solution was added to 50 μL enzyme solution and mixed to equilibrate components. 50 μL of reaction

mixture was added onto each slide and the sections were incubated for 60 mins at RT in a humidified atmosphere chamber in the dark. Lack of TdT in the TUNEL mix completely abolished labeling under all working conditions and therefore served as TUNEL negative control. Tissue sections were analyzed in a drop of PBS buffer under a fluorescence microscopy at an excitation wavelength of 450 nm and detection range of 515-565 nm. The photomicrographs were obtained. Further, signal conversion was performed using converter-POD included in the kit. 50 μL of it was added onto each slide and incubated for 30 mins at RT in a humidified atmosphere chamber in the dark at RT. Slides were rinsed 3 times with PBS and 50 μL of 0.05% 3-3'-diaminobenzidine tetrahydrochloride (DAB) substrate was added onto each slide and incubated for 10 mins at RT. Next, slides were washed with PBS buffer 3 times and counterstained with hemotoxylin and eosin for 45-50 seconds each. The samples were mounted in an aqueous solution (VectaMount AQ, Vector Laboratories, Inc.) and covered with a coverslip to analyze under light microscope.

6.3.7 Microscopy

A Nikon Eclipse TE2000U inverted phase contrast microscope system was used to scan sections subjected to immunohistochemical staining. Sections were also viewed and photographed using a digital Nikon camera DXM1200F. An oil immersion lens and Motic Images 1.2 software was used to view Mac-1 stained slides.

6.3.8 Statistical Analyses

The Statistical Analysis System (SAS Enterprise Guide 4.1 [4.1.0.471] by SAS Institute, Cary, NC, USA) was used to analyze the data. Data were expressed as means ± SEM. Differences in cytokine levels were analyzed statistically by ANOVA Mixed Models. Data were considered significant at $p<0.05$.

6.4 Results

6.4.1 Animal Body Weights

Using the multi-step preparation procedure described above we obtained spherical alginate microcapsules with a narrow size distribution of 433±67 μm and high homogeneity. They were able to retain *L. acidophilus* bacterial cultures. Using optimal settings each microencapsulation yielded a consistent bacterial cell load of approximately 10^{10} cfu/mL. Bacterial cells were able to survive during the encapsulation process and grow normally (data not shown). After an acclimatization period of one week, the mice were randomly assigned using a block design into 3 groups, each consisting of 7 animals. Body weights were recorded on a weekly basis. Results show that all animals gained weight steadily up to week 13 in control group (23±0.5g) and week 14 in treatment group (23±0.5g). The treatment animals maintained constant weight until 20 weeks of age. However, control group animals maintained a constant weight only till week 16 after which there was a constant drop up to week 20 (20±0.6g) (data not shown).

6.4.2 IL-6 and IL-12 Levels in the serum

Results show the average levels of anti-inflammatory interleukin-6 (IL-6) were 22.63±0.93 for the control group and 13.80±1.15 pg/mL for the treatment group at the time of sacrifice on week 15 (Figure 6.1a). IL-6 expression levels in the treatment group were significantly reduced with the respect to the control group on week 7, 11 and 15. The IL-6 concentration for the treatment group reached a peak at week 7 after which the levels decreased till week 15. In contrast, the IL-6 concentration among control animals increased gradually throughout the treatment period. The release of interleukin 12 into blood results show that the average levels of proinflammatory cytokines IL-12*p*40 were not statistically significant between control animals and treatment group animals during the length of the experiment ($p>0.05$) (Figure 6.1b).

6.4.3 Fecal Bile Acid Analysis

To determine the effect of microencapsulated probiotic bacteria on luminal bile acids, the levels of bile acids in samples of feces from individual cages of each group

were measured. Using a bile acid standard with a concentration of 100-µmol/L, correlation of the determinant factor (R^2) of 0.9815 was obtained and used in this experiment. There was a constant drop in fecal bile acid observed in both groups (Figure 6.1c). After 15 weeks of administering treatment daily, the greatest bile acid reduction was observed in treatment group animals receiving microencapsulated *L. acidophilus* bacterial cells in 0.85% saline, it was 33±3.70 (µmol/g fecal sample/100g BW). The untreated animals were found to have a concentration of 91.5±4.38 (µmol/g fecal sample/100g BW) of bile acids at the time of sacrifice.

The averaged total fecal bile acid values with their respective groups and *p* values are presented in Figure 6.1d. Results show an average decrease of 122.93±56.49 (µmol/g fecal sample/100g BW) in treatment group compared to control animals 161.95±19.04 (µmol/g fecal sample/100g BW) receiving no treatment.

6.4.4 Luminal IL-12, IgA, TNF-α and IFN-γ Levels

Within the treatment group, luminal IL-12 concentrations were lowest in the jejunum (13.61±11.57 pg/mL) and the highest in the proximal ileum (43.55±8.97 pg/mL). In contrast, among the control group, luminal IL-12 concentrations were lowest in the duodenum (8.97±8.48 pg/mL) and highest in the distal ileum (22.79±4.76 pg/mL) (Figure 6.2a). The concentration range of luminal IgA was observed to be between 28.68±3.48 and 33.07±3.29 (pg/mL) and therefore it did not differ within each intestinal section between the groups (Figure 6.2b). The exception was the control animal group where the IgA concentration in the duodenum was 22.06±4.45 (pg/mL). The concentration of luminal TNF-α was the highest in the distal ileum of the control group, 30.12±9.08 (pg/mL). In contrast, treatment group animals had much lower concentration in all intestinal sections (Figure 6.2c). Furthermore, control group animals had a significantly higher concentration of luminal IFN-γ (151.52±22.86, 163.46±18.77, 156.15±17.80 and 146.76±19.09 pg/mL in duodenum, jejunum and proximal and distal ileum, respectively) when compared to the treatment group (103.77±12.07, 92.42±7.05, 107.21±17.95 and 106.06±6.18 pg/mL in duodenum, jejunum and proximal and distal ileum, respectively) (Figure 6.2d).

6.4.5 Adenoma Reduction in the Treated Animals: Classification and Histology

The number of adenoma, low grade dysplasia, high grade dysplasia, and gastrointestinal intraepithelial neoplasias (GIN) were scored in treated and control groups in both the small and large intestine and the data are included in Figure 6.3c. The numbers were averaged per animal in a given group.

In the small intestine, there were 3.5 adenomas and 10 GIN in the control group versus 1 adenoma and 9 GIN in the treatment group. In the large intestine of control animals, there were 0.5 adenomas and 2.25 GIN versus 0.2 adenomas and 0.2 GIN in treatment group. The total number of tumors per animal as well as number of adenomas and GIN's alone was statistically lower in treatment group ($p<0.05$). This is 36.62% decrease in total number of lesions in treatment receiving animals when compared to control group animals.

Histopathology further revealed analysis of tumors. There was considerably lower polyp occurrence in treated animals. Figure 6.3 displays representative photographs obtained from control and treatment animals. Photograph 6.3a shows multiple lesions found in untreated animal classified as papillary adenomas with high grade of dysplasia. Figure 6.3b reveals neoplasia found in treated animals with much higher nuclear to cytoplasmic ratio. The displayed lesions were characterized by a low grade of dysplasia and a smaller polyp size.

6.4.6 CD8, Mac-1 Immunohistostaining

Staining of frozen-OCT cross sections with CD8+ T cells shows a higher cell population in control group (Figure 6.4). Similarly, CD8+ T cells were identified in all parafinized tissue samples and were more abundant in control groups than treated animals (Figure 6.5a). Mac-1 cells were found in all adenomas (Figure 6.5c). The Mac-1 cells were located in the lamina propria area of the villi. In addition CD8+ T cells were more numerous than Mac-1 cells. A minimum of 4 villi/sample was counted for positively stained cells.

6.4.7 The TUNEL Method

Protease digestion was necessary to make the DNA accessible for incorporation of nucleotides and was done with proteinase K. Labeling of cells using this technique was shown to be correlated with typical morphological criteria of apoptosis. The detected cells generally exhibited a clear-cut nuclear staining and mostly showed signs of apoptotic cell death, except for some cells that were obviously at very early stages of apoptosis and therefore had minimal nuclear condensation (Figure 6.6). The highest concentration of apoptotic cells was observed in healthy tissue, localized along intestinal villi. The absence of apoptotic cells was noted within polyps except for their base and surface.

6.5 Discussion

Inflammation is a complex process involving numerous mediators of cellular and plasma origin with elaborate, interrelated biological effects. The inflammatory process distorts the colonic epithelium. The present study confirms that probiotic bacteria have immunomodulatory effect.

The Min mouse has been established as an animal model of genetic intestinal neoplasia [381] but is criticized as to its relevance to human disease [382] because its tumors are sited predominantly in the small bowel, as opposed to the colon in the human. However, detailed histological morphology of these Min mouse adenomas is a valuable tool for our understanding of polyp morphology occurring in the gut. The main objective of this study was to understand the immunomodulatory impact of probiotic bacteria *L. acidophilus* delivered in microencapsulated form to the gut on polyp formation in the small intestine of Min mice.

Diet and disease development are closely linked and have been proposed to play a role in colon carcinogenesis. The weight gain and its steady maintenance is representative of adequate food intake, proper metabolism and excretion. To study these effects, we monitored the animal's weekly body weight and recorded their food intake. The animals

in both groups exhibited a steady increase in body weight up to 12 weeks of age for control group and 14 weeks of age for treatment group. After 12th week of age, control animals maintained their weight till week 16 after which we observed a steady decrease suggestive of disease progression. On contrary, treatment animals were able to preserve their weight from week 14 onward without weight loss. According to the Animal Care Committee of McGill University and the Canadian council on Animal Care (CCAC) any animal which lost >20% of their body weight should be euthanized. At the time of sacrifice, the control animals were on an average 15% lighter than the treatment animals. This implies that the treatment had a beneficial effect on animals. The control animals, in comparison, showed a higher rate of disease progression.

Cytokines play a vital role in coordinating inflammatory responses. They are involved in responses to viral and bacterial infections, immunity and hemopoiesis. Intestinal epithelial cells express and respond to several active cytokines, including IL-1, IL-2, IL-6, IL-12, TNF-α , IFN-γ and TGF-β [376,383-387]. Inflammation is associated with epithelial cell transformation and the process of carcinogenesis. Results in the present study indicate an overall increasing level in blood plasma of IL-6 in control animals and a decreasing trend in treated animals. Similar results were obtained by measuring concentration of IL-12 in blood plasma throughout the experimental period of time. These cytokines can in turn promote T-cell responses and macrophage activation. It is known that interleukin-12 (IL-12) is a very potent antitumoral cytokine. Maximum levels of IL-12 correlate with the maximum inhibitory effect on tumor growth [388]. This indicates that a pro-inflammatory state correlates with the beneficial effect of the probiotic bacteria on the involved immunomodulatory mechanisms. The IL-12:IL-6 ratio is an effective discriminator of infectious from non-infectious state and although the overall decreasing trend was observed in both animal groups, the treatment group showed a slower progression indicating the lag phase in polyp formation. These results could be due to the beneficial action of probiotic bacteria employed in the gut acting by regulating the pathogenic bacterial growth, binding to or penetration of pathogens to mucosal surfaces, stimulating the mucosal barrier function, or altering immunoregulation (modulating the normal epithelial architecture by promoting protective molecules).

As IL-12 is a proinflammatory cytokine that enhances IFN-γ-producing T cell responses with the ability to form a link between innate and adaptive immunity by inducing IFN- γ production, we expected to obtain similar results after performing ELISA assays of this cytokine. The assay showed a higher IFN- γ concentration levels in control mice. Since IL-12 is a potent inducer of IFN- γ, this indicates that inflammatory cytokines were recruited at a larger scale in animals with higher inflammation levels.

IL-12 also enhances CD8 T cell homeostatic proliferation and provides a third signal that promotes full activation and survival of activated CD8 T cells. Recently it has been shown that it regulates the generation of CD8 cell memory which provides greater protective immunity against infection [389]**.** Results in the present study further indicate that untreated animals at the time of sacrifice yielded a greater inflammatory condition of their intestines than treatment receiving group.

CD8 cells are cytotoxic T lymphocytes which secrete molecules that recognize infected or malignant cells. The lack of intense staining of intestinal sections with CD8 in treatment group indicates decrease in T cell population which further indicates lesser local inflammation. Mac-l, also known as the integrin CDl lb/CD18, plays a critical role in monocyte adhesion. The absence of inflammation was further supported by fewer stained cells found in treatment group. Mac-1 cells were observed in all adenomas, and they were often relatively abundant which indicates increased proliferation of T cells in untreated animals. Overall, CD8 + T-cells were more numerous than Mac-1 T-cells.

Tumor necrosis factor and lymphotoxin-α were isolated more than 10 years ago, on the basis of their ability to kill tumor cells in vitro and to cause hemorrhagic necrosis of transplantable tumors in mice [390]. TNF-α was originally identified as a mediator responsible for endotoxin-induced tumor necrosis and was utilized for the treatment of patients with advanced localized solid tumors [391]. Antitumor properties of TNF rely on direct induction of apoptosis in cancer cells as well as on its antivascular effects [392] however, blood vessel necrosis and induction of a strong inflammatory response limits the clinical use of this cytokine. In the study by Moore *et al.* TNF-α–deficient mice developed fewer tumors than wild-type mice when exposed to several types of carcinogens [393]. In the present study at the time of sacrifice, treatment group animals had lower concentrations of TNF-α compared to control in all intestinal segments.

Murine IFN-γ is a regulatory protein produced by activated NK cells and CD4+ TCR $\alpha\beta^+$, $CD8^+$TCR $\alpha\beta^+$, and TCR-γ σ^+ T cells. IFN-γ has an intricate and complex role in the development of both innate and adaptive immune responses as it promotes robust inflammation that is capable of clearing many bacterial infections [394,395]. In addition, IFN-γ can upgrade the number of lymphoid cell functions including the anti microbial and anti tumor responses of macrophages, NK cells, and neutrophils. Increased production of IFN-γ and IL-12 is seen in Crohn's Disease tissue [396,397]. IFN-γ has been associated almost invariably with inflammatory responses; therefore it was expected to see higher concentrations of this cytokine in untreated animals. In detecting concentration levels in different segments of the small intestine, we obtained similar values in all 4 sections: duodenum, jejunum, proximal and distal ileum.

Secretory IgA (sIgA) released from the mucosal surface has a central role in the mucosal immune system in inhibiting the adherence of pathogenic bacteria and neutralizing biologically active antigens (e.g., bacterial toxins, viruses and enzymes) [398]. Luminal digesta obtained from the intestines were used to give an indication of IgA levels in the gastrointestinal tract at the time of sacrifice. Relatively similar and considerably high concentrations were obtained in the jejunum and proximal and distal segments of ileum. However, in the duodenum, treatment animals had a greater amount of IgA. As previously noted bacterial interactions with either IgA or mucin, secreted on mucosal surfaces, are important for survival of bacteria within the lumen of the gut. IgA mediates the formation of biofilm in the gut, which further facilitates the growth of beneficial microbes. The abundance of IgA in the gut has a protective effect; in disease state it prevents the opportunistic infections by pathogenic organisms as well as provides beneficial effects to the host [399]. Thus the delivery of naturally occurring bacteria, *L. acidophilus*, and not genetically modified strains, acts as a beneficial component of natural microflora.

Fecal occult blood is one of many signs that may indicate the presence of polyps in the colon or rectum, or cancer. The fecal occult blood test (FOBT) is a common and cheapest method of screening. Rectal bleeding was observed in animals on arrival and the test was repeated at the end of experiment to verify whether the treatment had an effect in decreasing the bleeding from the GI tract. Occasional bloody stools were observed in

some cages towards the end of the experiment, but no differences were observed in the incidence of bloody stool among the groups. No liquid stools were observed during the experimental period. The Hemoccult SENSA qualitative test detected the presence of blood in the feces in all animal cages at the end of the treatment, but did not reveal any significant differences among groups. Although this test is more sensitive than other occult blood tests, it is considered to be a qualitative screening rather than decisive test; therefore it cannot provide conclusive evidence of the presence or absence of gastrointestinal bleeding or pathology.

More than 95% of the conjugated bile acids are reabsorbed in the small intestine, mostly in the terminal ileum. The bile acids that escape absorption in the ileum (2%–3% per cycle) are metabolized by colonic bacteria. Part of these secondary bile acids (about 30%) are absorbed in the proximal colon and enters the enterohepatic circulation, and part (about 70%) is excreted in the feces [400]. High levels of bile acids have been linked to oxidative stress, DNA damage, and mitochondrial membrane instability which lead to cancer formation [401]. Past studies have indicated that bile acids contribute to colonic carcinogenesis by disturbing the fine balance between proliferation, differentiation, and apoptosis in colonic epithelial cells [402]. Therefore it was important to assess the effect of our treatment on animals and compare it with the control mice. Previous testing in the lab revealed that the *L. acidophilus* strain used in this study had no significant BSH activity (data not shown).Therefore we postulate that this trend could result in the replacement of BSH positive colon flora with one that exhibits lesser BSH activity, over the course of the experiment. In our study the treatment resulted in a significant lowering of fecal bile acid concentration. Thus, the overall decreasing trend may indicate that a small amount of primary bile acids were deconjugated to secondary bile acids.

After investigating histology slides of both small and large intestines and scoring them for presence of polyps we concluded that all tumors were adenomas. The number of adenomas found in the small intestines of the treatment group indicates that the probiotic formulation had an attenuating effect on polyp growth. This was valid in both adenomas and GINs amounts. Most of the adenomas found in the untreated group exhibited a high grade of dysplasia. This is probably due to the fact that the colonic wall, including the mucosa and the submucosa, is much thinner than that of other organs. Macrophage

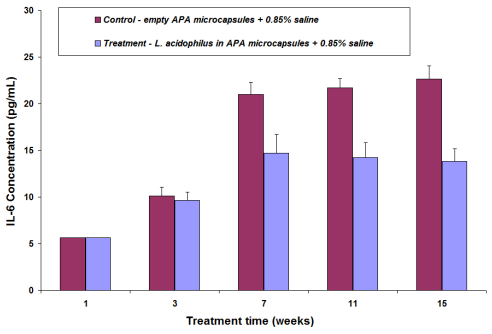
induction at mucosal surfaces is an early trigger of an inflammatory cascade that leads to destruction of the intestinal wall. In addition, some grossly normal colonic mucosas displayed atypical hyperplasia. In general, the treatment animals had a lower incidence of polyps, both in the small and large intestines. The nuclear/cytoplasmic ratio was greater and on average the villi were better defined.

Terminal deoxynucleotidyl Transferase-mediated dUTP nick end labeling (TUNEL) is an in situ method for detecting the 3'-OH ends of DNA exposed during the internucleosomal cleavage that occurs during apoptosis. Growing evidence suggests that apoptosis is relevant in some infectious diseases by regulating immune responses and, less commonly, by directly affecting microbial proliferation [403,404]. As expected, we observed a high occurrence of cell turnover in healthy, well defined villi and substantially lesser fluorescence intensity in tissue bearing polyps. This observation further proves that the process of apoptosis is suppressed in inflamed tissue which allows cells with precancerous genetic mutations to live and grow into a cancer instead of programming cell death.

One of the important aspects of the above study is that a daily dose of microencapsulated bacterial cells exhibited a beneficial effect on the intestinal inflammatory state in Min mice evidenced by reduced number of adenomas. Our study also suggests that polyp formation may occur independently of specific segments in the small intestine. Since Min mice are genetically predisposed to intestinal tumors, this study does not address whether probiotic bacteria delivery might suppress tumorigenesis before this stage in humans or other animal models. Nevertheless, this probiotic formulation will be valuable not only to study pathogenetic mechanisms involved in the disease process but also to explore possibilities for prevention and for therapeutic intervention.

6.6 Acknowledgements

We acknowledge post graduate scholarship from NSERC to A. Urbanska, post graduate scholarship from CIHR to J. Bhathena and post graduate scholarship from NSERC to C. Martoni. We also acknowledge Micropharma for financial support to S. Prakash.



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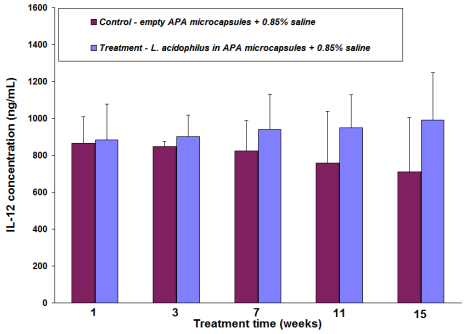

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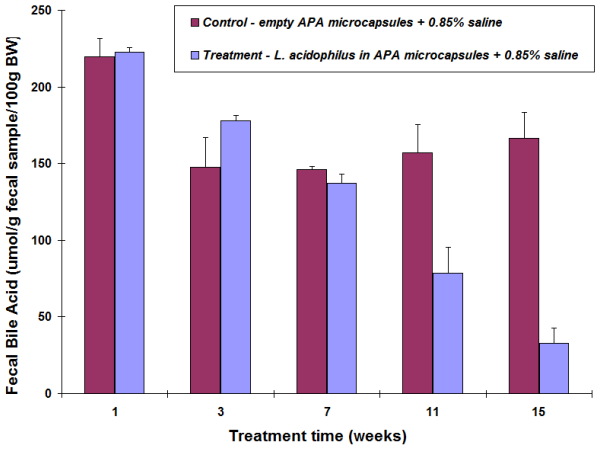


(C)

	Average Average Total Fecal Bile acid (umol/g fecal sample/100g BW)	Repeated measures ANOVA using Mixed Models Analysis *p* values
Control (C)	161.95±19.04	0.026
Treatment (T)	122.93±56.49	0.017

(D)

Figure 6.1: The effect of treatment on IL-6 (A) and IL-12 (B) concentrations in plasma blood and total fecal bile acid levels (C). Data represent the mean ± SEM of expression levels per group, *n*=7. Comparison of average total fecal bile acid per group and their *p* values (D).

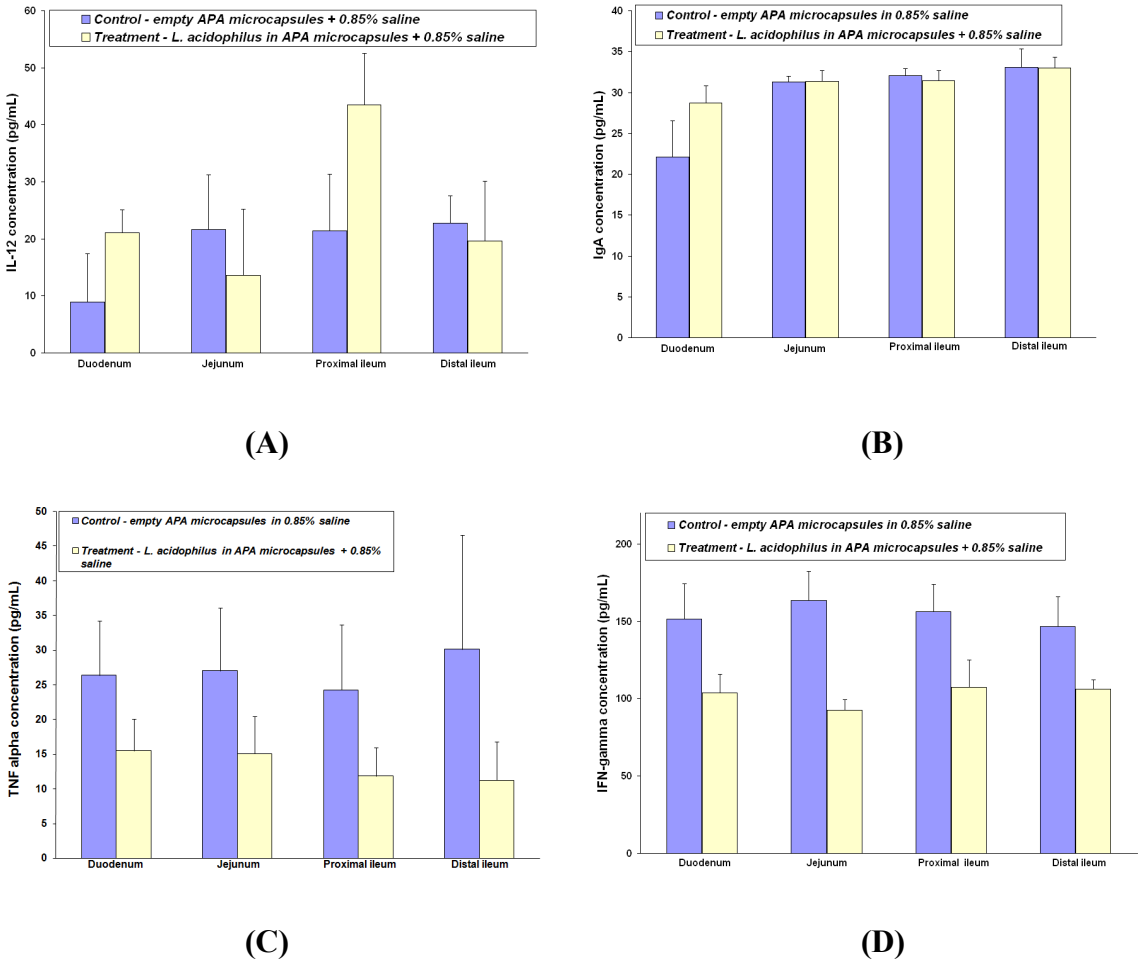
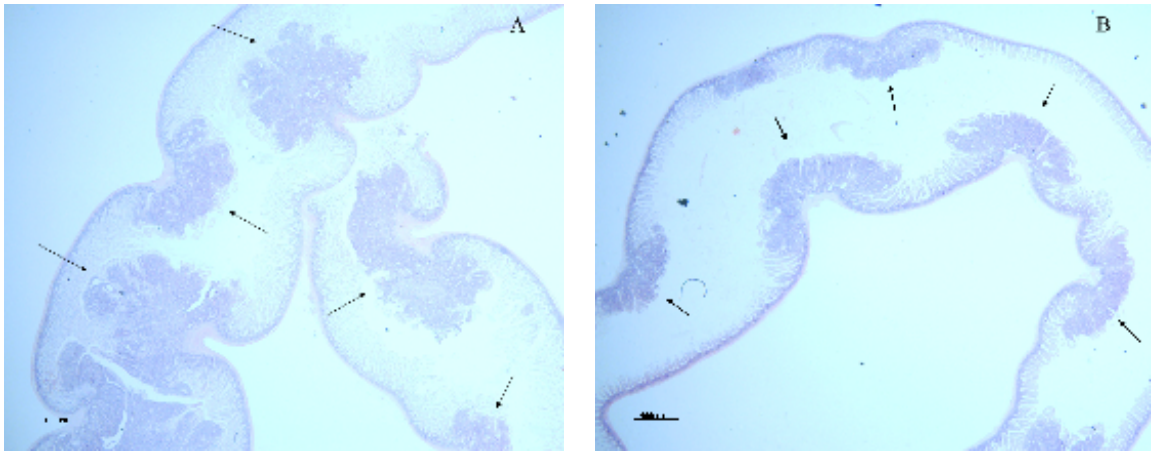


Figure 6.2: The effect of treatment on luminal IL-12 (*A*), IgA (*B*), TNF-α (*C*) and IFN-γ (*D*) concentrations in small intestine. Data represent the mean ± SEM of expression levels per group, *n*=7.

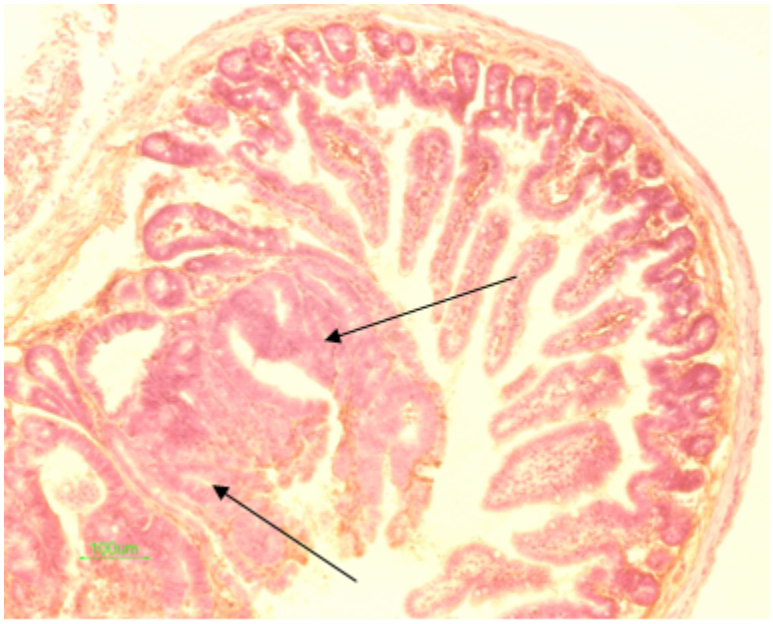


(A) **(B)**

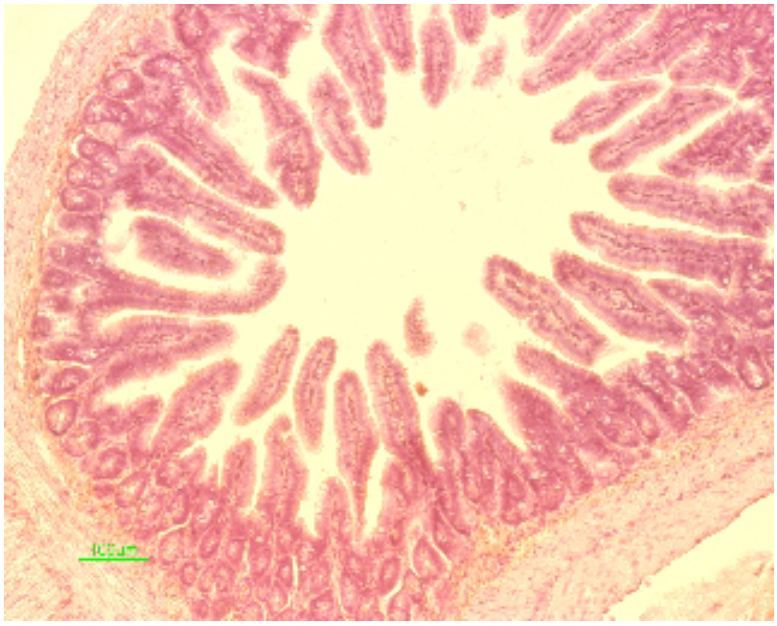
Group (*n*)	No. tumors per mouse			No. mice with adenoma			No. mice with GIN		
	Small intestine	**Large intestine**	**Total**	**Small intestine**	**Large Intestine**	**Total**	**Small intestine**	**Large Intestine**	**Total**
Control (4)	16 13 13 12	4 2 2 3	20 15 15 15	14	2	26	40	9	39
Avg/ animal	13.5±1.73	2.75±0.95	**16.25±2.5**	3.5	0.5	6.5	10	2.25	9.75
Treat. (6)	11 2 13 12 8 14	0 1 0 0 0 1	11 3 13 12 8 15	6	1	7	54	1	55
Avg/ animal	10±4.47	0.3±0.52	**10.3±4.27**	1.0	0.2	1.17	9.0	0.2	9.17

(C)

Figure 6.3: Histological longitudinal sections showing intestinal changes in *Apc*Min mice. (*A*) Multiple lesions found in control animal. Arrows point to papillary adenomas with high grade of dysplasia. Original magnification 16*X*. (*B*) Multiple neoplasias (microadenoma), sessile with low grade of dysplasia (arrows) (Sessile adenomatous polyps) found in a treatment mouse. Note the increased Nuclear / Cytoplasmic ratio. Original magnification 16*X*. All tissues were stained with hematoxylin and eosin. *(C)* Table comparing adenoma enumeration and classification.



(A)



(*B*)

Figure 6.4: Immunohistochemical staining of frozen-OCT cross sections of small intestines stained with CD8+. Staining with CD8 antibody shows positive cells brown stained *(A)* the cross-section of control animal, arrows point to cell-depleted epithelium and crypt abscesses leading to formation of adenoma, *(B)* the cross-section of treated animal with well defined and not obstructed villi. Sections were counterstained with hematoxylin. Original magnification 200*X*.

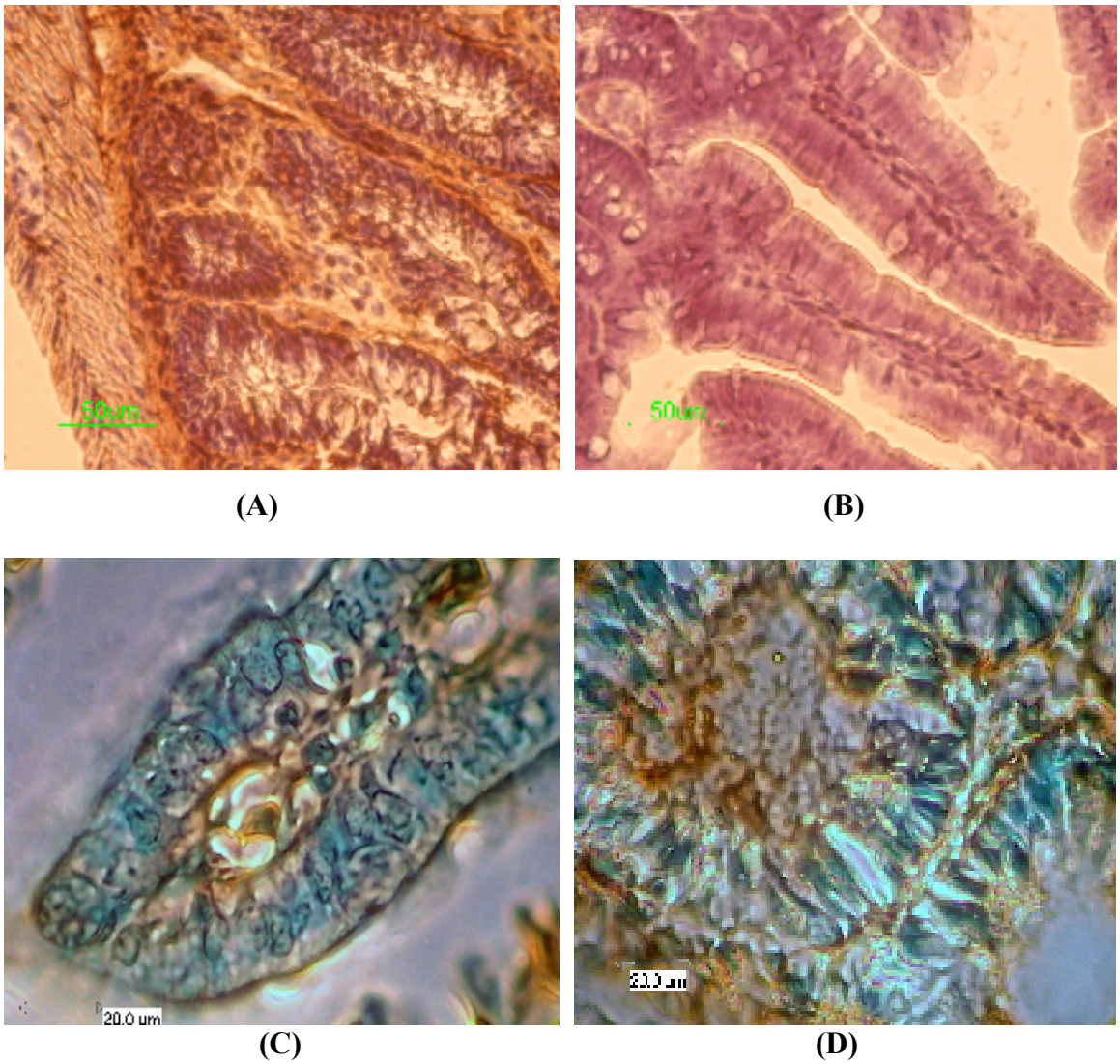
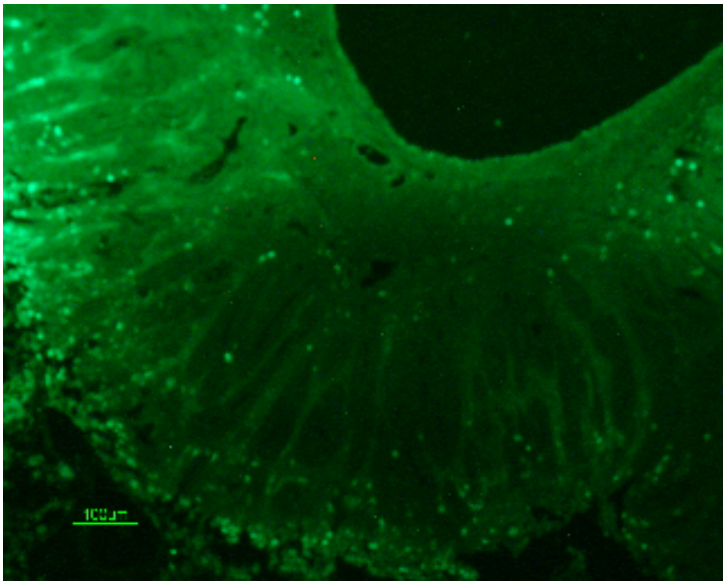


Figure 6.5: Immunohistochemical analysis of samples ileal mucosa stained for (*A*) $CD8^+$ the positive cells show brown staining, (magnification 400*X*); (*B*) negative control sample without the primary antibodies, (magnification 400*X*); (*C)* Mac-1^+ cells the positive cells show black staining, (magnification 1250*X*); *(D)* negative control sample without the primary antibodies, (magnification 1250*X*).



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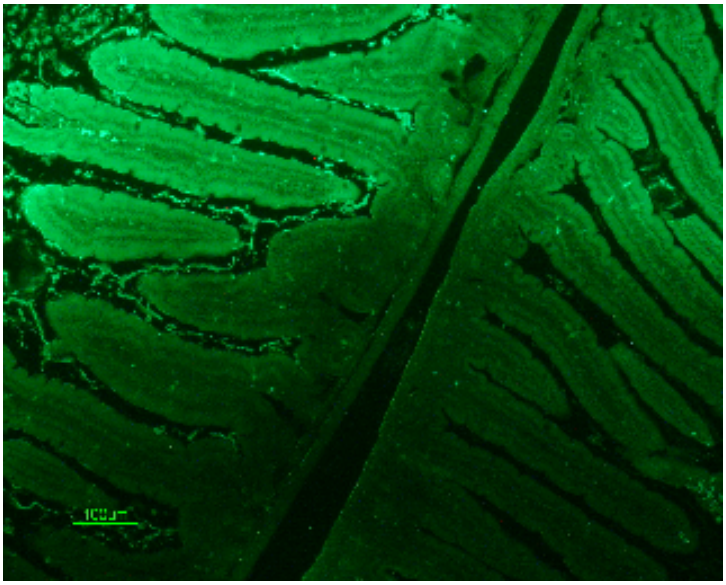

(B)

Figure 6.6: Immunofluorescent detection of apoptotic cells in the small intestines of mice using TUNEL with Proteinase K pretreatment. TUNEL positivity (green fluorescence) *(A)* image of polyp found in a control animal showing apoptotic cells protruding into the lumen of intestine, proliferating cells at the base and within the tumors are not fluorescent *(B)* image of treatment animal showing well defined villi lined with non-proliferating cells undergoing apoptosis (magnification 100*X*).

CHAPTER 7
Original paper 5

Suppression of tumorigenesis in *Apc*Min mice: down-regulation of inflammatory cytokines by administration of microencapsulated probiotic yogurt formulation

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Original paper to be submitted to: ***BMC Microbiology***

Preface

The main objective behind this study is to further illustrate the impact of oral administration of APA microencapsulated probiotic yogurt formulation.
In this in vivo study of 17 week duration, two treatment formulations are being evaluated: APA microencapsulated bacterial cells in probiotic 2% M.F. yogurt and 0.85% saline solution. The following inflammatory cytokines are measured: IL-1β, IL-6, IL-12, PGE_2, Thromboxane B2, TNF-α, IFN-γ and CRP. The samples are obtained from intestinal digesta and blood collection throughout the treatment period. The biomarker studies are carried out using ELISA and other methods. In order to complete the analysis and verify the potential of probiotic formulation, the expression of COX-2 is being compared in control and treatment group animals. COX-2 play important role in tumor invasiveness. The Western Blot method was performed to qualitatively show the expression of this enzyme in homogenized distal ileum of small intestine tissues in control and treatment animals and Image J was used to quantify its expression.

7.1 Abstract

The present study describes a novel microencapsulated probiotic yogurt formulation showing high ability to suppress the intestinal inflammation in *Apc*Min mice. We assessed its anticancer activity by screening biomarkers such as interleukin-1, 6, and 12 as well as measured the secretory levels of tumor necrosis factor-alpha, interferon-gamma, prostaglandin E_2, and thromboxane in the digesta obtained from the duodenum, jejunum, proximal and distal segments of the ileum. Treatment group animals showed consistently lower pro-inflammatory cytokines' levels when compared to control group animals. The concentrations of interleukin-12 found in serum in control and treatment group animals were significant towards end point of the experiment. At the time of sacrifice the average levels were 46.58±16.96 pg/mL and 158.58±28.56 pg/mL for control and treatment animals, respectively.

Plasma C-reactive protein was determined by enzyme-linked immunosorbent assay and a significant change was observed: 81.04±23.73 ng/mL in control group as compared to 64.21±16.64 ng/mL in treatment group. Western blots showed a 71%

downregulation of Cox-2 protein in treatment group animals compared with control. Oral administration of the probiotic formulation ameliorated biochemical evaluations directly related to intestinal inflammation whereas the saline suspension was ineffective in this regard. The novel probiotic yogurt formulation described in the present paper, upon further characterization, can be developed into a useful probiotic therapeutic aimed at the treatment of patients suffering from gut related disorders.

Key words: Microencapsulation, Probiotics, Cyclooxygenase, Cytokines, Colon Cancer.

7.2 Introduction

The burden of colon cancer in Western countries is overwhelming, amounting to 50,000 deaths per year in USA alone [405]. Much effort is being devoted to the development of effective therapies for this disease as well as to its prevention. Inflammation plays a major role in pathogenesis of colorectal cancer and its evaluation is a powerful tool in screening and understanding the key components that lead to this complex disorder. Most inflammation occurs in areas with the highest density of intestinal bacteria: the gastrointestinal tract.

Cyclooxygenase (COX) is an enzyme which catalyzes the first step in the formation of prostaglandins (PGs), the conversion of arachidonic acid to PGH_2, followed by the metabolism of PGH_2 to biologically active end products, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, or thromboxane A_2 (TxA_2) via specific synthases [406]. Two cyclooxygenase isoforms, COX-1 and COX-2, have been identified. Cox-2 is critical for the development of colorectal neoplasia [407]. COX-2 inhibitors can reduce intestinal inflammation leading to tumorigenesis [408] and are therefore used to treat Familial Adenomatous Polyposis (FAP) patients [409] and patients with colorectal cancer [410]. Accumulating evidence have shown that prostaglandin E_2 (PGE_2), the main product of cyclooxygenase-2 (COX-2) activity, can promote a number of molecular mechanisms involved in colorectal carcinogenesis [411] in particular tumor cell proliferation and angiogenesis [412-415]. C-reactive protein (CRP) is an acute-phase systemic protein produced primarily in the liver in response to stimulation by interleukin 6 (IL-6) [416]. In addition to studies which show consistency in demonstrating an increased risk of mortality due to inflammation and

subsequent cancer development, CRP and IL-6 have been shown to be associated with total and noncardiovascular mortality [417-420]. Tumor necrosis factor-α (TNF-α) and interleukin 1-β (IL1-β) seem to play an important role in ulcerative colitis (UC) in relevant experimental models [421].

The recognition of the compelling association between intestinal inflammation leading to such disorders as Crohn's Disease (CD), ulcerative colitis (UC) and colon cancer has led to an abundance of studies investigating the therapeutic potential of altering luminal bacteria using probiotics and/or probiotics.

Probiotics are defined as living organisms in food and dietary supplements which, upon ingestion, improve the health of the host beyond their inherent basic nutrition [422]. Probiotic bacteria have beneficial effects on the intestinal epithelia both directly and indirectly, including enhanced barrier function, modulation of the mucosal immune system, production of antimicrobials, and alteration of the intestinal microflora [423]. There is a vast interest in testing probiotics performance in gastrointestinal disorders both in animal studies and clinical trials [424,425]. Data is now emerging which suggests that probiotics are capable of preventing relapse of chronic intestinal inflammation and have beneficial contributions in disorders such as diarrhea, gastroenteritis, irritable bowel syndrome and inflammatory bowel disease [426,427].

Microencapsulation in specialized ultra-thin semi-permeable polymer membranes successfully protects live bacterial cells and offers protection to bacteria from the harsh gastrointestinal environment during transit [428,429]. It also limits stimulation of the host immune response, as well as minimizes risks of systemic infections, the replacement of the normal intestinal flora and gene transfer [430].

In this study we investigate the potency of microencapsulated probiotic bacterial cells contained in a yogurt formulation in reducing intestinal inflammation in *Apc*Min mice. We provide evidence that the daily gavage of the probiotic formulation to mice reduces expression of Cox-2 as well as lowers plasma C reactive protein (CRP) levels. We have quantified the levels of secretory cytokines IL-1β, IL-6, IL-12, PGE_2, TNF-α, IFN-γ and Thromboxane B2 in ileal contents. In addition, animals' body weight along with their food consumption profile was obtained.

7.3 Materials and Methods

7.3.1 Materials

7.3.1.1 Chemicals

Sodium alginate (low viscosity), poly-L-lysine hydrobromide (MW=27,400) and calcium chloride (A.C.S. reagent) were purchased from Sigma-Aldrich, Canada. Difco ™ Lactobacilli MRS AGAR and Difco ™ Lactobacilli MRS BROTH were purchased from Becton, Dickinson and Company Sparks, USA. Liberty plain yogurt 2% M. F. containing active *Acidophilus* and *Bifidus* cultures was procured from a local grocery store.

7.3.1.2 Bacteria and Culture Conditions

Lactobacillus acidophilus cells were purchased from ATCC (314) (Manassas, VA) and were cultivated and serially propagated in the MRS medium before experimental use. Incubations were performed at 37°C in a Professional Sanyo MCO-18M Multi-Gas Incubator under anaerobic conditions (1-2% CO_2, Atmosphere Generation System AnaeroGen™; Oxoid Ltd., Hampshire, England). Bacteria were harvested after 20 hours of the 3rd passage for encapsulation.

7.3.1.3 Microencapsulation method

The bacterial strains were microencapsulated into Alginate-Poly-L-Lysine-Alginate (APA) membranes. All membrane components were filter sterilized through a 0.22 μm Sterivex-GS filter (Millipore, Bedford, MA, USA) prior to use. Grown cultures were centrifuged at *3000* xg for 15 minutes at 25ºC and the supernatant broth was decanted. The pellet of wet cells was weighed and suspended in 0.85% saline, pooled and slowly added to a gently stirred sterile 3.3% sodium alginate solution (final concentration adjusted to 1.65% with 0.85% saline). The entire procedure was performed under sterile conditions in a Microzone Biological Containment Hood (Microzone Corporation ON, Canada) and all solutions were autoclaved with the exception poly-L-lysine which was 0.22 μm sterile-filtered prior to usage. APA microcapsules were prepared aseptically using an Inotech Encapsulator® IER-20 (Inotech Biosystems Intl. Inc. Switzerland).

Freshly prepared microcapsules were washed twice with 0.85% saline and stored at 4°C. Parameters for microencapsulation were as follows: gelation time in $CaCl_2$ – 30 minutes, coating time – 10 minutes, nozzle diameter - 300 μm, vibrational frequency – 918 Hz, voltage > 1.00kV and current 2 amp.

7.3.1.4 Preparation of probiotic formulation

APA microcapsules loaded with *L. acidophilus* bacterial cells were carefully mixed with Liberty plain yogurt 2% M.F. and 0.85% saline in the proportions of 3:1, respectively. Empty APA microcapsules were suspended in 0.85% saline using the same formulation and stored at 4°C until use.

7.3.2 Animals

Male C57BL/6J-*Apc*$^{Min/+}$ mice[338] 5 or 6 weeks old were obtained from The Jackson Laboratory (Bar Harbor, ME). The animals were housed in the McIntyre Medical Sciences Building Animal Care Facility in a room with a 12-hour light-dark cycle and controlled humidity and temperature. The mice were maintained in a barrier facility. They were allowed sterile water and the laboratory rodent diet 5001 from Purina Land O'Lakes *ad libitum*. Overall health of the animals was monitored daily. The animal use protocol was approved by the Animal Care Committee of McGill University and animals were cared for in accord with the Canadian Council on Animal Care (CCAC) guidelines.

7.3.2.1 Experimental design

Upon arrival, animals were kept in a sterile environment in individual ventilated cages (IVC) which filter the air with HEPA filters (μ size). The cages, food, water bottles, etc. were autoclaved. Animals were randomly placed in the cages and allowed one week of acclimatization. Based on initial serum IL-6 values the animals were ranked and assigned to groups according to a randomized block design. The mice were separated into two experimental groups: Control (n=24) - animals were gavaged 0.3 mL of 0.85% saline solution and treatment animals (n=24) were gavaged with APA microencapsulated

L. acidophilus bacterial cells blended in 2% M.F. yogurt. Animals were weighed individually every week and their food consumption was weighed per cage of 4 animals. Blood was collected from the saphenous vein was performed every 4 weeks. Blood was separated using 5000xg at 4°C for 10 minutes.

7.3.2.2 Luminal Digesta

At the time of sacrifice, the small intestine of each animal was measured and cut into 4 equal segments, approximately representing duodenum, jejunum as well as proximal and distal ileum. Each segment was flushed with cold D-PBS buffer (Gibco) and the contents were collected and frozen at -85°C. Before analyses, the digesta samples were thawed and treated with 1.0% BSA-50 mM Tris buffer (pH 7.5) for 60 min at room temperature to separate the food matrix and cellular material. The samples were then centrifuged at 50,000 x*g* for 15 min. The supernatants were stored at -85°C and used later for measurements.

7.3.3 Analytical methods

7.3.3.1 Quantification of IL-1β, IL-6, IL-12, PGE_2, Thromboxane B2, TNF-α, IFN-γ and CRP expressions using ELISA

IL-1β, IL-6 and IL-12 were quantified using kits from Biosource, Invitrogen, USA according to manufacturer's recommendations. PGE_2 was measured using a competitive enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) as described previously [431]. Briefly, 96-well plates were precoated with the capture Ab (goat anti-mouse Ab). 100 µL enzyme immunoassay (EIA) buffer was loaded to non-specific binding (NSB) wells. 50 µL EIA buffer was loaded to maximum binding (B_0) wells. 50 µL PGE_2 standards were loaded into appropriate wells. 50 µL of samples or standards and incubated with 50 µL of PGE_2 tracer and 50 µL of PGE_2 mAb overnight at 4°C. After three washes in wash buffer, 200uL of Ellman's reagent was added to the plate and allowed to incubate for 1 h for the color to develop. The Optical Density (OD) of each

well at 405nm was determined using a Perkin Elmer Victor microtiter plate reader and PGE_2 production was expressed as picograms per milligram.
Thromboxane B2 (Express EIA kit-monoclonal, Cat.No. 10004023, Cayman Chemical, Ann Arbor, MI) was measured according to manufacturer's instructions. TNF- α was measured using a competitive enzyme immunoassay (Cat.No. KMC3012, Immunoassay Kit, Biosource Int., Inc, USA) according to manufacturer's instructions. Biotin gamma rabbit anti-mouse interferon-γ was purchased from Cedarlane, (Hornby, ON, Canada) and reconstituted from sterile form to 50ug/mL with PBS solution containing 0.1% BSA. Murine IFN-γ ELISA kit was purchased from Peprotech (Rocky Hill, NJ), and used as recommended by the manufacturer. CRP was measured in plasma using a mouse CRP ELISA kit (Life Diagnostics, Inc., USA).

7.3.3.2 Immunoblotting of Cox-2

Intestinal tissue samples (flushed with cold PBS) were flash frozen in liquid nitrogen before storing at -85ºC. Frozen samples were weighed and 3 mL RIPA buffer (Santa Cruz Biotech, CA) (with PMSF in DMSO, protease inhibitor cocktail and sodium orthovanadate) was added per gram of tissue. The samples were homogenized, pooled, sonicated and centrifuged at 4ºC for 10 minutes at 10,000xg. The protein content was determined using Quant-iT protein assay kit (Invitrogen, Burlington, Canada) with bovine serum albumin (BSA) as the standard.

Twenty micrograms of total proteins, as evaluated by Quant-iT protein assay, from tissue were used for Western blot. Aliquots containing protein were fractionated on 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) at 120V for 2 hours. After electrophoresis, proteins were transferred from the gel to a nitrocellulose membrane (Whatman, Maidstone, Kent, UK) using Novex Semi-Dry Blotter (Invitrogen, Carlsbad, CA). COX-2 mouse monoclonal antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) was used as the primary antibody. Horseradish peroxidase-conjugated goat anti-mouse IgG was used as the secondary antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was then developed with chemiluminescent agents (ECL, BM Chemiluminescence Blotting Substrate (POD), Roche Diagnostics, IN) and visualized in a

Versa Doc Imaging System using software Quantity One-4.5.1 (Model 5000, Bio-Rad Laboratories (UK). Western blot images were analyzed using Image J software (http://rsb.info.nih.gov/ij/ (accessed in December 2005, NIH, USA).

7.3.4 Statistical Analyses

All results in this article are means calculated using Excel and expressed as means ± SEM or SD. Student t test was used to assess the statistical significance of the differences between test and control groups. Data was considered significant at $p<0.05$.

7.4 Results

Artificial cell microcapsules containing *L. acidophilus* were prepared using the multi-step preparation methods described and were stored at 4ºC for use in experiments. Sterile conditions and procedures were strictly adhered to during the process of microencapsulation. Results show that bacterial cells were able to survive the encapsulation process and grow normally when obtained supernatant was plated after breaking of the microcapsule membrane. The microcapsules contained, on an average, 10^{10} cfu/mL of bacteria. Freshly prepared microcapsules were spherical and opaque on account of bacterial density. Morphological studies by microscopic analysis revealed that the mean capsule diameter was 433±67 μm and high homogeneity. Bacterial cells were able to survive during the encapsulation process and grow normally (data not shown).

Food intake and body weights

Results show (Figure 7.1a) that all animals gained weight steadily up to week 16 of age in control group (25±0.5g) and in treatment group (25±1.4g). All the animals maintained constant weight until 17 weeks of age after which the weight in control group animals decreased to 20±0.4g whereas treatment group animals continued to increase in their weight up to 27±0.3g at the time of sacrifice ($p<0.05$). The food consumption was consistent with body weight gain and loss. Every week after week 14 of age, mice in control group had a lower consumption than treatment group animals ($p<0.05$). Normal distribution of animal body weights further confirms the weight loss in control animals

versus treatment group (Figure 7.1B). MatLab analysis revealed that the population weight histogram was unimodal and near Gaussian, which proves that ANOVA tests were applicable (Figure 7.1c). This clearly supports the treatment effect on animals in the last 6 weeks.

Serum IL-12

During the 17-week experimental period animal serum was used to measure the levels of inflammatory interleukin 12. Results show the concentration levels were significantly higher (weeks 5, 13 and 17, $p<0.05$) in treatment animal group compared to control group animals (Figure 7.2). At the time of sacrifice the average levels were 46.58±16.96 pg/mL and 158.58±28.56 pg/mL for control and treatment animals, respectively.

Luminal IL-12

Concentrations of luminal IL-12 were measured in 4 distinct parts of the small intestine: duodenum, jejunum, proximal and distal ileum. In the control group similar concentrations were found in all 4 intestinal sections (12.35±5.55 pg/mL) (Figure 7.3a). Among the treatment group, luminal IL-12 concentrations were lowest in the duodenum 35.79±16.13 pg/mL and the highest in proximal ileum, 53.74±14.29 pg/mL. All measurements were significant when compared to control ($p<0.05$).

Luminal IL-6

The concentration of luminal IL-6 was measured in the same sections of the small intestine as IL-12. Control group animals had statistically higher levels of IL-6 in all intestinal sections when compared to treatment group animals ($p<0.05$). The IL-6 concentration was especially high in the duodenum and jejunum of control group animals 115.07±27.12 pg/mL and 116.29±38.92 pg/mL, respectively (Figure 7.3b).

Luminal TNF-α

The concentration of luminal TNF-α was measured in the same manner as described for IL-6 and IL-12. Higher concentrations were detected in control group

animals and were relatively comparable in all intestinal sections (Figure 7.3c). The highest concentration was in proximal ileum, 24.08±10.59 pg/mL. The concentrations of TNF-α in proximal ileum in treatment group animals were statistically lowest, 9.38±4.23 pg/mL.

Luminal IFN-γ

There were significantly higher concentrations of luminal IFN-γ in control group animals when compared to the treatment group (Figure 7.3d). The highest concentration of luminal IFN-γ was found in the jejunum of the treatment group, 168.55±11.55 pg/mL. The lowest concentration was found in the jejunum of control group animals, 51.08±24.59 pg/mL.

Luminal IL-1β

The difference in concentration of luminal IL-1β was statistically significant in jejunum, $p<0.05$, (Figure 7.4a). In control group animals it was found to be 460.4±68.45 pg/mL whereas in the treatment group it was found to be 180.09±43.56 pg/mL. In the duodenum, proximal and distal ileum the concentration levels did not differ statistically from control.

Luminal Thromboxane B2

The thromboxane B2 concentration was especially high in all intestinal sections in control animal group and its range was from 51.27±23.53 pg/mL in duodenum to 35.50±13.16 pg/mL in distal ileum (Figure 7.4b). On contrary, the levels of thromboxane B2 found in treatment animal group were relatively low, ranging from 3.31±1.75 pg/mL to 1.52±0.62 pg/mL. All the concentration levels between groups in each intestinal section were statistically significant, ($p<0.05$).

Luminal PGE_2

The concentration of luminal PGE_2 was the highest in control animals in all intestinal sections, especially in duodenum 1836.55±389.88 pg/mL, (Figure 7.4c). The concentration of PGE_2 correlated positively with the total number of adenomas, adenoma

burden, and the relative proportion of medium-sized and large adenomas. PGE_2 correlated negatively with the relative proportion of small adenomas in treatment group. The lowest concentration of PGE_2 was found in treatment group in distal ileum 248.57±126.88 pg/mL.

C reactive protein (CRP)

C-reactive protein concentrations were measured using ELISA kit from plasma stored at -85°C obtained from animals by cardiac puncture at the time of sacrifice. The levels between control and treatment group animal were not significant, (Figure 7.5). They were found to be 81.04±23.73 ng/mL in control group and 64.21±16.64 ng/mL in treatment group.

Cox-2 expression

Intestinal lysates obtained from distal ileum were analyzed by western blotting employing antibodies specific for the cox-2 isoform. Figure 7.6 shows representative Western Blot bands of the 72 kD cox-2 protein. A higher expression level of Cox-2 was found in control group animals. Using Image J software, the bands were analyzed and relative intensities for control and treatment group animals measured. It was found that the Cox-2 in treatment group animals was 71% lower than in control group animals.

7.5 Discussion

Cancer is a chronic pathologic process. Inflammation is considered to be a particularly important factor in the pathogenesis of colorectal cancers. A period of time is required for a cancer to develop, invade or metastasize, and eventually kill the host. Diet and disease development are strongly related in disease incidence. For instance, the so called “Western diet”, containing red meat, is considered to be the one leading to a higher risk to develop colorectal cancer. The main objective of this study was to maintain test animals in a healthy condition ensuring that the levels of inflammatory cytokines are as low as possible. The inhibitory effect of microencapsulated probiotic bacterial cells

may be an important mechanism that reduces the growth of malignant tumors. Thus the animals were administered with microencapsulated probiotic bacterial cells daily. The assessment of animal health was achieved by measuring the various inflammation biomarkers at the time of sacrifice.

Animals which received daily treatment with probiotic yogurt formulation were able, not only to maintain their body weight, but also to slightly increase it which is an indication of overall health. They also had a higher food intake comparing to control animals. This may further imply that the rate of disease progression was slower.

Biomarkers are very beneficial to identify pathological processes before individuals become symptomatic or to identify individuals who are susceptible to cancer [432]. Luminal digesta obtained from the intestines were used to give an indication of IL-1β, IL-6, IL-12, PGE_2, Thromboxane B2, TNF-α, IFN-γ and CRP levels in the gastrointestinal tract and CRP in the plasma at the time of sacrifice. Our results showed an overall trend indicating notably lower inflammation in the small intestines in the animals receiving daily treatment. As *Apc*Min mice develop spontaneous neoplasia predominantly in the small intestine as opposed to the colon, this study investigated and validated the inflammatory state of that organ. In the intestine, IL-1 has been shown to be an important inflammatory mediator whose levels are increased in inflammatory bowel disease [433,434]. IL-1β is solely active in its secreted form, whereas IL-1α is mainly active in cell-associated forms (intracellular precursor and membrane-bound IL-1α). IL-1α is only rarely secreted in a limited manner [435]. The assay showed higher concentrations of IL-1β in all sections of the small intestine in control group animals. In addition, IL-6 lower concentration and IL-12 higher levels were indicative of a slower progress of the disease in treatment group animals. Similar results were obtained for TNF-α and IFN-γ. These findings are consistent with those from previous studies [436] that raise the possibility that inflammation could play a role in the development of cancer.

Both thromboxane B2 and PGE_2 play critical roles in tumor invasiveness. Therefore, we compared the levels of thromboxane B2 and PGE_2 in the small intestines. PGE_2 is one of the primary prostaglandins formed from the coupled metabolism of arachidonic acid by the COX-1 and COX-2 and PGE synthases (microsomal and/or cytosolic). Moreover, its activity influences inflammation, fertility and parturition, gastric

mucosal integrity and immune modulation [437,438]. Accumulating evidence suggests that PGE_2 has direct effects in enhancing colonic epithelial cell survival by stimulating cell proliferation and survival, tumor cells invasiveness and production of angiogenic agents [439]. Higher concentrations of PGE_2 in control group animals' further show intestinal inflammation of a greater extent when compared to treatment group animals. As it was postulated before, the inducible cyclooxygenase isoenzyme, COX-2, is significantly over-expressed at sites of inflammation and in various malignant tissues, with concomitant overproduction of the major arachidonate metabolite, PGE_2 [440].

Increased expression of CRPs has been described in several different malignancies, including colorectal [441], gastric [442], lung [443], renal [444], and breast [445] cancers. We have measured plasma concentrations of C-reactive protein from animals at the time of sacrifice. Elevated levels of CRP were detected in control group animals when compared to animals receiving treatment. C-reactive protein remains significantly associated with a higher risk of colon cancer in *Apc*Min mice. Nonetheless, CRP is a nonspecific marker of inflammation, and additional studies of specific cytokines or factors that regulate acute-phase response are necessary to elucidate the mechanisms by which inflammation increases the risk of colon cancer.

COX-2 expression has a large impact on adenoma growth in *Apc*Min mice, where treatment with a COX-2-specific inhibitor is known to markedly reduced both the numbers and growth of adenomas [446]. Marked upregulation of COX-2 occurs in various cells including endothelial cells during stress and in inflammatory conditions such as sepsis. As COX-2 expression is induced by a number of cytokines including TNF-α and IL-1, mitogens or growth factors, lipopolysaccharide (LPS), and other inflammatory stimuli it was of crucial importance to verify its expression levels. Cox-2 levels were different between control and treatment groups, (we obtained 71% reduction of averaged inflammation level in treatment receiving animals) as expected further providing evidence that the animals in treatment group have lower intestinal inflammation when compared to control group animals.

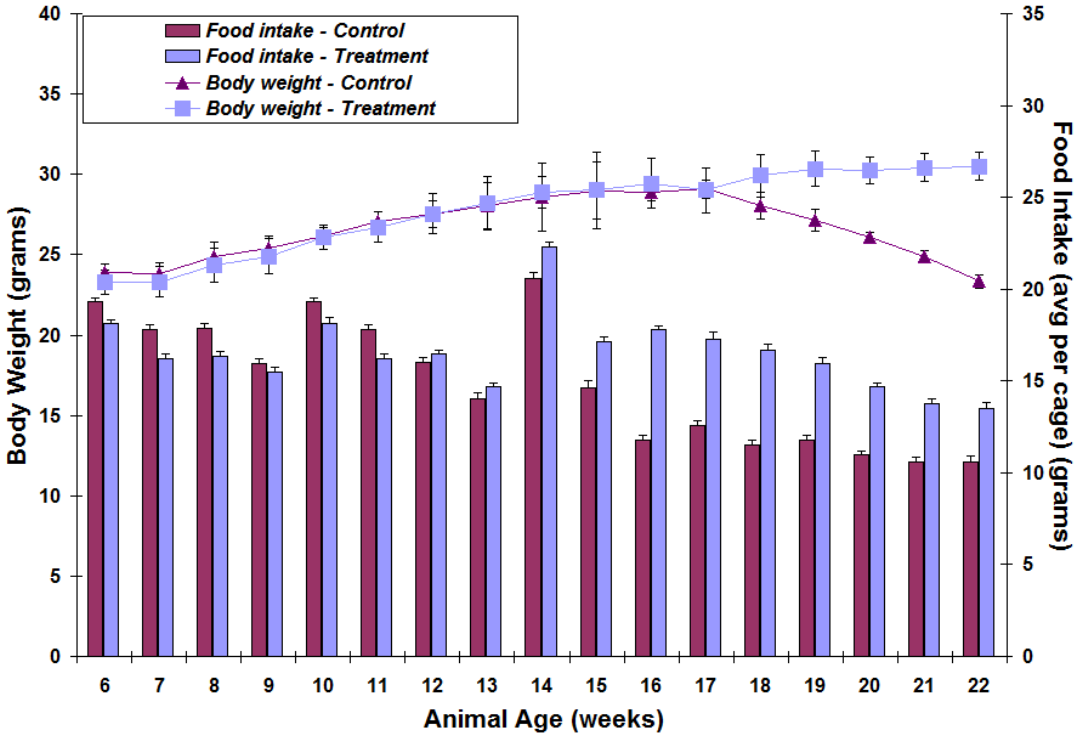
Several factors should be considered in the interpretation of our findings. A major strength of the current study is that it is a prospective study, and thus, we can more confidently infer a temporal association between inflammation and the occurrence of

colon cancer. Compared to other studies [447-449] we obtained greater reduction in *Apc*Min mice due to oral treatment with formulation of microencapsulated probiotic bacterial cells and yogurt. This could be due to the beneficial action of probiotic bacteria on gastrointestinal system such as the innate immune response [450]. Based on the results of the present study, it is not possible however, to explain the tumorigenesis in the *Apc*Min mouse model by immunological responses. Although confirmation of these results is clearly warranted, this finding, if true, could have implications for prevention strategies. Additional studies are needed to clarify the mechanism of bacterial activity and its impact on immunomodulating gastrointestinal tract.

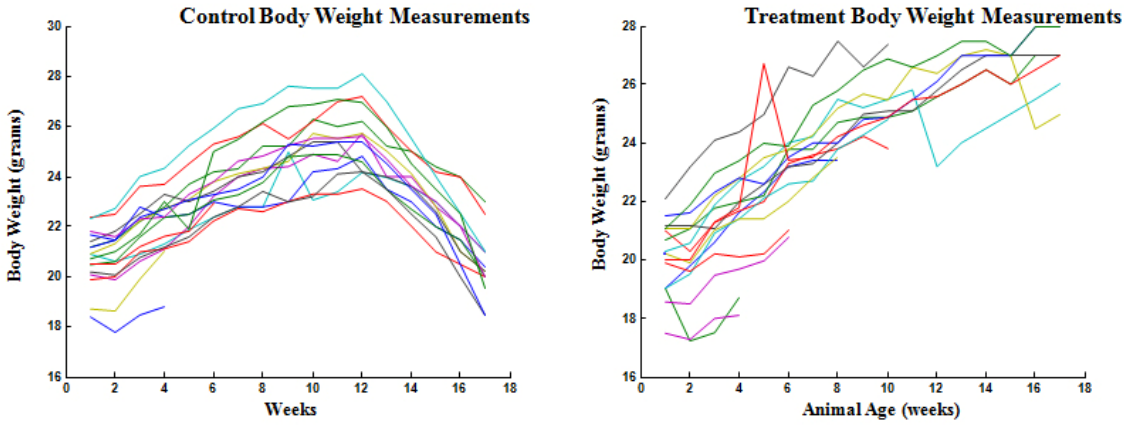
In conclusion, daily oral administration of the microencapsulated probiotic formulation results in an improvement of the intestinal microbial balance and general functioning which leads to increasing host protection against pathologies. This study supports the role for supplemental probiotics as a strategy both for suppressing inflammation and for preventing colon cancer.

Acknowledgements:

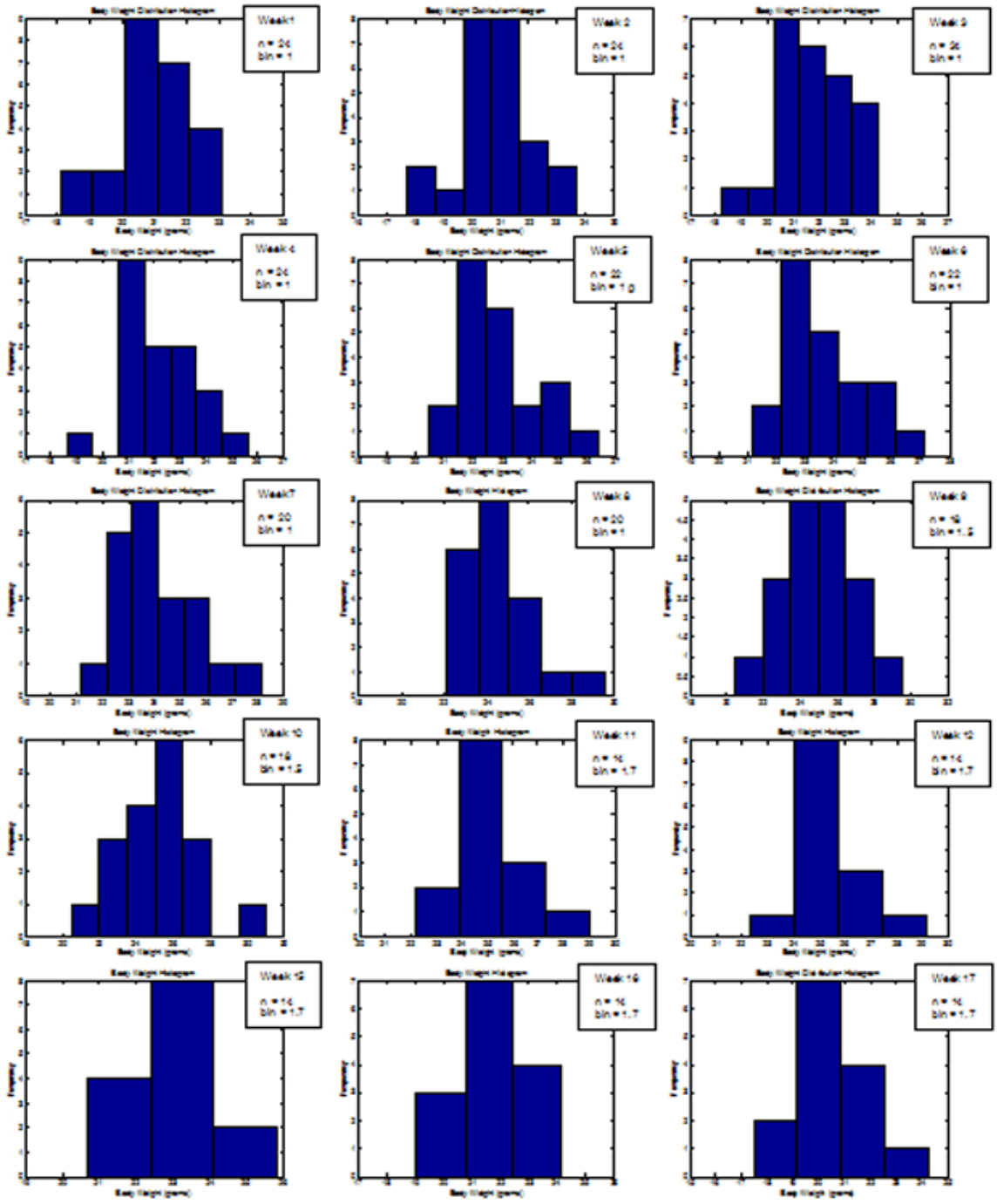
We acknowledge Canadian Institute of Health Research (CIHR) to S. Prakash as well as NSERC scholarship to A. Urbanska and A. Paul; CIHR to J. Bhathena. We also would like to thank Melina Narlis for her help with preparation of histological samples and Anna Jimenez for professional help with handling animals.We also acknowledge Micropharma for financial support to S. Prakash.


Food intake - Control
Food intake - Treatment
Body weight - Control
Body weight - Treatment
Body Weight (grams)
Food Intake (avg per cage) (grams)
Animal Age (weeks)

(A)


Control Body Weight Measurements
Body Weight (grams)
Weeks
Treatment Body Weight Measurements
Body Weight (grams)
Animal Age (weeks)

(B)



(C)

Figure 7.1: The effect of daily gavage of microencapsulated *L. acidophilus* cells in 2% M.F. yogurt in *Apc*Min mice on animal body weights and food intake (weekly food intake averaged per cage (A); Normal distribution of body weights for each animal (B); Histograms representing weekly animal body weight distribution (weeks:1-12,15-17, *n*=sample size, b=bin (g); size selected so the histogram of the simulated Gaussian function exhibits the expected normal distribution (C). Data represent the mean ± SEM per group; *n=24*.

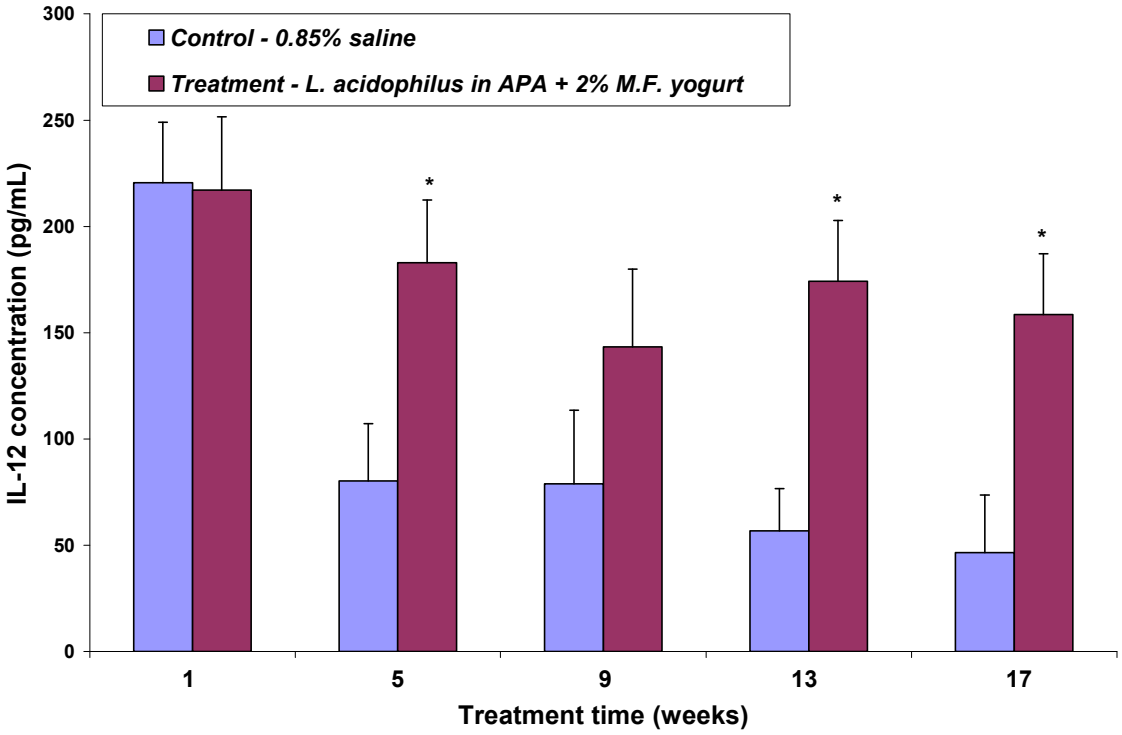


Figure 7.2: The effect of treatment on IL-12 concentrations in serum. Data represent the mean ± SD of concentration levels per group; *n=24*. Asterisks, statistical differences ($p<0.05$) when compared to control.

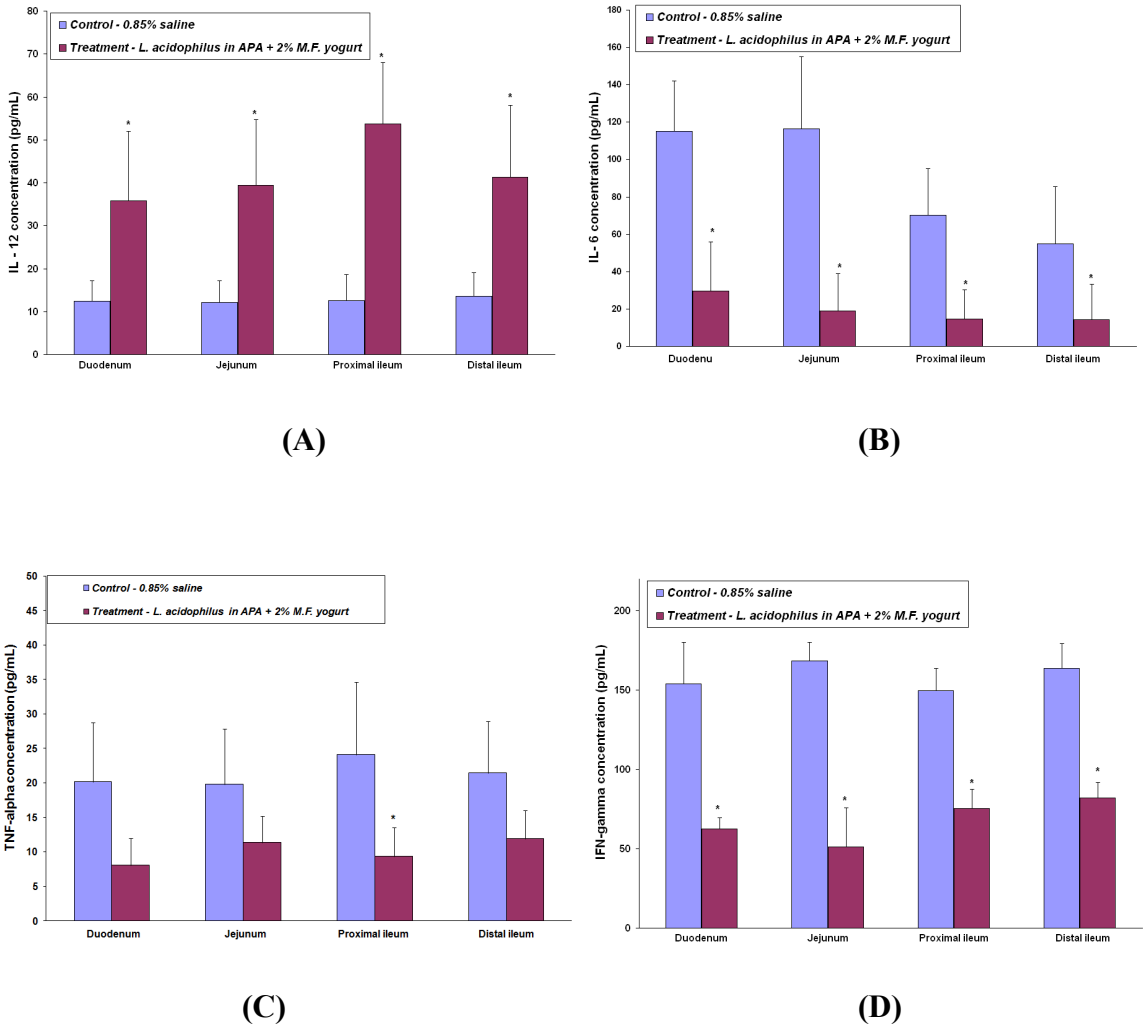
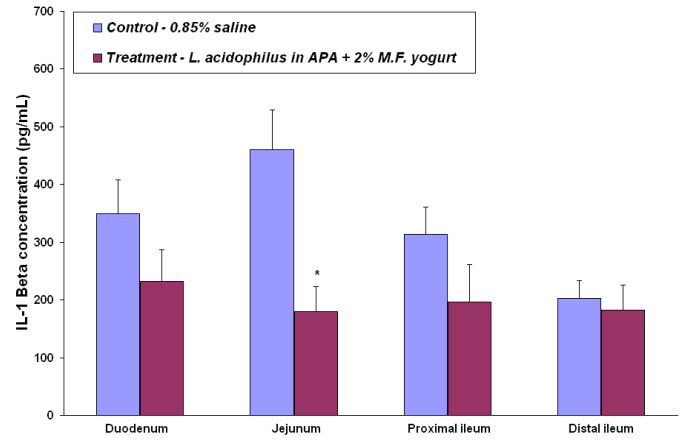
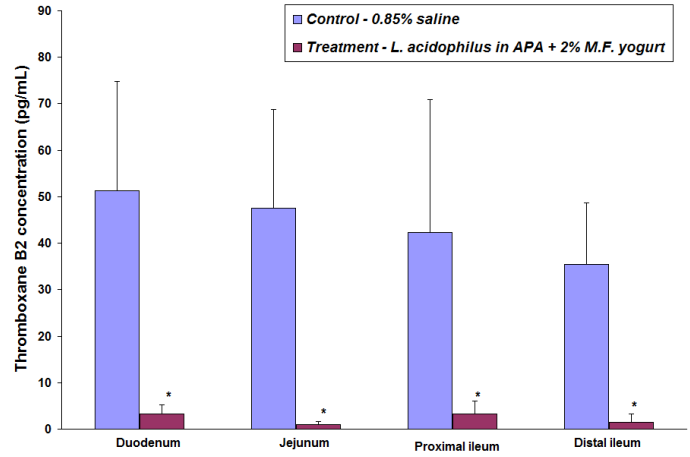


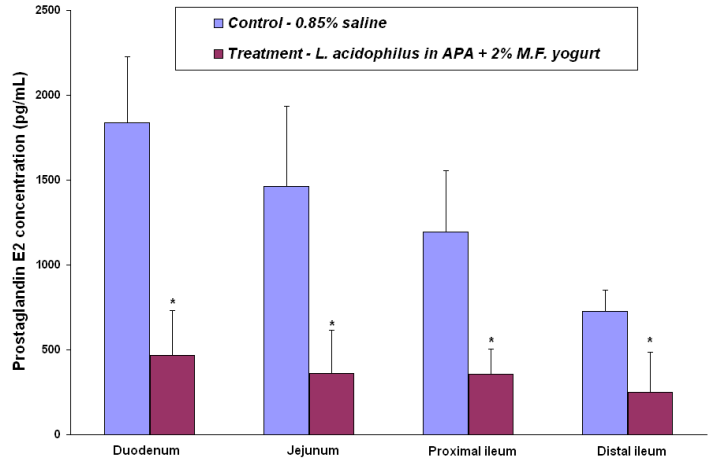
Figure 7.3: The effect of treatment on luminal cytokine concentration levels found in duodenum, jejunum, proximal and distal ileum: IL-12 (*A*), IL-6 (*B*), TNF-α (*C*) and IFN-γ (*D*). Data represent the mean ± SD of concentration levels per group; *n=24*. Asterisks, statistical differences ($p<0.05$) when compared to control.



(A)



(B)



(C)

Figure 7.4: Luminal IL-1β (A), Thromboxane B2 (B) and Prostaglandin E_2 (C) concentration levels found in duodenum, jejunum, proximal and distal ileum. Data represent the mean ± SD of concentration per group; *n=24*. Asterisks, statistical differences ($p<0.05$) when compared to control.

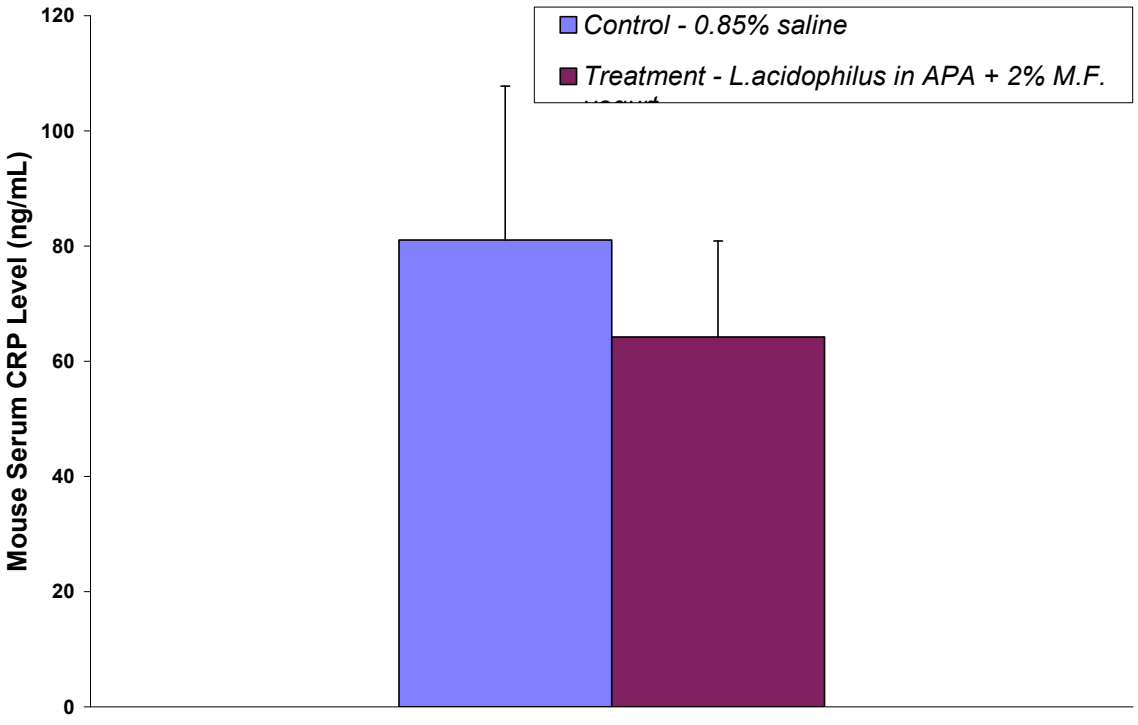


Figure 7.5: Comparison of plasma C reactive protein (CRP) levels between control and treatment mice measured by enzyme-linked immunosorbent assay at the time of sacrifice. n=24 and error bars represent SD. Asterisks, statistical differences (P<0.05) when compared to control.

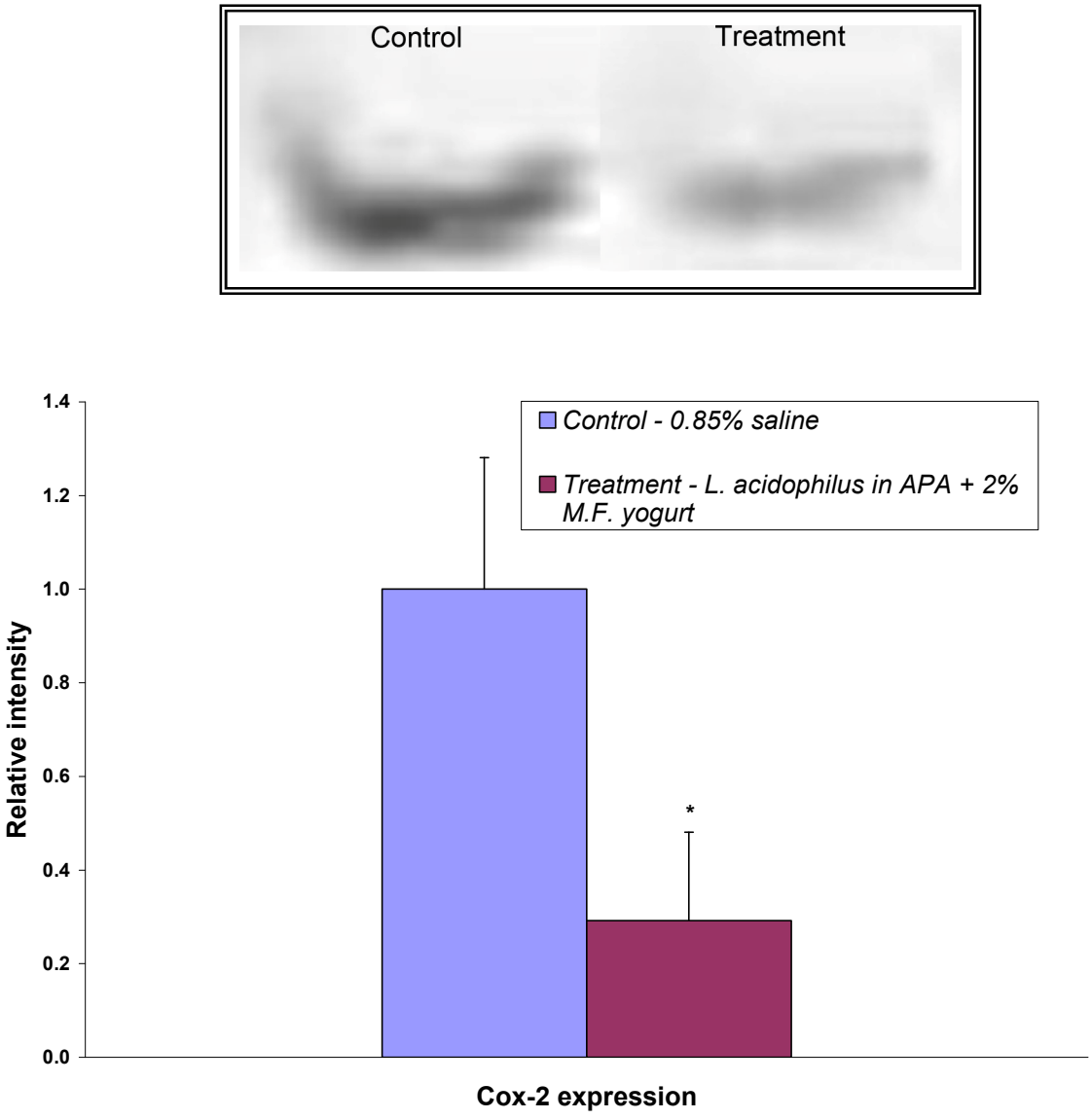


Figure 7.6: Western Blot showing COX-2 expression found in homogenized distal ileum of small intestine tissues in control and treatment animals, MW of COX-2 – 72kD. Samples were pooled per treatment and the average expression was obtained for the total of 24 per group. The relative band intensities were calculated with Image J software. (p=0.018).

CHAPTER 8
Original paper 6

Oral administration of microencapsulated probiotic yogurt formulation ameliorates polyp incidence in *Apc*Min mice: histological and immunohistochemical analysis

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Original paper to be submitted to: ***Journal of Pathological anatomy and histopathology***

Preface

In this paper, the importance of the study and the impact of oral administration of probiotic yogurt formulation are being evaluated using immunohistochemical and histological analyses. Two treatment formulations are being evaluated: APA microencapsulated bacterial cells in probiotic 2% M.F. yogurt and 0.85% saline solution. The treatment duration lasts 17 weeks. Histological analysis involves count of polyps, its classification, evaluation and assigninig scoring. Also, all intestinal samples are being examined and most representative ones are being studied. To further investigate the treatment effect on recruitment of T cells in animal tissues, the TUNEL method is being conducted to show cell proliferation and apoptosis. Immunohistochemical markers such as CD8, Mac-1, Ki-67 and cleaved caspase-3 are being used on animal samples and quantified using cutom designed MatLab algorithm. This chapter and its numerous experiments further validate the effect of probiotic treatment on polyp formation and attenuation.

8.1 Abstract

One of the advantages of conducting animal trials is the ability to perform histological evaluation of diseased organs. In our protocol, we administered the *Apc*Min mice with probiotic yogurt formulation containing microencapsulated *Lactobacillus acidophilus* cells. We compared the incidence of animals' polyp formation in the small and large intestines in control and treatment receiving animals. Using in situ apoptotic cell labeling method we detected apoptotic cells in intestinal tissues. Furthermore, using a custom designed MatLab program, we quantified immunohistochemical markers, namely: CD8, Mac-1, Ki-67 and cleaved caspase-3.

Mice receiving probiotic treatment formulation developed fewer tumors in the intestines overall. A significant decrease in the average number of tumors per animal was seen in treatment group animals, 2.5 when compared to 4.5 in control group animals. In the large intestine, however, there was no significant difference observed.

MatLab analysis revealed the following cell expressions in control and treatment groups: 6.87 and 1.93% for CD8, 6.02 and 5.43% for Mac-1, 8.72 and 5.00% for Ki-67, 11.36 and 6.09 % for cleaved caspase-3, respectively.

Our results suggest that administration of probiotic formulation ameliorates histological scores directly related to intestinal inflammation and may have a potential as dietary supplement for the prevention and treatment of various gastrointestinal infectious and inflammatory conditions.

Key words: Microencapsulation, Probiotic, *Apc*Min Mice, Immunohistochemistry, Adenoma.

8.2 Introduction

Immunological interactions between cancer and host are believed to play an important role in regulating tumor cell growth. In addition, they are easily detectable and screened for using various biomarkers designed specifically for individual reactions.

The *Apc*Min mouse, a well-established in literature model for colorectal cancer and it is used to study the impact of such factors as genetics, diet or different chemical compounds on the incidence of polyp formation in the intestines [451]. Min mice have a germ-line nonsense mutation at codon 850 of adenomatous polyposis coli (*Apc*) gene and spontaneously develop multiple polyps in the small and large intestines at the age of 10–12 weeks [338]. *Apc*Min mice are, therefore, considered to be a useful preclinical animal model to study and understand in the prevention of human familial adenomatous polyposis (FAP) and sporadic colorectal cancers [452].

Histology is considered the gold standard of pathological evaluation. It serves as a prepotent tool in comprehension of the disease progression and development.
The intestinal epithelium is a highly proliferative single-layer epithelium that enables terminal digestion and absorption of nutrients while maintaining a barrier to antigens and to the abundant gut microflora [453]. It is inhabited by large and dynamic populations of bacteria species. The generation of inflammatory changes is largely dependent upon the activities of T cells and is mediated through the release of cytokines [454]. There are many

cytokines that are involved in a complex process of inflammation. However, making a clinical diagnosis without obtaining histological examination of the tissue of interest may miss potentially serious conditions, including malignant lesions.

According to Su *et al*. this animal model allows studying the effects of probiotic treatment on polyp incidence [338]. In an attempt to assess the relative contribution of probiotic yogurt formulation containing microencapsulated bacterial cells in prevention of intestinal polyp formation, we have conducted study screening the overall inflammation in the *Apc*Min mice. Studies show that apoptosis has been evaluated immunohistochemically by antibodies recognizing single-stranded DNA (ssDNA) and cleaved (activated) caspase-3 in normal and neoplastic tissues [455,456]. Terminal deoxynucleotidyl Transferase-mediated dUTP nick end labeling (TUNEL) is an in situ method for detecting the 3'-OH ends of DNA exposed during the internucleosomal cleavage that occurs during apoptosis. Growing evidence suggests that apoptosis is relevant in some infectious diseases by regulating immune responses and, less commonly, by directly affecting microbial proliferation [403,404]. As the gut epithelial apoptosis is involved in the pathophysiology of multiple diseases, we detected apoptotic cells using TUNEL[457]. Caspase-3 is an enzyme crucial to the apoptotic process. Detection of its activated form is a valuable tool to identify dying cells even before morphological features of apoptosis are present [458].

This current research extends results from our previous studies in which using various cytokines and other parameters of evaluation we demonstrated that the administration of the microencapsulated probiotic yogurt formulation has an immunomodulatory effect in addition to overall lowering incidence of polyp formation in the intestines (article in press). The aim of this study was to classify adenomas found in animals' intestines and score them according to size and location. In addition, we used MatLab to quantify immunohistochemical markers, such as CD8, Mac-1, Ki-67 and cleaved caspase-3 for overall intestinal inflammation.

8.3 Materials and Methods

8.3.1 Chemicals

Sodium alginate (low viscosity), poly-L-lysine hydrobromide (MW=27,400) and calcium chloride (A.C.S. reagent) were purchased from Sigma-Aldrich, Canada. Difco ™ Lactobacilli MRS AGAR and Difco ™ Lactobacilli MRS BROTH were purchased from Becton, Dickinson and Company Sparks, USA. Liberty plain yogurt 2% M. F. containing active *Acidophilus* and *Bifidus* cultures was procured from a local grocery store.

8.3.2 Bacteria and Culture Conditions

Lactobacillus acidophilus cells were purchased from ATCC (314) (Manassas, VA) and were cultivated and serially propagated in the MRS medium before experimental use. Incubations were performed at 37°C in a Professional Sanyo MCO-18M Multi-Gas Incubator under anaerobic conditions (1-2% CO_2, Atmosphere Generation System AnaeroGen™; Oxoid Ltd., Hampshire, England). Bacteria were harvested after 20 hours of the 3rd passage for encapsulation.

8.3.3 Microencapsulation method

The bacterial strains were microencapsulated into Alginate-Poly-L-Lysine-Alginate (APA) membranes. All membrane components were filter sterilized through a 0.22 μm Sterivex-GS filter (Millipore, Bedford, MA, USA) prior to use. Grown cultures were centrifuged at *3000* xg for 15 minutes at 25ºC and the supernatant broth was decanted. The pellet of wet cells was weighed and suspended in 0.85% saline, pooled and slowly added to a gently stirred sterile 3.3% sodium alginate solution (final concentration adjusted to 1.65% with 0.85% saline). The entire procedure was performed under sterile conditions in a Microzone Biological Containment Hood (Microzone Corporation ON, Canada) and all solutions were autoclaved with the exception poly-L-lysine which was 0.22 μm sterile-filtered prior to usage. APA microcapsules were prepared aseptically using an Inotech Encapsulator® IER-20 (Inotech Biosystems Intl. Inc. Switzerland). Freshly prepared microcapsules were washed twice with 0.85% saline and stored at 4°C. Parameters for microencapsulation were as follows: gelation time in $CaCl_2$ – 30 minutes,

coating time – 10 minutes, nozzle diameter - 300 μm, vibrational frequency – 918 Hz, voltage > 1.00kV and current 2 amp.

8.3.4 Treatment formulation preparation

APA microcapsules loaded with *L. acidophilus* bacterial cells were carefully mixed with Liberty plain yogurt 2% M.F. and 0.85% saline in the proportions of 3:1, respectively. APA microcapsules loaded with *L. acidophilus* bacterial cells were carefully mixed with Liberty plain yogurt 2% M.F. and 0.85% saline in the proportions of 3:1, respectively. They were stored at 4°C until use.

8.3.5 Animals

Male C57BL/6J-*Apc*$^{Min/+,}$ abbreviated *Apc*Min ^{338} mice 5 or 6 weeks old were obtained from The Jackson Laboratory (Bar Harbor, ME). The animals were kept in the McIntyre Medical Sciences Building Animal Care Facility in the room with a 12-hour light-dark cycle and controlled humidity and temperature. Mice were maintained in a barrier facility. They were allowed sterile water and the laboratory rodent diet 5001 from Purina Land O'Lakes *ad libitum*. Animals overall health was monitored daily. The animal use protocol was approved by the Animal Care Committee of McGill University and animals were cared for in accord with the Canadian Council on Animal Care (CCAC) guidelines.

8.3.6 Experimental design

Upon arrival, animals were kept in a sterile environment in individual ventilated cages (IVC) which filter the air with HEPA filters (μ size). The cages, food, water bottles, etc. were autoclaved. Animals were randomly placed in the cages and allowed one week of acclimatization. Based on initial serum IL-6 values the animals were ranked and assigned to groups according to a randomized block design. The mice were separated into two experimental groups: Control (n=24) animals were gavaged 0.3 mL of 0.85%

saline solution and treatment (n=24) animals were gavaged with APA microencapsulated *L. acidophilus* bacterial cells blended in 2% M.F. yogurt. Animals were weighed individually every week and their food consumption was weighed per cage of 4 animals.

8.3.7 Histology

Tissue Sampling

The mice were euthanized by CO_2 asphyxiation. The small intestines were excised and separated from the stomach, cecum and large intestine. The length of the small intestine was measured and 4 equal sections were cut. The section representing the distal ileum was flushed with PBS buffer, cut in a half and Swiss rolled into a cassette and placed in 10% Phosphate Buffered Formalin (PBF) as a fixative. The remaining portion of tissue was subjected to OCT.

Preparation of Microscopic Sections

In order to preserve the cell and tissue structure, specimens were fixed with formaldehyde. A formaldehyde solution (final concentration, 10% [*wt/vol*] paraformaldehyde) in phosphate-buffered saline (130 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7.2) was used. The material was then incubated overnight in the refrigerator (4°C) for fixation. After fixation, the tissues were immersed in 15% sucrose/PBS (~x10 volume) at 4°C with gentle rocking for 4 hours. Overnight incubation in 30% sucrose/PBS (~x10 volume) at 4°C with gentle rocking followed. The usual protocol for preparing cryosections of tissue material includes embedding the material in OCT tissue-freezing medium (OCT Tissue-Tex [Miles, Elkhart, Ind.]). A Cryotome cryostat (ThermoElectron Corporation) was used to prepare the cryosection.
The samples were flash frozen with ice acetone after being transferred from OCT. Sections with a thickness of 6 μm were cut in the cryostat with a manual cryotome. Sections were directly picked up onto an adhesive, electrostatically charged microscope slides (Fisherbrand Superfrost Plus Microscope Slides, USA). This was done immediately after the microscope slides were introduced into the cryostat. It was

important that the microscope slides be at room temperature during this procedure in order to allow adhesion and smoothing out of the thawing microtomic sections. Subsequently, the mounted tissue holder was incubated at −22°C for 1 h (precooling) in the cryostat. Slides were then stored at −20°C prior to immunostaining.

Polyp Scoring

Polyp scoring was performed by a person blinded to the treatment. The lesions observed were found in a section of distal ileum and were divided into two categories mostly based on the size of the lesion: gastrointestinal intraepithelial neoplasia (GIN)(<1mm) and adenoma (>1mm). The standards for the histological assessment were established from the MMHCC-sponsored symposium and are detailed on the MMHC web site: (http://emice.nci.nih.gov/emice/mouse_models/organ_models/gastro_models/murine_intestinal_neoplasia/models_colorectal_cancer). In addition, the counted polyps were graded as follows: (small intestines) no polyps: score 0; up to 5: score 1; up to 10: score 2; from 11 to 15: score 3 and above 15: score 4; (large intestines) no polyps: score 0; up to 1: score 1; up to 2: score 2; up to 3 score: 3; up to 4 score: 4 and above 5: score 5.

8.3.8 Immunohistochemistry

CD8, Mac-1 staining

Embedded tissue blocks were cut serially. Slides were stained for hematoxylin and eosin for histopathological evaluation and the remaining were used for immunohistochemistry. A standard avidin-biotin complex (ABC) method was used for immunohistochemistry (Vector Laboratories, Burlingame, California, USA). Briefly, 4 μm thick cryostat sections were fixed in ice cold acetone and rehydrated in 10 mM sodium phosphate, pH 7.5, 0.9% saline (PBS). Non-specific binding sites were blocked with normal serum from the kit for 10 minutes. 3% hydrogen peroxidase treatment was applied to samples for 2 minutes. Samples were rinsed with PBS buffer and incubated with 2% normal serum in PBS to avoid background staining. The slices were incubated

with the biotinylated antimouse CD8a monoclonal antibody or the biotinylated antimouse Mac-1 monoclonal antibody, both from Cedarlane (Hornby, Ontario, Canada). The slices were washed in TBS and incubated with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). The slides were again washed in TBS, and the staining was developed with 3,3'-diaminobenzidine peroxidase (DAB) substrate kits of Vector Laboratories or Zymed Laboratories (South San Francisco, CA) for CD8 and Mac-1 analysis, respectively. All of the kits were used according to the manufacturers' instructions. The slides were counterstained with hematoxylin and methyl green for CD8 and Mac-1 analysis, respectively. They were mounted with VectaMount AQ mounting medium and VectaMount mounting medium (VectaMount AQ, Vector Laboratories, Inc.) for CD8 and Mac-1 analysis, respectively. Negative control samples were produced by incubating the samples in TBS plus 1% BSA without either of the biotinylated primary antibodies.

Ki-67 and cleaved-caspase-3 staining

Deparafinization of tissues was performed using xylene for 10 minutes (x3 changes), and was followed by rehydration in ethanol baths of descending ethanol concentrations; 100% EtOH (5 mins), 90% EtOH (2mins), 80% EtOH (2mins), 70% EtOH (2mins) and 30% (2 mins) followed by $_{d}$H2O. Next, the slides were washed in Tris Buffered Saline with 0.05% Tween 20. Slides were placed in a hot target retrival solution (95ºC) and incubated for 20 minutes. They were allowed to cool down and rinsed with distilled water. Diluted (1:40) in Tris-HCl anti-mouse Ki-67 was applied to tissue and incubated for 30 minutes at room temperature. Further, slides were washed in TBST and biotinylated rabbit anti-rat antibody (Dako, Canada Inc.) (dilution 1:200) was applied to the slides and incubated for 30 minutes at room temperature. Wash in TBST followed. Slides were incubated in Streptavidin/HRP (Dako, Canada Inc.) (dilution 1:300) for 30 minutes. After washing in TBST, slides were incubated with DAB+ chromogen for 10 minutes at room temperature. Final wash was with deionized water. Slides were counterstained with Hematoxylin for 5 minutes and dehydrated with graded alcohol and clered with xylene. Slides were mounted with a permanent mount fluid. Proliferative cells were identified by staining with antibodies against Ki-67 (Dako Canada Inc.) Apoptotic

cells were stained with an antibody against cleaved-caspase 3 (Cell Signalling Technology, Inc., Danvers, MA). Negative controls were performed by omitting the primary antibody.

8.3.9 In Situ Apoptotic Cell Labeling Using the Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Method

Deparaffinization of tissues was performed as described above. The slides were suspended in PBS buffer for 30 mins at RT. Subsequently, tissues were digested for 30 minutes with Proteinase K (Roche). The TUNEL method was carried out according to manufacturer's instructions (Roche). Briefly, slides were rinsed with PBS twice. 450 μL of label solution was added to 50 μL enzyme solution and mixed to equilibrate components. 50 μL of reaction mixture was added onto each slide and the sections were incubated for 60 mins at RT in a humidified atmosphere chamber in the dark. Lack of TdT in the TUNEL mix completely abolished labeling under all working conditions and therefore served as TUNEL negative control. Tissue sections were analyzed in a drop of PBS buffer under a fluorescence microscopy at an excitation wavelength of 450 nm and detection range of 515-565 nm. The photomicrographs were obtained. Further, signal conversion was performed using converter-POD included in the kit. 50 μL of it was added onto each slide and incubated for 30 mins at RT in a humidified atmosphere chamber in the dark at RT. Slides were rinsed 3 times with PBS and 50 μL of 0.05% 3-3'-diaminobenzidine tetrahydrochloride (DAB) substrate was added onto each slide and incubated for 10 mins at RT. Next, slides were washed with PBS buffer 3 times and counterstained with hemotoxylin and eosin for 45-50 seconds each. The samples were mounted in an aqueous solution (VectaMount AQ, Vector Laboratories, Inc.) and covered with a coverslip to analyze under light microscope.

8.3.10 MatLab analysis of immunohistochemical images

For all specimens, control and treatment tissues for each biomarker were processed identically and at the same time. All specimens were evaluated using a

ScanScope XT from Aperio. Images of four randomly selected animals (from each group) for each set of biomarkers with same resolution and magnification were compared. The quantification of was performed by using custom made algorithm in Matlab 7.0.4 (The MathWorks, Inc., Natick, MA). For the analysis, in all cases, files were saved in tagged-image file format (TIFF), which allows Lempel-Ziv-Welch (LZW) compression without discarding any data, as occurs when the more popular joint photographic experts group (JPEG) format is used. Consequently, the average file size of each original digital image was 3 MB and the processed image resolution was 1072x910 pixels. Images were processed in MatLab using Image Processing Toolbox. The algororithm consisted of converting the RGB colored images into a gray scale images. Each segment was visualized and thresholds were manually selected. Threshold was set based on intensity of picture's pixels such that the darker cells of interest were separated from the rest of the tissue. The red area represents stained cells and the blue area is discarded, not processed area. Our procedure was to reduce the threshold until extracellular noise became excessive and then raise the threshold enough to clearly isolate the segment.

The ratio was calculated by dividing the number of red pixels divided by the total number of pixels in the image minus the discarded pixels. We ran Matlab code to generate the output image containing red and blue areas.

8.3.11 Microscopy and Analysis Software

A ScanScope XT from Aperio along with software Image Scope v9.1 (Aperio Technologies, Inc., Vista, CA was used to scan sections subjected to immunohistochemical staining.

8.3.12 Statistical Analyses

Statistical significance was calculated using the unpaired Student's *t*-test assuming unequal variance, and a *P*-value <0.05 was considered to indicate significant difference.

8.4 Results

Artificial cell microcapsules were prepared using the optimized methods described above and the bacterial concentration was verified by serial dilutions and spread plate count. The microencapsulation yielded spherical microcapsules which were able to retain bacterial cells, which were able to grow normally when obtained supernatant was plated after manually breaking the microcapsule membrane.

The administration of probiotic yogurt formulation significantly reduced the total number of intestinal tumors when compared to saline receiving animals ($p<0.05$). There was on average of 4.5±1.46 tumors found per animal in control group and 2.5±1.60 tumors in treatment group (Table 8.1a). Most found lesions were small GIN: a total of 66 in small intestine of control group and 42 in large intestine of treatment receiving animals. In general, adenomas found in colon of control group (4) and treatment group animals (2) were less numerous. This is 44% decrease in total number of lesions in treatment receiving animals when compared to control group animals. The number of tumors was also scored according to 0-5 scale, and the scores are presented in Table 8.1b.

There was a difference in body weights and food consumption between animal groups; treatment group animals had a higher food intake, thus their body weight was slightly increasing whereas control group animals had a lower food intake and corresponding body weight loss was observed (data not shown).

Tumors in the small intestine were mainly less than 1mm in diameter. The control group animals had significantly higher polyp count. Figure 8.1 shows longitudinal sections of small intestine of representative control and treatment group animals along with their magnified areas of interest. Figure 8.2 depicts large intestines obtained from the animals from control and treatment groups. The multiplicity of the adenomas found in the large intestine is significantly lower than that found in the small intestine. From a thorough analysis of intestinal polyp morphology of all 48 animals, only one lesion resembling malignant tumor was observed in animal from control group. It was characterized by cribriform, sieve-like pattern with nuclear pleiomorphism and loss of cell polarity. Additional pathology was also observed in a form of intraglandular papillary projections and the presence of flat destructed villi. The remaining animals, on

average had sessile/broad-based adenomas with either low or high grade of dysplasia. They were composed of papillary projections of lamina propria covered by an epithelium.

The TUNEL method was used to determine the apoptotic cells. Figure 8.3a shows a photograph of control group animal small intestine with the apoptotic cells distributed at the base and along the surface of the adenoma. The photograph of well defined villi obtained from the representative animal of the treatment group is depicted in Figure 8.3b. Since the TUNEL is a semi-qualitative method, we used staining against cleaved caspase-3 cells.

Figure 8.4a shows a photograph processed using our customized MatLab program. The algorithm for analysis of immunohisstochemical staining was designed to count stained cells with antibodies, namely CD8, Mac-1, cleaved caspase-3 and Ki-67. Their expression profile was quantified in Figure 8.4b.

In the present study, the tissue of treatment group animals expressed higher content of CD8 cells than control group, 1.93 and 6.87 %, respectively (Figure 8.4b). Adenoma tissues, on average contained higher CD8 cells indicating efficient recruitment of the T lymphocytes. Relatively similar Mac-1 expression was found in animal tissues from both control and treatment groups, 6.02 and 5.43%, respectively (Figure 8.4b). Antibodies against CD8, Mac-1, Ki-67 and cleaved caspase-3 were used in immunohistochemistry to determine the treatment effect on recruitment of T cells as well as cell proliferation and apoptosis (Figure 8.5). Cleaved caspase-3 was expressed in apoptotic cells, apoptotic bodies and in some tumor cells, mostly at the base and surface (Figure 8.5e, f). In treatment group animals, cleaved caspase-3 expression was localized to the nucleus and to the cytoplasm diffusely, thus is was much less in the normal mucosa. Quantification using MatLab yielded 11.36 and 6.09 % expression of cleaved caspase-3 in control and treatment group animals, respectively (Figure 8.4b). The Ki-67 staining was observed to be lower in adenomas of treatment group as compared with adenomas of the control group (Figure 8.5g, h). There was 8.72 and 5.00% of expressed Ki-67cells in control and treatment group animals, respectively (Figure 8.4b).

8.5 Discussion

In this study we assessed the potential of probiotic yogurt formulation on intestinal tumorigenesis in *Apc*Min mice using various immunochemical markers. In the evolution of colon rectal cancer (CRC) the imbalance between cell proliferation and apoptosis is considered one of the prominent causes of tumor induction and/or progression. Therefore the studies involving the thorough evaluation of apoptotic cells are quite valuable. We used TUNEL as a most common and most reliable apoptosis detection method in tissue sections [457,459,460]. However, this method fails to detect the early stages of apoptosis [461]. In comparison with TUNEL method, cleaved caspase-3 staining was able to identify more apoptotic cells, most likely even pre-apoptotic cells. Therefore, this study shows that cleave caspase-3 is a very useful tool for detecting apoptosis on the formalin-fixed, paraffin-embedded tissue sections.

An important step of immune response system is the recruitment of lymphocytes into target tissues. We obtained higher expression levels of CD8 cells than Mac-1 indicating that the recruitment of T lymphocytes was more efficient in the former. Ki-67, a marker of proliferating cells, was highly expressed in adenomas of the control group. We did not observed significant correlation in detection of apoptotic cells stained with cleaved caspase-3 and Ki-67. However, the proposed link between cell proliferation and susceptibility to colorectal cancer is still being questioned by the differences in the background proliferation indices in different regions of the intestinal tract [462,463].

The quantitative distribution generated by MatLab can contribute detailed information that will further demonstrate the specific antibody occurrence with respect to its size and location. Although a more diligent program is needed to fully assess the immunohistochemical analysis, we were able to show relative biomarkers distribution between control and treatment groups.

We further demonstrated the inhibitory activity of microencapsulated yogurt formulation on polyp incidence, the significant difference in total number or polyps per animal. These might be due to the suppression of cell proliferation, enhancement of apoptotic cells and restoration of microbial gut flora.

It is known that the microflora of the gut stimulates the proliferation and differentiation of epithelial cells [464]. In addition, the colonization of the gut with commensal microflora influences the development of the immune system [465]. The basic functions of the mucosal immune system include protection against pathogens, prevention of the penetration by foreign antigens, induction of oral tolerance, and maintainance of mucosal homeostasis [466]. The knowledge of the different requirements to induce an effective immune response at the mucosal sites would help to define immunotherapeutic approaches and to prevent diseases. Probiotic bacteria would be a good choice to improve the mucosal immune system; however, the mechanisms through which they work such as network of signals involved in GI immune response are still unknown.

Although *Apc*Min mice provide a genetically valid model for studying and understanding intestinal tumorigenesis, its major drawback is that the variation in individual mice' tumor incidence is large and therefore it is difficult to draw consistent conclusions when comparing different animals batches.

As gastrointestinal intraepithelial neoplasias (GIN) are the precursors of adenomas and later of intestinal carcinoma, it was interesting to note highest polyp counts of these lesions. To improve the effectiveness of polyp enumeration and classification along with immunohistochemical analysis, one needs to design an automated system (protocol) that would allow consistency by implementing universal guidelines and standards.

In theory, oral administration of probiotic yogurt formulation is a promising alternative modality for polyp suppression. However, the major drawbacks of this study is the use of *Apc*Min mice which while being the most suited mouse model of the human colorectal cancer syndrome, it differs from the cancer development in humans. For instance, in human, carcinogenesis is a complex, multistep, often including metastasis, process. Most colorectal cancers in human are regarded as sporadic and only a small percentage is due to an autosomal-dominant inherited syndrome [467]. The polyps found in *Apc*Min mice do not undergo the process of metastasis [468]. Furthermore, adenomas in *Apc*Min mice occur primarily in the small intestine, whereas tumors in human are generally restricted to the colon and rectum. Additional studies are needed to clarify the

mechanism of bacterial activity and its impact on immunomodulion of gastrointestinal tract in the mouse and/or humans.

Acknowledgements

We acknowledge post graduate scholarship from NSERC to A. Urbanska. We also acknowledge Micropharma for financial support to S. Prakash. We also thank Melina Narlis for histological samples preparation.

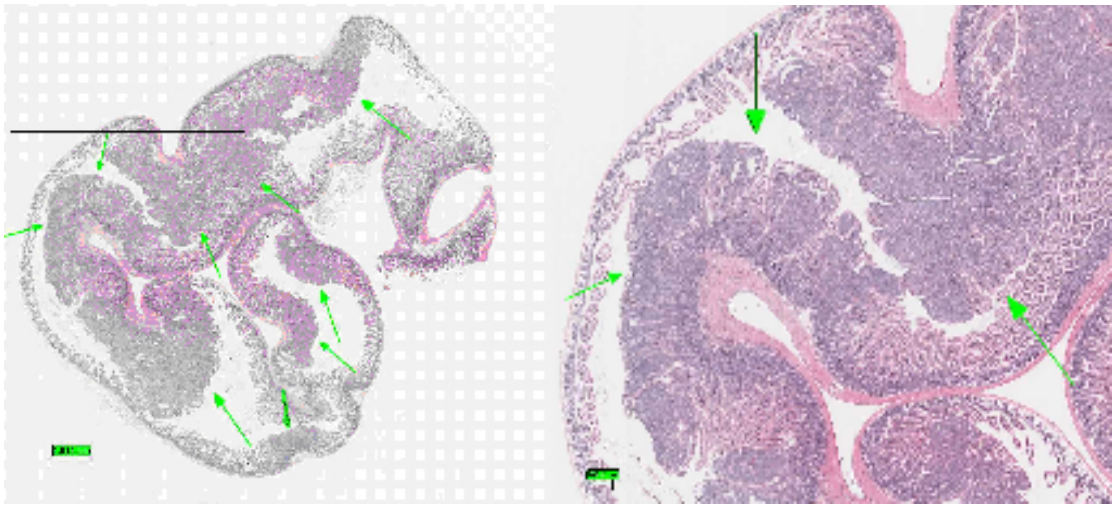
(A)

Group	No. tumors per cage			No. mice with GIN			No. mice with adenoma		
	Small intestine	**Large intestine**	**Total**	**Small intestine**	**Large Intestine**	**Total**	**Small intestine**	**Large Intestine**	**Total**
Control (6 cages, 4 animals per cage)	20 24 14 12 8 18	2 3 0 1 5 3	22 27 14 13 13 21	66	10	76	30	4	34
Avg/ animal	4.0±1.45	0.6±0.44	**4.5±1.46**	2.8	0.4	3.2	1.3	0.2	1.4
Treat. (6 cages, 4 animals per cage)	9 2 9 6 20 10	0 0 1 0 1 2	9 2 10 6 21 12	42	2	44	14	2	16
Avg/ animal	2.3±1.50	0.2±0.20	**2.5±1.60**	1.8	0.08	1.8	0.6	0.08	0.7

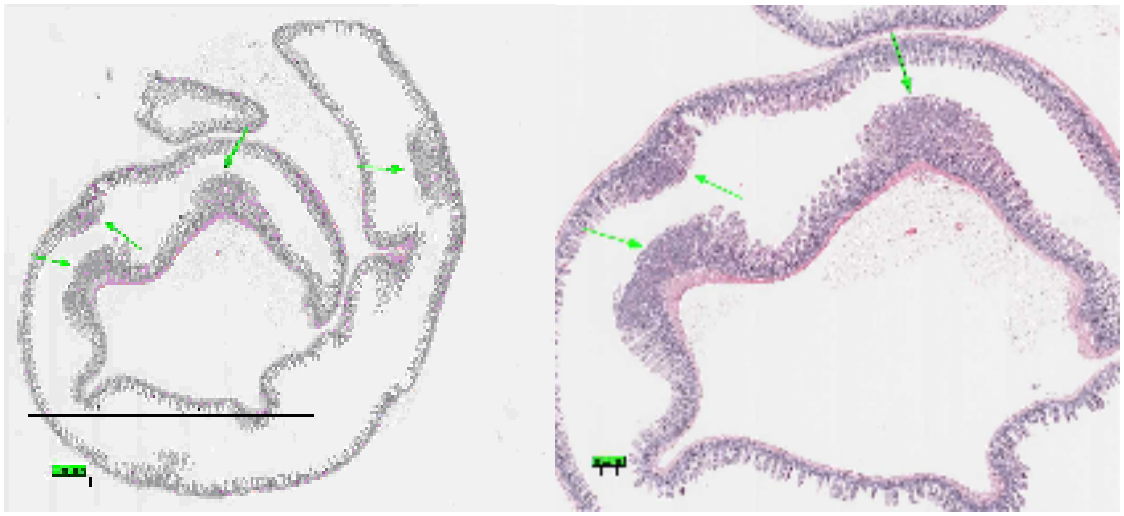
(B)

Group (*n*)	Tumor scores per cage		
	Small intestine	**Large intestine**	**Total**
Control (24)	4	2	4
	4	3	4
	3	0	4
	3	1	4
	2	5	4
	4	3	4
Avg/animal	0.8±0.20	0.6±0.44	**1±0**
Treat. (24)	2	0	2
	1	0	1
	2	1	3
	2	0	2
	4	1	4
	2	2	2
Avg/animal	0.5±0.25	0.2±0.20	**0.6±0.26**

Table 8.1: Enumeration, classification (A) and scoring (B) of tumors in *Apc*Min mice.

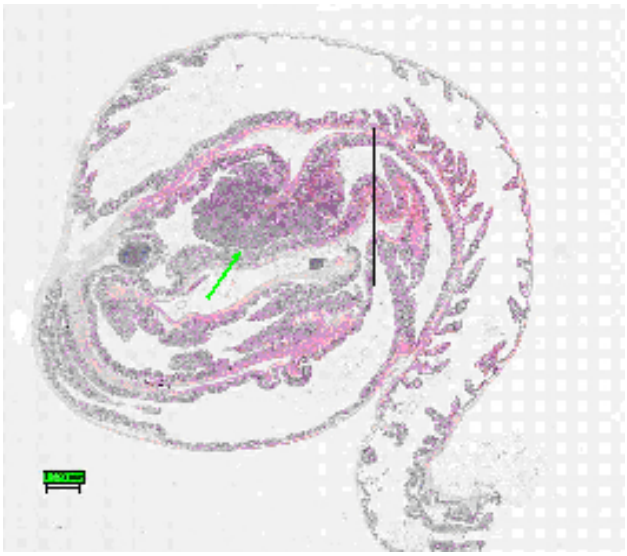


(A)

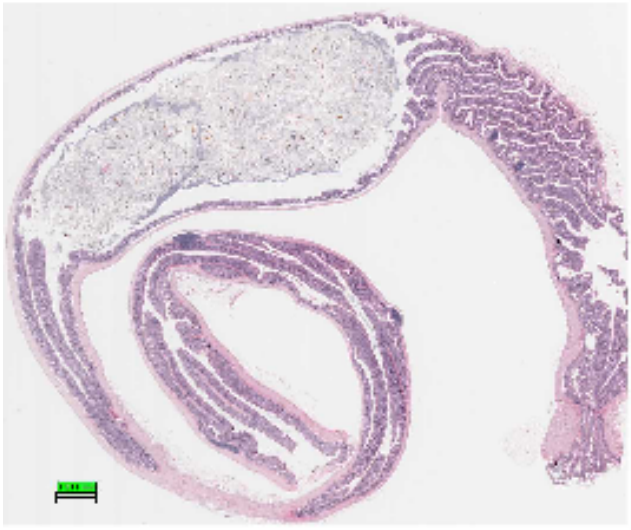


(B)

Figure 8.1: Histological evaluation: intestinal changes in the small intestines of *Apc*Min mice; (A) Multiple lesions found in control group animal, arrows point to high and low grade adenomas, note magnified tumor show resembling adenocarcinoma, with the initiation of cribriform pattern features (top arrow); Diagnosis of possible adenocarcinoma was based on cellular and nuclear pleiomorphism and loss of cell polarity. Architectural abnormalities such as the presence of intraglandular papillary projections and cribriform and solid areas were also present; the presence of flat destructed villi and intestinal glands and was also observed. Magnification: 16*X*, 50*X*. (B) Microadenomas found in treatment group animal, note the unobstructed lumen and well defined villi. Magnification: 16*X*, 30*X*. All tissues were stained with hematoxylin eosin.

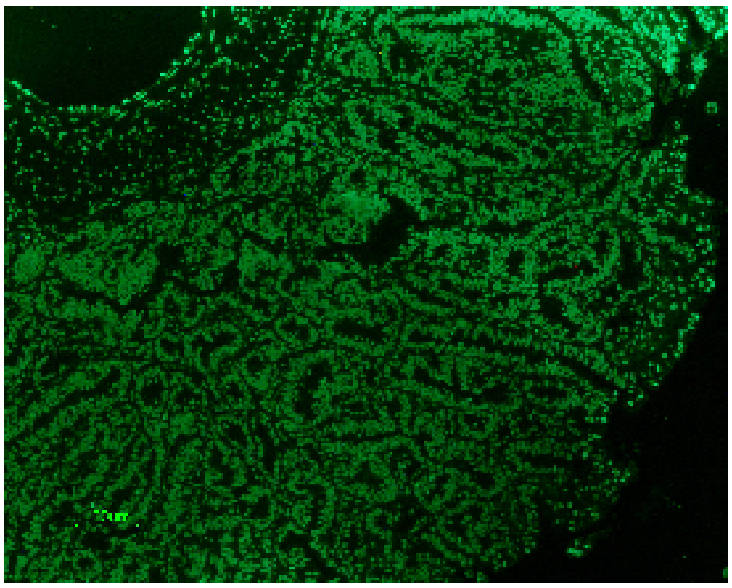


(A)

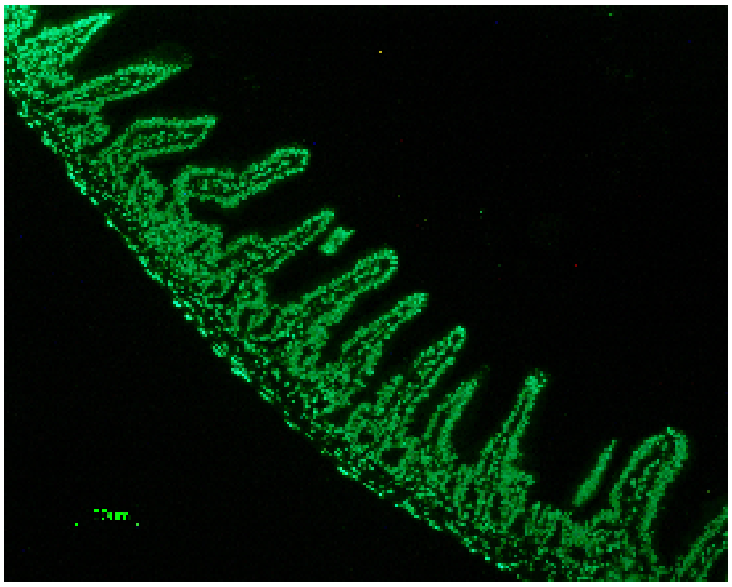


(B)

Figure 8.2: Histological evaluation: intestinal changes in the large intestines of *Apc*Min mice; (A) a representative tumor found in a control group animal shows features of an invasive adenoma. Note absence of Lieberkühn crypts around the adenoma. (B) unobstructed large intestine of treatment group animal showing absence of lesions Original magnification: 16*X*. All tissues were stained with hematoxylin eosin.

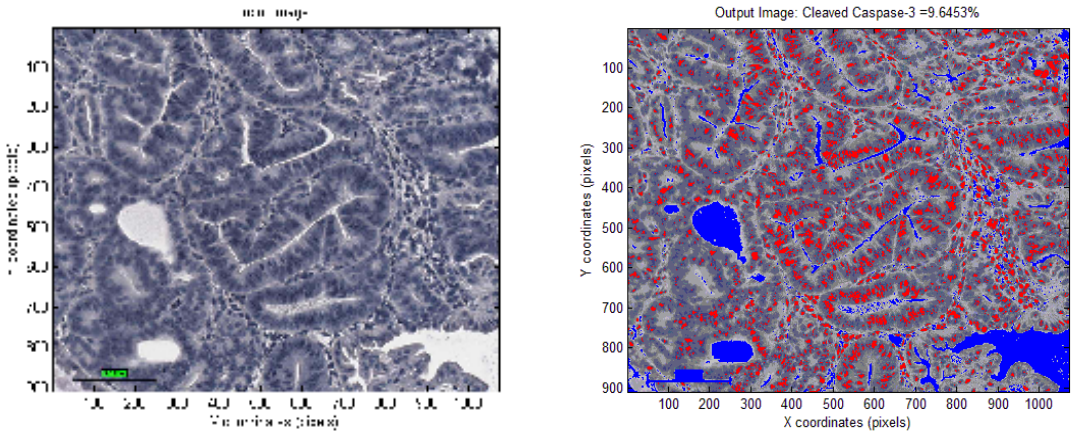


(A)

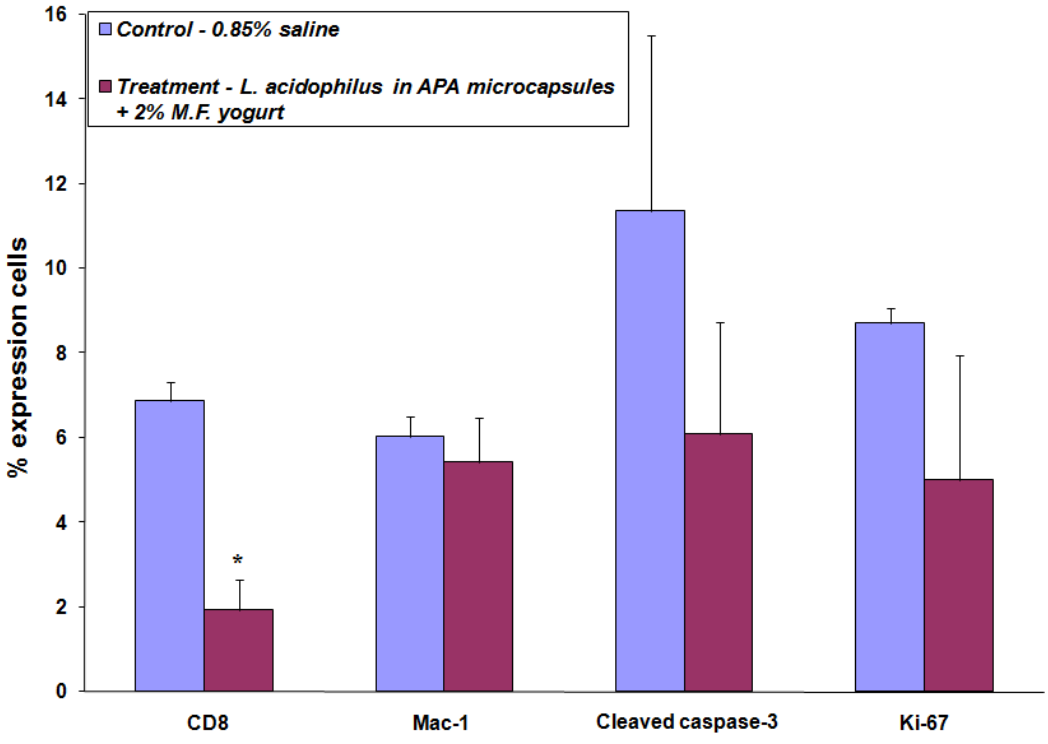


(B)

Figure 8.3: Determination of apoptotic cells in lamina propria of the small intestine of *Apc*Min mice using In Situ Apoptotic Cell Labeling Method (TUNEL); (A) photography of control animal tissue, note presence of apoptotic cells at the base and surface of the tumor; magnification 100*X* (B) photography of treatment animal tissue showing abundance of apoptotic cells localized along the villi; magnification 100*X*.

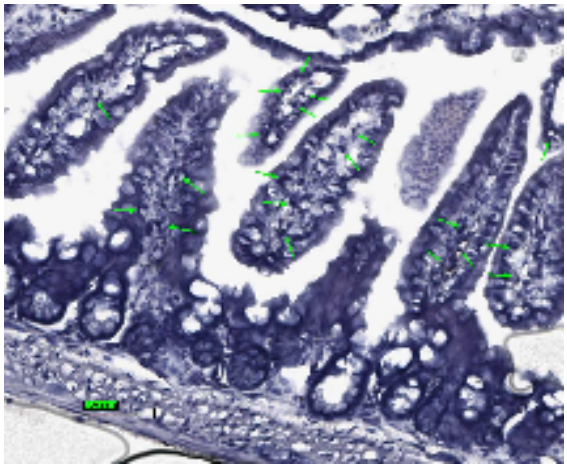


(A)

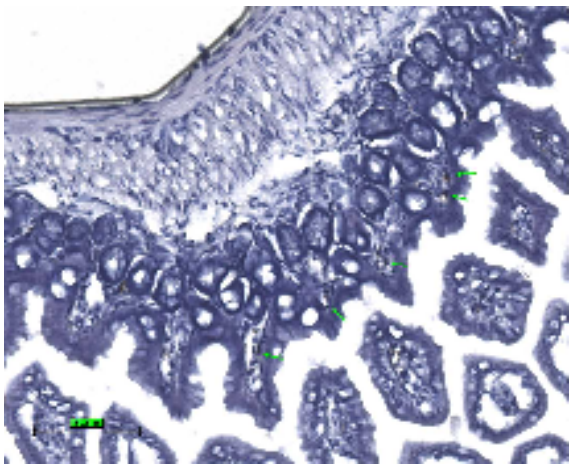


(B)

Figure 8.4: MatLab processing and analysis; (A) representative photograph showing processed image; (B) the percent expression of CD8, Mac-1, Cleaved caspase-3 and Ki-67 cells in control and treatment animals; asterisks, statistical differences ($p<0.05$) when compared to control.



(A)



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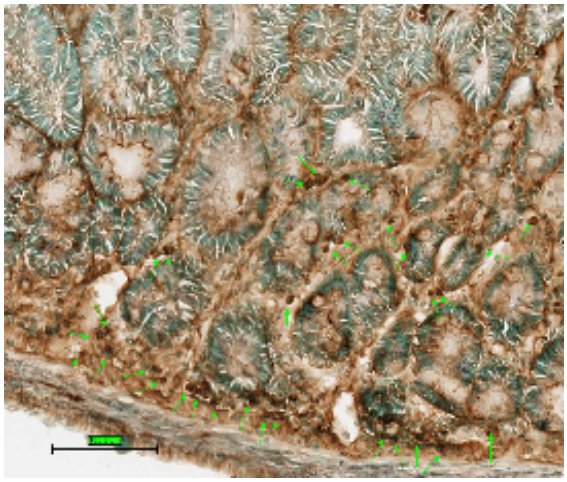

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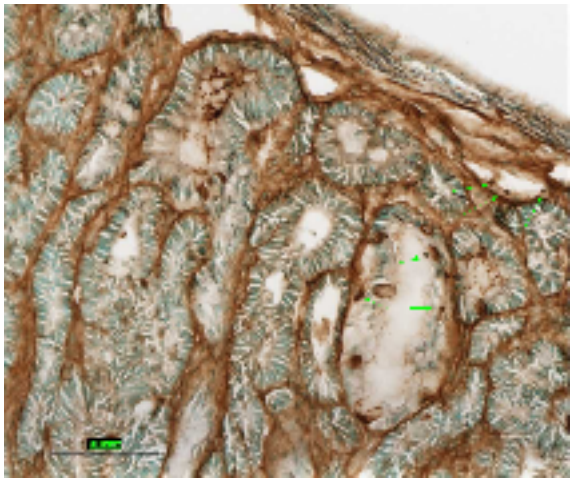

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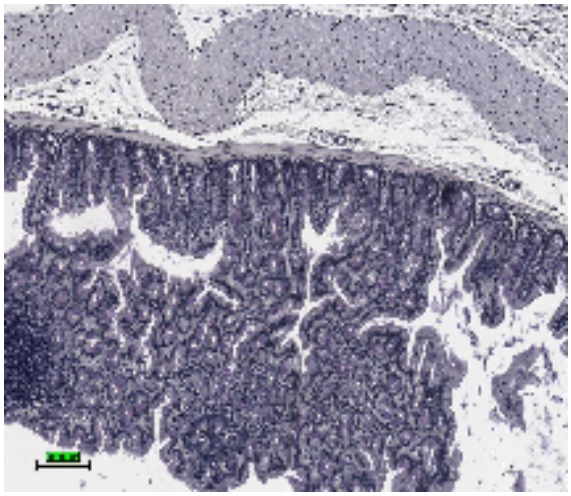

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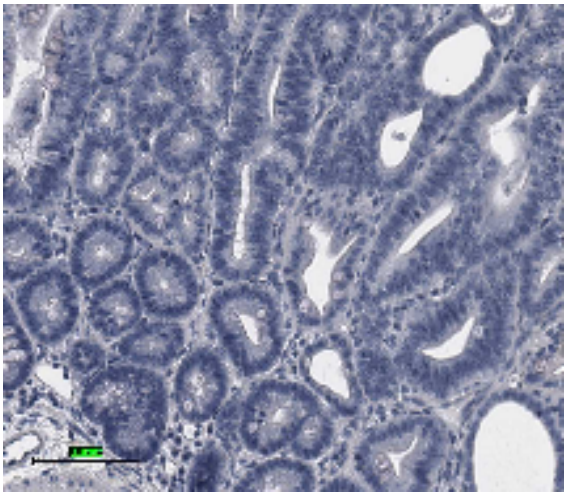
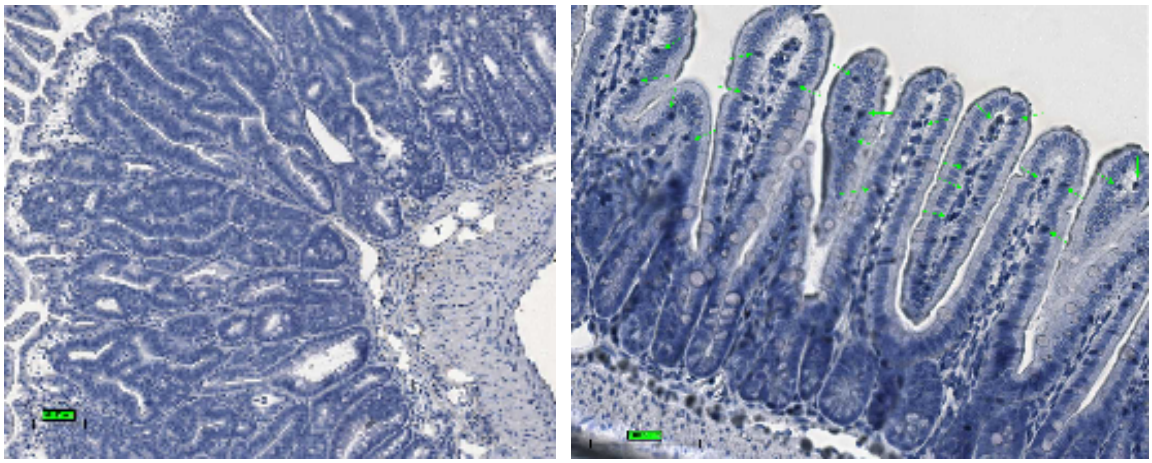


(F)



(G) **(H)**

Figure 8.5: Immunohistochemical staining for CD8, Mac-1, cleaved caspase-3 and Ki-67. (A) higher abundance of positive CD8 cells in control group animal; magnification 400*X* (B) localization of CD8 cells within and around a tumorous overgrowth; magnification 400*X* (C) expression of Mac-1 cells in control group animal; magnification 400*X* (D) scarce positive Mac-1 cells detected in treatment group animal; magnification 400*X* (E) staining for cleaved caspase-3: high expression of apoptotic cells in the area of the tumor; magnification 200*X* (F) lower expression of cleaved caspase-3 cells in normal mucosa; magnification 400*X* (G) proliferation cells marker Ki-67 greatly present in adenoma of control group animal; magnification 200*X* (H) fewer Ki-67 positive cells seen within villi of the small intestine of the treatment group animal, see pointing arrows; magnification 200*X*.

CHAPTER 9
Summary of Accomplishments, Conclusions and Claims to Original Contributions

9.1 Summary of accomplishments

In this thesis research, a novel yogurt formulation with a microencapsulated probiotic system containing *Lactobacillus acidophilus* bacterial cells for oral delivery in the suppression of intestinal tumorigenesis in *Apc*Min mice was designed, prepared and characterized. The potential of this probiotic yogurt formulation for use in human health in general and colon health in particular was tested in experimental animals.

The following is a summary of accomplishments and observations from in vitro and in vivo studies:

1. Selection of a suitable bacterial strain and microcapsule polymeric membrane to design a novel microencapsulated microbial system.

Based on an extensive literature review we concluded that the most suitable bacterial strain should be non-pathogenic, gut-friendly, with the capacity to modulate gastrointestinal inflammation, to sustain bioactivity during the gastric transit and be safe. *Lactobacilus acidophilus* met all these requirements in addition to being tested with successful results in studies investigating such disorders as: diarrhea, gastrointestinal inflammation caused by *Helicobacter pylori,* mucosal immunity, and Crohn's disease.

The selection of membrane materials was performed based on previous studies in addition to their physicochemical properties and membrane molecular weight cut-off point. Alginate is an FDA-approved food additive, cell and tissue compatible and it gels in presence of calcium ions. Poly-L-lysine provides membrane with controlled porosity and environmental protection. Although chitosan is non-toxic, biocompatible, and biodegradable and it is projected as a promising supporting polymer for gene delivery, cell culture, and tissue engineering, the AC membrane was less robust, characterized by lower bacterial retention capacity, preserved worse bacterial culture viability and had a

lower resistance to gastrointestinal environment. Therefore the APA membrane was selected as more suitable for our application.

2. Optimization of microencapsulation parameters for APA and AC microcapsule bacterial cell oral delivery systems.

The APA microcapsule preparation involved ionotropic gelation of the alginate-calcium core, coating with poly-L-lysine and covalent cross-linking with the additional layer of alginic acid. All membrane components were filter-sterilized through a 0.22 μm Sterivex-GS filter prior to use. Using an Inotech Encapsulator® IER-20 microcapsules were prepared aseptically in a Microzone Biological Containment Hood. Freshly prepared microcapsules were washed twice with 0.85% saline and stored at 4°C.

The optimized parameters for APA microencapsulation were as follows: alginate concentration – 1.65%, gelation time in 0.1 M $CaCl_2$ – 30 minutes, coating time – 10 minutes (Poly-L-lysine concentration - 0.1%, alginate concentration - 0.1%) , nozzle diameter - 300 μm, vibrational frequency – 918 Hz, voltage > 1.00kV, current 2 amp and pump speed > 22.

The optimized parameters for AC microencapsulation were: 1.5 % (w/v) sodium alginate (low viscosity), gelation time in 0.1 M $CaCl_2$ – 30 minutes, nozzle size - 300 μm vibrational frequency – 918 Hz, voltage > 1.00kV, current 2 amp and pump speed > 24. Coating - 0.5 %, 0.25 % and 0.1 % chitosan 10 or 100 solution dissolved in dilute acetic acid at a pH of 5.3 for 30 min.

Both microencapsulation techniques yielded spherical microcapsules with a narrow size distribution (APA - 433±67 μm, AC - 550±26 μm) and high homogeneity. It also yielded a consistent bacterial cell load of approximately 10^{10} cfu/mL.

3. Design and preparation of a novel probiotic yogurt formulation for the oral delivery of *Lactobacilus acidophilus* bacterial cells.

L. acidophilus cells were inoculated and cultured in MRS Broth at 37 ºC using anaerobic jars and gas generating kits in a Professional Sanyo MCO-18M Multi-Gas

Incubator. Microcapsules containing live bacteria were homogenized manually for dilution and plating. Cell count was determined by anaerobic spread plate on MRS agar after 48 hours and was kept constant throughout the experiment. Grown cultures were centrifuged at 3,000 *x*g for 15 minutes at 25ºC and the supernatant broth was decanted. The pellet of wet cells was weighted and suspended in 0.85% saline, pooled and slowly added to a gently stirred sterile 3.3% sodium alginate solution (adjusted to 1.65% with 0.85% saline). APA microcapsules loaded with *L. acidophilus* bacterial cells were blended with Liberty plain yogurt 2% M.F. containing active *Acidophilus* and *Bifidus* cultures (procured from a local store) and 0.85% saline in the proportions of 3:1, respectively.

4. Evaluation of physical stability of microcapsule systems in simulated gastric fluids

An evaluation of the GI stability of APA microcapsule was carried out by exposing the APA microcapsules containing live LAB cells to simulated gastric fluid (SGF) solutions (pH 1.98) at 37ºC for 3, 12 and 24 hours with 150 rpm mechanical shaking. Results showed that APA microcapsules were sturdy after exposure and remained intact in SGF for up to 24 hours at pH 1.98 and with 150 rpm shaking. After being exposed to simulated intestinal fluid (SIF) at 37ºC and with 150 rpm mechanical shaking, APA microcapsules remained intact. Up to 3% of the APA microcapsules were found to have been damaged after treatment in SGF for 3 hours and SIF for 24 hours. In comparison, the AC microcapsules preserved 97.42 %, 91.11 %, 88.43 % and 84.19 % integrity after being treated in SGF for 3 hours, SGF and SIF for 3 hours, SGF 3 hours followed by 12 hours in SIF and SGF 3 hours followed by 24 hours in SIF, respectively.

5. Testing of the yogurt formulation in dynamic computer controlled simulated human gastrointestinal (GI) model

Microcapsules containing live bacterial cultures blended with yogurt formulation were tested using computer controlled simulated human GI model. In comparison to the

AC microcapsules which showed 8.05, and 7.96 log (cfu/mL) bacterial survival in vessel representing stomach and small intestine, respectively; APA microcapsules showed 7.10 log cfu/g survival when tested in a vessel representing stomach (120 min exposure time) and 9.18 log cfu/g viability when tested in a vessel representing small intestine (360 min exposure time).

6. Evaluation of microbial stability in microencapsulated systems in GI pH conditions in vitro

The survival of live *L. acidophilus* cells in difference pH environments was tested over the 72-hour study. In the APA microcapsule system, the lowest survival rates were found at the acidic pH of 2 (5.38 log cfu/g) and at pH 3 (5.52 log cfu/g) after 72 hours of exposure. At pH 6 (representative of the small intestine) the viability was seen to be 8.43 log cfu/g after 72 hours, and 6.41 log cfu/g at pH 4. While there was a steep drop in the total bacterial count during the first 3 hours, a slower decline was observed from the 6^{th} hour onward until the 72^{nd} hour.

In the AC microcapsule system, the survival of microencapsulated live *L. acidophilus* cells in buffers of pH 2, 3, 4, 6 and 8 supplemented with 2 % M.F. yogurt showed the highest survival at pH 8, 10.34 log (cfu/mL), and lowest at pH2 of 7.48 log (cfu/mL) after 72 hours of exposure. Moreover, at pH 6 cells reached 10.07 log (cfu/mL), at pH 4, 7.56 log (cfu/mL) and 7.82 log (cfu/mL) at pH 3.

7. Selection of *Apc*Min mice as a study model

In scientific literature, *Apc*Min mice is a well-established colon cancer animal model [334-338]. Animals have a mutation in *ApcMin* allele of the adenomatous polyposis coli (*Apc*) gene that is similar in its type and location to mutations found in the inherited CRC syndrome and familial adenomatous polyposis (FAP). Their mutation is chemically induced and the animals are become highly susceptible to development of spontaneous intestinal adenomas throughout their lifespan.

8. Evaluation of various biomarkers for inflammation and intestinal tumorigenesis

We conducted three successful animal trials to test the potential of microencapsulated probiotic yogurt formulation. The following biomarkers were investigated: IL-1β, 6, and 12, TNF-α, IFN-γ, IgA, PGE_2, CRP, Cox-2, thromboxane and fecal bile acids. In general, the inflammatory marker levels were lower in treatment animal groups compared to control animals. In comparison, the average levels of IL-6 in blood were 11.17±1.59 pg/mL for animals receiving APA microencapsulated *L. acidophilus* bacterial cells blended in 2% M.F. yogurt , 17.45±2.74 pg/mL for animals receiving APA microencapsulated *L. acidophilus* bacterial cells suspended in 0.85% saline and 18.33±1.46 pg/mL for the control group animals receiving empty APA microcapsules suspended in 0.85% saline, at the time of sacrifice.

C-reactive protein concentrations were measured using ELISA from plasma and they were found to be 81.04±23.73 ng/mL in control group animals receiving 0.85% saline solution and 64.21±16.64 ng/mL in treatment group animals gavaged with APA microencapsulated *L. acidophilus* bacterial cells blended in 2% M.F. yogurt ($p<0.05$).

The digesta obtained from the duodenum, jejunum, proximal and distal segments of the ileum were used to measure the secretory levels of IL-12, TNF-α, IFN-γ, IgA, PGE_2 and thromboxane.

Within the treatment group, luminal IL-12 concentrations were lowest in the jejunum (13.61±11.57 pg/mL) and the highest in the proximal ileum (43.55±8.97 pg/mL). In contrast, among the control group, luminal IL-12 concentrations were lowest in the duodenum (8.97±8.48 pg/mL) and highest in the distal ileum (22.79±4.76 pg/mL).

The concentration of luminal TNF-α was the highest in the distal ileum of the control group receiving empty APA microcapsules suspended in 0.85% saline, 30.12±9.08 (pg/mL) ($p<0.05$). In contrast, treatment group animals receiving APA microencapsulated *L. acidophilus* bacterial cells suspended in 0.85% saline had much lower concentration in all intestinal sections. Furthermore, same control group animals had a significantly higher concentration of luminal IFN-γ (151.52±22.86, 163.46±18.77, 156.15±17.8, 146.76±19.09 pg/mL in duodenum, jejunum and proximal and distal ileum, respectively) when compared to the same treatment group (103.77±12.07, 92.42±7.05,

107.21±17.95, 106.06±6.18 pg/mL in duodenum, jejunum and proximal and distal ileum, respectively)(p<0.05).

The concentration range of luminal IgA was observed to be between 28.68±3.48 and 33.07±3.29 (pg/mL) and therefore it did not differ within each intestinal section between the groups. The exception was the control animal group where the IgA concentration in the duodenum was 22.06±4.45 (pg/mL).

The concentration of luminal PGE_2 was the highest in control animals receiving 0.85% saline solution in all intestinal sections, especially in duodenum 1836.55±389.88 pg/mL. The thromboxane B2 concentration was especially high in all intestinal sections in the same control animal group and its range was from 51.27±23.53 pg/mL in duodenum to 35.50±13.16 pg/mL in distal ileum. On contrary, the levels of thromboxane B_2 found in treatment animal group receiving APA microencapsulated *L. acidophilus* bacterial cells blended in 2% M.F. yogurt were relatively low, ranging from 3.31±1.75 pg/mL in duodenum to 1.52±0.62 pg/mL in distal ileum.

In addition, fecal bile acid analysis was performed to determine the effect of microencapsulated probiotic bacteria on luminal bile acids. It was found that the greatest bile acid reduction was observed in treatment group animals receiving microencapsulated *L. acidophilus* bacterial cells in 0.85% saline, it was 33±3.7 (µmol/g fecal sample/100g BW). The untreated animals were found to have a concentration of 91.5±4.38 (µmol/g fecal sample/100g BW) of bile acids at the time of sacrifice.

The cox-2 analysis was performed by western blotting. Using distal ileum lysates we obtained a 71% lower expression of this enzyme in treatment group animals gavaged with APA microencapsulated *L. acidophilus* bacterial cells blended in 2% M.F. yogurt than in control group animals gavaged with 0.85% saline solution (p=0.018).
Based on these results we postulated that oral administration of live encapsulated bacterial cells has an overall immunomodulatory effect.

9. Investigation of the overall efficacy of microencapsulated probiotic cells on intestinal physiology using histology evaluation

The adenomas and gastrointestinal intraepithelial neoplasias (GIN) enumeration and classification were performed on all animals using histology evaluation.
In an animal trial with two experimental groups: Control (n=7) animals gavaged with empty APA microcapsules suspended in 0.85% saline and Treatment (n=7) animals gavaged with APA microencapsulated *L. acidophilus* bacterial cells suspended in 0.85% saline we obtained the following scores: in the small intestine, there were 3.5 adenomas and 10 GIN in the control group versus 1 adenomas and 9 GIN in the treatment group. In the large intestine of control animals, there were 0.5 adenomas and 2.25 GIN versus 0.2 adenomas and 0.2 GIN in treatment group.

In another animal trial including two experimental groups: Control (n=24) animals were gavaged 0.3 mL of 0.85% saline solution and treatment (n=24) animals were gavaged with APA microencapsulated *L. acidophilus* bacterial cells blended in 2% M.F. yogurt we obtained on average, 4.5±1.46 polyps per animal in control group compared to 2.5±1.60 polyps per animal in treatment group.

The morphology of tumors revealed mainly well differentiated pedunculated adenomas with high grade of dysplasia in a representative animal of the control group gavaged with empty APA microcapsules suspended in 0.85% saline compared to treatment groups receiving microencapsulated *L. acidophilus* bacterial cells blended in saline and 2% M.F. yogurt, respectively. A clear trend in controlling colon lesions was observed in the treatment groups. Histological analysis of treatment group receiving microencapsulated *L. acidophilus* bacterial cells blended in saline indicates that the animals had tumors in a more advance stage characterized by low grade of dysplasia and broad-based adenomas. In contrast, the animal in treatment group receiving microencapsulated *L. acidophilus* bacterial cells blended in 2% M.F. yogurt had mostly microadenomas. In general, we observed a decrease in incidence of polyp formation and pathologic tissue in treated animals with beneficial effect of microencapsulated probiotic cells in presence of yogurt.

10. Analyses of animals' intestinal tissues using immunohistochemical evaluation

Using CD8, Mac-1, Ki-67 and cleaved caspase-3 immunostaining as well as the Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Method we investigated the treatment effect on recruitment of T cells in animal tissues as well as cell proliferation and apoptosis.

In treatment group animals (gavaged with APA microencapsulated *L. acidophilus* bacterial cells blended in 2% M.F. yogurt) the levels of expressed CD8 cells were higher than control group (gavaged 0.85% saline solution), 1.93 and 6.87 %, respectively. Mac-1 expression was found in animal tissues from both control and treatment groups and it was 6.02 and 5.43%, respectively.

Cleaved caspase-3 was expressed in apoptotic cells, apoptotic bodies and in some tumor cells. Quantification using MatLab yielded 11.36 and 6.09 % expression of cleaved caspase-3 in control and treatment group animals, respectively.

Ki-67 staining was observed to be lower in adenomas of the treatment group as compared with adenomas of the control group. There were 8.72 and 5.00% of expressed Ki-67cells in control and treatment group animals, respectively.

The TUNEL results showed the highest concentration of apoptotic cells in healthy animal tissue, localized along intestinal villi. The absence of apoptotic cells was noted within polyps except for their base and surface.

11. Toxicology evaluations of novel yogurt formulation

When conducting separate animal trials we evaluated and recorded toxicology parameters. Appetite and general health was monitored daily in all animal trials. We recorded body weights on weekly basis. Results showed a steady drop in the body weight of control group animals (24.6±0.48 to 22.0±1.47 grams at the 12^{th} week of the treatment). However, a stable body weight was observed in animals receiving treatment; 24.2±0.47 to 24.0±1.32 grams (group receiving APA microencapsulated *L. acidophilus* bacterial cells blended in 2% M.F. yogurt) and 24.3± 0.36 to 24.0±0.71 grams (group receiving APA microencapsulated *L. acidophilus* bacterial cells suspended in 0.85% saline). After 17 weeks, rapid decline in body weight was observed in control group animals as compared to treated animals. All animals had a free access to water.

We also monitored animals' integument daily and paid special attention to such conditions as erythema, haircoat condition, status of hydration and pruritus. We did not observe any significant changes. Moreover, we monitored animals' equilibrium: unsteadiness on legs, coordination of legs and/or abnormal reflexes. Based on daily observations of animal overall health, we noticed lack of coordination along with slight body shivering in some animals of control group receiving empty APA microcapsules suspended in 0.85% saline. In addition, they had a body weight decrease of over 20% within a week. They were scheduled for euthanasia.

No changes in muscular disturbances such as generalized tremors, lip drooping and/or paralysis were noted in any animal. We also did not observe any cardiovascular problems such as unusual heart rate or high/low blood pressure.

The aforementioned control group animals were characterized by lack of energy and decreased motion rate. They acted restless with curled bodies and lowered heads. No aggression was ever observed in any of the experimental animals.
We also had no problems with animals' respiratory system.

At the end of study animals were sacrificed, dissected and all organs were analyzed. The majority of enlarged spleens was observed in control group animals. We recorded all animals' spleen lengths. The morphology of heart and kidneys was never altered. In a few cases in the control group animals we observed enlarged mammary glands filled with pus. No alterations in the liver were observed.

In addition to weekly fecal sample collection, we monitored animals for any blood presence in their rectum and feces. The results using fecal samples were recorded using the Hemoccult SENSA test.

9.2 Conclusions

Cancer, in general is a chronic pathologic process and colon cancer, in particular, has become one of the major causes of death in the industrialized world. Chronic intestinal inflammation is caused by an excessive immune response to mucosal antigens and elements of the normal bacterial microflora, inappropriately controlled by the normal counter regulatory mechanisms. In the pathogenesis of colorectal cancers inflammation is

considered to be the central player. The burden of colorectal cancer patients on the population is very high. Despite available screening, therapies and considerable effort to develop new and better treatment modalities, colorectal cancer still remains a major killer. The need for an alternative treatment method has never been more pertinent. The inspiration for this thesis was to design, formulate and characterize a novel probiotic yogurt based treatment containing microencapsulated live *L. acidophilus* bacterial cells capable of lowering the gastrointestinal inflammation by oral administration. Overall this thesis introduces a novel approach in colon cancer therapy by oral administration of a probiotic yogurt formulation.

9.3 Claims to original contributions

1. A novel probiotic yogurt based treatment containing microencapsulated live *L. acidophilus* bacterial cells was developed.

2. The 2% M.F. plain yogurt was used for the first time as an oral delivery system for microencapsulated live bacterial cells. It has been modified and characterized.

3. A method for encapsulating live bacterial cells in semipermeable capsule for use in in vitro and in vivo have been established and optimized.

4. Optimized physical and mechanical APA microcapsules properties were used in treatment preparation formulation.

5. Optimized APA microcapsules empty and loaded with live bacterial cells were successfully gavaged to animals.

6. The biomarkers analyses reveal that oral delivery of the novel probiotic formulation had an overall immunomodulatory effect on gastrointestinal tract in animals receiving treatment.

7. Histology evaluation showed a lower polyp incidence in animals receiving APA microencapsulated *L. acidophilus* bacterial cells blended in 2% M.F. plain yogurt.

8. Immunohistochemical analysis further revealed lowered GI inflammatory levels by expression of regulatory T cells which have a critical role in the suppression of immune responses and the presence of apoptotic cells.

9. The novel probiotic formulation containing microencapsulated *L. acidophilus* bacterial cells has been shown as an effective method in suppression of intestinal tumorigenesis in *Apc*Min mice.

10. Numerous toxicology evaluations proved this novel probiotic formulation safe.

11. This novel probiotic formulation has a potential in other GI related disorders such as Crohn's disease, inflammatory bowel disease and others.

CHAPTER 10

Recommendations

This thesis presents a novel and alternative approach with a potential in colon cancer treatment. To ensure the continued success and future development of probiotic yogurt formulation in this field, there are a number of issues that require further investigation. The following are suggestions of future initiatives that need to be addressed in depth in order to complete, determine and validate this project as a valid and functional anti colon cancer treatment:

- Understanding the complex mechanism of action of probiotic bacteria in yogurt formulation
- Understanding mechanisms involved in the modulation of the gut by the immune system, which consists of the innate and the acquired immune systems that induce both the systemic and the mucosal immune responses
- Studying the cross talk between the immune cells associated with the lamina propria and the intestinal epithelial cells and probiotic formulation – cellular studies
- Studying the microbial recognition via pattern recognition receptors (PRR) such as mannose receptor (MR) and Toll-like receptors (TLRs)
- Understanding the recognition of indigenous bacteria, including probiotic bacteria, by intestinal epithelial cells and their influence on homeostasis of the gut microflora
- Understanding how defective NF-κB signaling in the gut epithelium initiates the outbreak of inflammation in the intestine causing pathogenesis of inflammatory bowel disease leading to carcinogenesis
- Validating anti- and pro-inflammatory behavior of both endothelial and epithelial cells by screening for the protease-activated receptors (PAR)
- Understanding the network of signals involved in the GI immune response (types on interactions, kinds of signals)

- Conducting more animal trials on a larger scale and screening for additional parameters to validate the research potential
- Incorporating the probiotic formulation into other functional foods and screening for colorectal impact
- Establishing how this biotherapy may be used in the management of patients with existing gastrointestinal problems in clinical trials
- Understanding this formulation's efficacy in other animal/human models
- Implementing new formulations by meeting Food and Drug Administration guidelines to determine safety and dosing followed by efficacy studies to support claims

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APPENDICES