PROTEIN SYNTHESIS AND GASTROINTESTINAL PATHOPHYSIOLOGY IN A PIGLET MODEL OF COLITIS: IMPORTANCE OF NUTRITION AND PROBIOTICS

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A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of Doctor of Philosophy (Human Nutrition).

This thesis is dedicated to the memories of my father,

Jack Harding. Jr.

my brother

John Harding

my grandparents

Jack and Mary Harding, Sr.

Jim and Lucy Vinnicombe

this achievement would have been nice to share with you all

and to the best friend a man could ever have

Einstein

Make me feel tiny if it makes you feel tall, but there's always someone cooler than you…

Ben Folds, 2003

THESIS ABSTRACT

Objectives. Adequate nutrition and probiotics have both been shown to reduce the severity of colitis but their impact on hepatic and gastrointestinal protein metabolism has not been studied. Our primary objective was to compare the independent effect of probiotics vs. providing adequate nutrition on protein synthesis in a macronutrient-restricted piglet model of colitis. The secondary outcomes of this study were to determine histological contrasts and changes in oxidative stress markers resulting from probiotics in the malnourished state or providing adequate nutrition. Finally, we also measured mass balance and plasma concentrations of copper, iron and zinc over 5 days of colitis to determine how trace element nutrition is impacted by both colitis and probiotics.

Design and Analysis. Twenty-four piglets, receiving $1g \bullet kg^{-1} \bullet d^{-1}$ dextran sulphate (DS), were randomized to receive a 50% macronutrient restricted diet without (MR) and with probiotics (MR+PRO) or a diet providing 100% NRC requirements for growing piglets (WN). Eight other piglets were randomized into a well-nourished group without colitis (REF) for histological and trace element comparisons. A primed constant infusion of the tracer L-[ring-$^{2}H_{5}$]phenylalanine was performed to determine the protein synthesis in small intestinal mucosa, colon, liver and of plasma proteins. Standard, *in situ* and immunohistological staining techniques were used for histological assessment and the ferric reducing antioxidant power assay, ELISA F_{2}-isoprostane assay and plasma copper:zinc ratio were used as oxidative stress markers.

Results. Providing adequate nutrition increased protein synthesis in colon, liver and plasma albumin pool and decreased colitis severity. Probiotics stimulated protein synthesis in the liver as well as synthesis of all liver-derived plasma proteins, without affecting GI protein synthesis. Iron and zinc appear to be affected by both colitis and colitis with superimposed malnutrition but copper status was unaffected.

Conclusions. While probiotics did not appear to affect the gut (protein synthesis or colitis severity), a clear signalling mechanism between the gut and liver would appear to be responsible for the stimulation of liver protein synthesis. A strategy for correcting compromised nutrition appears to be more beneficial than using probiotics only for reducing damage during colitis accompanied by mild malnutrition.

RESUME

Objectifs La bonne nutrition et les probiotiques ont prouvé réduire la sévérité de la colite. Cependant, leur impact sur le métabolisme des protéines gastro-intestinales et hépatiques n'a pas encore été étudié. Notre objectif premier était de comparer l'effet indépendant des probiotiques à celui d'un régime alimentaire adéquat, sur la synthèse des protéines, chez le porcelet présentant une colite, recevant une diète restreinte en macronutriments. Le but secondaire de cette étude était de déterminer les contrastes histologiques ainsi que les variations au niveau des marqueurs du stress oxydatif, résultant de l'effet des probiotiques dans les deux cas de malnutrition et de nutrition adéquate. Nous avons aussi mesuré l'équilibre et les concentrations de cuivre, fer et zinc dans le plasma durant les 5 jours de colite, dans le but de déterminer comment les probiotiques et la colite affectent tous deux la balance des oligo-éléments.
Méthodes et analyses Vingt-quatre porcelets, recevant 1g/kg/jour de sulphate de dextran (DS), ont été randomisés en quatre groupes : deux groupes recevant une diète appauvrie en macronutriment à 50%, avec (MR+PRO) ou sans (MR) probiotiques, et un groupe recevant une diète contenant 100% des besoins nutritionnels pour porcelets, suivant les standards NRC (WN). Un quatrième groupe comprenant huit porcelets recevant une diète adéquate et ne présentant pas de colite (REF) a été inclus à des fins de comparaisons histologiques ainsi qu'au niveau des oligo-éléments. Une infusion constante du traceur L-[ring-$^{2}H_5$]phenylalanine a été effectuée pour déterminer la synthèse des protéines de la muqueuse de l'intestin grêle, colon, foie et plasma. Les techniques de « staining » standard, in situ et immunohistologiques ont été utilisées pour les déterminations histologiques, et des analyses du pouvoir antioxydant, ELISA F_2-isoprostane assay, ainsi que le ratio cuivre:zinc ont été utilisé comme marqueurs du stress oxydatif.
Résultats : La nutrition adéquate a augmenté la synthèse des protéines au niveau du colon, foie et l'albumine du plasma. Elle a aussi réduit la sévérité de la colite. Les probiotiques ont stimulé la synthèse des protéines hépatiques ainsi que les protéines du plasma dérivées du foie. Cependant les probiotiques n'ont pas affecté la synthèse des protéines gastro-intestinales. Les niveaux de fer et de zinc sembleraient être affectés par la colite seule ainsi que par la colite accompagnée de malnutrition. Le cuivre lui n'a pas été affecté.

Conclusions Malgré que les probiotiques semblent ne pas affecter l'appareil digestif (synthèse de protéines et sévérité de la colite), il existe un mécanisme de communication entre celui-ci et le foie qui serait à l'origine de la stimulation de la synthèse de protéines au niveau du foie. Il serait donc plus avantageux de corriger la malnutrition que d'administrer des probiotiques, le seul but étant de réduire les dégâts causés par la colite accompagnée d'une malnutrition modérée.

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LIST OF ABBREVIATIONS

5-aminosalicylic acid	5-ASA
Absolute Synthesis Rate	ASR
Acute Phase Proteins	APP
Acute Phase Response	APR
Crohn's Disease	CD
Dextran Sulphate	DS
Dinitrobenzene Sodium	DNBS
Ethylenediaminetetraacetic Acid	EDTA
Fractional Synthesis Rate	FSR
Gamma glutayl leucine	GL
Gastrointestinal	GI
Inflammatory Bowel Disease	IBD
Insulin Like Growth Factor 1	IGF-1
Interleukin	IL
Lamina Propria Mononuclear Cells	LPMC
Macronutrient Restricted with colitis	MR
Macronutrient restricted with colitis and probiotics	MRP
Macronutrient restricted groups combined	MR-C
Metallothionein	MT
Myleoperoxidase	MPO
Nitric Oxide	NO
Non-Alcoholic Fatty Liver Disease	NAFLD
Perinuclear Anti-Neutrophil Cytoplasmic Antibodies	p-ANCA
Polyethylene glycol	PEG
Protein Energy Malnutrition	PEM
Reactive Nitrogen Species	RNS
Reactive Oxygen Species	ROS
Reference Group – Well-nourished, no colitis	REF
Resting Energy Expenditure	REE
Saacharomyces cerevisae Antibodies	ASCA
Toll Like Receptor 9	TLR-9
Trinitrobenzenesulfonic Acid	TNBS
Tumour Necrosis Factor Alpha	TNF-α
Ulcerative Colitis	UC
Well-nourished piglets with colitis	WNC

CONTRIBUTIONS OF AUTHORS

Scott V. Harding*

Mr. Harding was the sole PhD student working on this study. He was responsible assisting in the study design, diet formulation, conducting the animal surgeries and trial, isotope infusions and sample analysis (including calculations) associated with this study. He carried out sample analysis including GC-MS analysis for protein synthesis, histological grading, immunohistological staining, TUNEL staining, FRAP assay, F_2-isoprostane assay and all trace element analysis using AAS. Mr. Harding was also responsible for all statistical analysis of data relating to this study.

Keely G. Fraser*

Ms. Fraser was the sole MSc student working on this study. She was responsible for conducting the animal surgeries and trial, isotope infusions and sample analysis (including calculations) associated with this study. She also carried out sample preparation, GC-MS analysis and protein synthesis calculations.

Dr. Linda J. Wykes

Dr. Wykes was the study and thesis supervisor for both Mr. Harding and Ms. Fraser. Dr. Wykes trained both students in piglet surgery, isotope infusion experiments, GC-MS analysis and protein synthesis calculations. The study was funded and fell under the wider range of research in her laboratory. Dr. Wykes was also responsible for study design and reviewing the manuscripts and other materials in this thesis.

**Contributed equally to the piglet surgeries, piglet trial, isotope infusions, GC-MS analysis and protein synthesis calculations.*

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STATEMENT OF ORIGINALITY

The study and subsequent sample analysis conducted and reported in this thesis has applied several well established techniques for assessing metabolic outcomes to test the use of probiotics during active colitis. The following aspects of the study design, to which the candidate contributed, are unique and constitute a distinct contribution to knowledge:

1. Further development of a persistent gastrointestinal inflammatory piglet model useful in the study of human nutrient-disease interactions.
2. Assisting in the development of a dual stable isotope amino acid infusion protocol, with the aim of measuring the synthesis rate of specific proteins while simultaneously measuring the synthesis rate of the tri-peptide antioxidant glutathione.
3. Design of a liquid based piglet diet which can be easily manipulated to study macronutrient and micronutrient deficiencies while being delivered enterally via gastric catheter.

Specific original findings of the studies presented in this thesis which make new contributions to knowledge are:

1. These studies are the first to assess the effect of probiotics on protein synthesis rates in the gastrointestinal tract, liver, skeletal muscle and for liver-derived plasma proteins and trace element balance during colitis.
2. These studies are also the first to explore the effect of dextran sulphate colitis on trace element balance and plasma concentrations.
3. Our finding of reduced disease severity in piglets receiving adequate nutrition during DS-induced colitis when compared to no treatment and probiotic treatment support revisiting the role of nutritional support during colitis as a means to control the inflammation.
4. Providing adequate nutrition during active colitis maintained healthy rates of protein synthesis in tissues and plasma proteins. These findings further support the importance of addressing nutritional status during the treatment of IBD.

5. Probiotic stimulation of protein synthesis in the liver, for both constitutive and secreted proteins, has not been previously demonstrated in colitis models. This unique finding requires further study to better determine the implications of this stimulation, especially in the malnourished state.
6. Iron and zinc status during colitis appears to be affected independently by both colitis and macronutrient restriction. These findings require closer study to determine the mechanism responsible the increased requirement of these trace element during DS-induced colitis.
7. In a more general sense the findings contained in this thesis have shown how probiotics can affect metabolism, specifically protein metabolism, in tissues other than the gastrointestinal tract. Also, these findings clearly demonstrate how avoiding macronutrient restriction during gastrointestinal inflammation reduces the severity of the insult.

Chapter 1

Introduction, Objectives and Hypothesis

INTRODUCTION AND RATIONALE

Inflammatory bowel disease (IBD) is a collection of several distinct inflammatory conditions of the gut with an assortment of potential triggers and risk factors (1). Current research directions of these diseases could be divided into two streams; 1) a search for a cure of each condition and 2) strategies to better control symptoms of the diseases (2). Research focusing on strategies that reduce disease activity such as maintaining nutritional status and probiotic administration provide valuable information for improving quality of life during active IBD.

Maintaining healthy body composition and nutritional status during IBD is important for reducing disease activity and improving disease control (3). However, the use of nutritional therapy as a primary treatment for the various forms of IBD is controversial (3, 4). Nutritional status is usually affected to some extent in all forms of IBD, ranging from micronutrient deficiencies to substantial lean body catabolism (5-9). Despite the fact that improving nutritional status has been shown to reduce disease activity in IBD, nutritional support is not routinely used until substantial changes in nutritional status occur (4, 10).

Probiotics are emerging as a potential companion therapy for conventional pharmaceutical and surgical management of IBD. The administration of probiotics, such as the commercial preparation VSL#3®, have been shown to induce remission in pouchitis and colitis patients (11-14). It has recently been proposed that probiotics exert an anti-inflammatory effect on the host's immune system, helping to reduce the extensive oxidative damage associated with the disease (15). Identifying the specific metabolic effects of these treatments during active disease is key to understanding their role in controlling IBD symptoms.

While clinical priority is given to the proven pharmaceutical and surgical treatments, complementary treatments such as probiotics are becoming more popular. Medical information from all areas of the world is easily accessible to the average person via the Internet. The general public is also exposed to media related to treatment options for

diseases like IBD which might not have been adequately tested. Probiotics and supplemental nutrition are 2 such treatment options with which individuals can self-medicate without medical supervision and without understanding the potential effects of doing so. A clearer understanding of the independent roles of both maintaining nutritional status and of probiotic use for treating IBD is needed. During active IBD the body is in a unique metabolic condition, proinflammatory cytokines and acute phase reactants lead to a catabolic state characterized by the loss of tissue protein and insulin resistance (16, 17). In addition, these individuals are usually reducing their food intakes (behaviour and cytokine driven) and possibly receiving glucocorticoid treatment (immunosuppressive but catabolic), which together would exacerbate the catabolic state (10).

Providing adequate nutrition or probiotics during inflammatory stress have both been shown to reduce proinflammatory cytokines and improve outcomes in clinical and experimental models of inflammation like colitis (11, 15, 18, 19). Nonetheless, there is a lack of information as to how either of these complementary treatments affects other metabolic and nutritional outcomes in IBD, such as protein synthesis and trace element status. Therefore, our global objective was to characterize how adequate nutrition and the probiotics mixture VSL#3 independently affect nutritional outcomes such as tissue protein synthesis, growth, lean body protein accretion and trace element status. We also intended to compare how each treatment affected histological damage, apoptosis, cell proliferation and inflammatory stress markers.

We have chosen the piglet model of colitis to conduct these investigations for several reasons. Firstly, the piglet is very well characterized as a model for nutritional, physiological and gastrointestinal research (20). Secondly, our choice to use the piglet colitis model was also based on the anatomical and physiological resemblance of the swine gut to that of humans, and also due to the similarities in amino acid metabolism (21, 22). Our laboratory has had previous success using this model to study protein synthesis during acute colitis (23). The current studies use a longer duration of dextran sulphate-induced colitis to mimic a chronic flare-up of the disease.

OBJECTIVES

The objectives of the work contained in this thesis were:

1. To compare protein synthesis rates of ileal mucosa, colon, liver and liver-derived plasma proteins in macronutrient restricted piglets receiving VSL#3 probiotics and piglets receiving adequate nutrition, both with dextran sulphate-induced colitis.
2. To compare growth and muscle tissue protein synthesis rate in macronutrient restricted piglets receiving VSL#3 probiotics and piglets receiving adequate nutrition, both with dextran sulphate-induced colitis.
3. To compare colon histological damage, epithelial colonocyte rates of apoptosis and cell proliferation and oxidative stress in the colon and whole body in macronutrient restricted piglets receiving VSL#3 probiotics and piglets receiving adequate nutrition, both with dextran sulphate-induced colitis.
4. To determine the effect of colitis on the mass balance of copper, iron and zinc in the well nourished state, and then to determine the impact of administration of VSL#3 probiotics in macronutrient restricted piglets with colitis.

THESIS HYPOTHESIS

Two main hypotheses are at the core of the stated objectives of this thesis:

1. The maintaining adequate nutrition during acute persistent dextran sulphate induced colitis will have a negligible effect on growth, trace element balance, protein synthesis rates and intestinal damage compared to healthy piglets without colitis.
2. Providing probiotic bacteria to piglets during acute persistent dextran sulphate induced colitis with superimposed macronutrient restriction will improve intestinal protein synthesis rates, decrease intestinal damage and improve trace element balance compared to macronutrient restricted with colitis not receiving probiotics.

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Chapter 2

Literature Review

INFLAMMATORY BOWEL DISEASE: A COLLECTION OF DISEASES

Inflammatory bowel disease is a collection of gastrointestinal diseases, most notably Crohn's disease (CD) and ulcerative colitis (UC), and also refers to intermediate colitis, pouchitis and microscopic colitides (1). Whereas all disease classifications involve inflammation of the gastrointestinal tract, they each have distinct diagnostic features. Classification of the specific type of IBD usually involves clinical, radiographic, endoscopic and histological examination, which in a number of cases is still not conclusive (2). Indeterminate colitis is diagnosed when conflicting diagnostic factors exist in the subject, preventing clear distinction between UC and CD (2). Pouchitis is an inflammation of the pouch in a colectomy with ileoanal anastomosis and pouch creation (1, 2). Microscopic colitide is characterized by chronic diarrhea with a normal macroscopic appearance of the colon; however upon biopsy microscopic lesions are identified (1).

Ulcerative Colitis

Ulcerative colitis is inflammation of the colon mucosa, always involving the rectum, usually other segments of the colon, and secondarily affecting the small bowel through backwashing of the colon contents. Inflammation of the colon during active UC is usually accompanied by bloody stool, reported in 80 – 90% of diagnosed subjects. Severe UC is usually associated with severe diarrhea (> 6 bowel movements per day), fever, tachycardia and overt anaemia (3). Ulcerative colitis can be associated with weight loss and growth failure but not to the same extent as in CD (4).

Crohn's Disease

Crohn's disease can affect any part of the GI tract from the mouth to the anus and usually manifests itself as multi-site lesions surrounded by sections of normal intestinal tissue (3). Crohn's disease usually affects the mucosa but can also affect the muscle, serosa and fat tissues, depending on severity. The inflammation during active CD can cause thickening of the GI tract wall , in turn leading to failure to properly digest nutrients. Active CD is usually always accompanied by weight loss as well as vomiting, diarrhea, constipation and cramping and growth failure is common in paediatric cases (4).

While both CD and UC have similar symptoms, treatments differ slightly; therefore correct diagnosis is essential for proper disease management (2). Perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA) and anti-Saacharomyces cerevisae antibodies (ASCA) are becoming more routinely assayed to confirm diagnosis of the disease (5, 6). Sixty to seventy percent of UC patients will have p-ANCA in a serum sample while only 10% of CD patients will have this antibody. Conversely, 60 – 70% of CD patients will present with the ASCA compared to only 10% of the UC patients (2). This serological marker is proving valuable in diagnosis of ambiguous IBD and has the potential to avert unnecessary endoscopy in paediatric cases of IBD (5).

Brief Epidemiology of IBD

The incidence of IBD has risen over the past 50 years as a result of better understanding and diagnosis of the disease leading to IBD becoming one of the biggest problems in gastrointestinal health for the developed world (7). The prevalence of UC and CD differ from each other but both are more prevalent in populations descending from Northern European and Jewish lineage. Prevalence of UC ranges from 35 - 100 per 100,000 (incidence: 2 – 10 per 100,000/year) while CD ranges from 10 - 100 per 100,000 (incidence: 1 – 6 per 100,000/year) (3, 7, 8). While women tend to be more at risk for developing CD, sex and age differences are not heavily weighted risk factors (9). Noteworthy is the almost non-existence of CD in children under 6 years of age, no rationale for this observation has been proposed (9). While the epidemiological evidence has characterized the risk of the disease it offers no clear insight to the etiology of either disease other then it is a complex interaction between environment and genetic predispositions. Six gene loci have been identified as being related to the incidence of IBD. It appears that the disease may be largely determined by genetic factors but not in a Mendelian pattern of inheritance (7).

Free Radical Mediated Damage in IBD

While the etiology and initial triggers of IBD are still the topic of vigorous debate, it is accepted that the damage to the intestinal tract is due, to a large extent, to reactive oxygen

and nitrogen species (ROS and RNS, respectively) (10-12). Normal antioxidant controls are overwhelmed and a dysfunction in normal immune control leads to lesion formation and further oxidative damage (13). The composition of the digestive milieu is likely to play in important role in the initiation and propagation of free radicals at the mucosal barrier. There are many compounds in our diet that are either oxidizing agents themselves or contribute to the production of free radicals in the gut (i.e. free or cheated iron complexes such as Fe-EDTA) (14). Care should also be taken in the treatment of anaemia which is common in IBD patients, supplemental iron could be deleterious to inducing remission (15). Once the antioxidant systems of the intestinal mucosa are overwhelmed, as is the cases in active flares of IBD, Fenton type chemistry leads to the proliferation of free radicals and subsequently damage to the surface of the intestinal barrier and within the interstitial spaces.

$$O_2^{\bullet -} + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad (1)$$

$$2O_2^{\bullet -} + 2H^{+} \rightarrow H_2O_2 + O_2 \quad (2)$$

$$H^{+} + Fe^{2+} + H_2O_2 \rightarrow HO^{\bullet} + Fe^{3+} + H_2O \quad (3)$$

Sustained inflammation will eventually lead to a loss of mucosal integrity and increases in oxidizing substances in the GI tract (i.e. bleeding releases heme iron and free iron in to GI tract). Also important to the generation of ROS in an active flare of IBD is the substantial infiltration of activated neutrophils, which produce high amounts of superoxide radical, $O_2^{\bullet -}$ (>90% of O_2 consumed by neutrophils is released as $O_2^{\bullet -}$). The activated neutrophils also contribute to the oxidative damage and ROS proliferation by the myleoperoxidase (MPO) activity they display. As shown below the hypochlorous acid formed by MPO reacts with primary amino groups forming N-chloroamino derivatives which are known to be cytotoxic (14).

$$H_2O_2 + Cl^{-} \xrightarrow{MPO} H_2O + HOCl \quad (4)$$

$$HOCl + RNH_2 \rightarrow H_2O + RNHCl \quad (5)$$

The oxidative damage and release of cytokines and chemokines from immune cells infiltrating the damaged areas elicits further recruitment of activated leukocytes to the

area, perpetuating the cycle (13). Production of nitric oxide (NO), a potent oxidizing agent, is regulated through the increased expression of inducible NO synthase and NO actually appears to have antioxidant properties in the IBD. Immune cell adhesion in the areas of inflammation is reduced by the production of NO (16). Despite NO concentrations being correlated to disease severity and NO contributing to the proliferation of free radicals in inflamed areas, its presence maybe more beneficial then harmful (12, 17).

Current Treatment Practices in IBD

The ultimate goal of UC and CD treatment is to achieve total remission and to prevent flare-ups or prolong the time between attacks. Currently, both UC and CD patients are prescribed oral and topical aminosalicylates. These drugs have been developed to act either in the colon only (Dipentum® or Colazal™) or in both small intestine and colon (Asacol®). In moderate to severe IBD aminosalicylates are used in concert with systemic and non-systemic corticosteroids to achieve remission of the disease. Antibiotics have also been used, with some success, to induce remission in moderate to severe colonic CD (Flagyl® and Cipro®). The potent immunosuppressant cyclosporine and other immunomodulators (6-mercaptopurine (Purinethol®) are also now used to treat IBD with increasing frequency. Infliximab (Remicade™), a chimeric (mouse/human) monoclonal antibody to TNF-α and is used in current therapeutic approaches to CD. In severe cases of UC surgical interventions or bowel rest through TPN are necessary. In paediatric cases, where growth failure is a risk, enteral feeding is also started. While not a treatment per se, enteral feeding and TPN are used to prevent growth failure by providing extra energy (18, 19). However there maybe some benefit in providing enteral nutrition during active disease with respect to inducing remission (18, 19). Nevertheless, the foundations for nutritional therapy are not based on randomized trials but clinical experience, making it difficult to identify a useful regime (20). Unfortunately, the lack of information as to the trigger(s) of the disease and incomplete knowledge of pathogenesis prevent a more patient specific therapy. Instead most IBD patients are treated globally using one if not all these treatments usually jointly with dietary consultation.

Possible future avenues for treatment of IBD include novel biochemical treatments, treatment delivery-enemas, and probiotics. Epidermal growth factor enemas in UC patients have promising potential but are still without proper validation (21, 22). Butyrate and other short chain fatty acid enemas have also been researched with favourable results (23). Animal studies of $ZnSO_4$ enemas have also shown promise as a treatment of colitis, however no human trials have been conducted (24, 25). Probiotics are emerging as possible treatment options however much work is still required to determine the efficacy and sustainability of changing the gut microflora (26). While these treatments are gaining much attention in terms of research none are mainstream alternatives to the current pharmaceutical approaches to treatment (27).

PROBIOTICS AND IBD

Role of Probiotics in Gastrointestinal Health

A number of definitions for probiotics have been proposed; in general they are live micro-organisms which exist naturally in the gastrointestinal tract of humans but are provided in supplemental form to enhance health (28-30). The beneficial health effects are attributed to their capacity to affect immune responses both in the intestinal mucosa and systemically, as well as exerting effects on nutritional and microbial factors in the GI tract (31). Lactobacilli and bifidobacteria are the most commonly studied species but *E. coli Nissle 1917* and streptococcus along with some fungi have also been investigated (31). While probiotics are commonly prescribed for specific gastrointestinal conditions (diarrhoea, inflammatory bowel disease) in Europe they are less commonly used by physicians in North America, being considered alternative or complementary treatments (28, 32, 33). The use of probiotics for the treatment of various types of diarrhoea is quite effective and has been well established but there is still debate as to their effectiveness in treating inflammatory bowel disease (28, 33).

Probiotic Effectiveness in Treating IBD: Clinical Studies

Patient with pouchitis and ulcerative colitis respond better to probiotic therapy than those with Crohn's disease (34). The Gionchetti and associates' randomized double blinded trial of the effectiveness of probiotics (VSL#3) to maintain pharmacologically induced

remission in patients with pouchitis clearly showed the positive role they play in long term treatment of the inflammation (35). Only 15% of the patients receiving probiotics relapsed in the 9 month follow-up period compared to 100% relapse in the placebo group (35). The results were confirmed in a second study with antibiotic-induced remission of pouchitis. This study reported 85% of patients receiving probiotics maintained remission over the 1 year follow-up versus only 6% of the placebo group (36). Probiotics have also been shown to be effective in prevention of inflammatory flare-ups in patients with ileal pouch-anal anastomosis surgery using VSL#3 as prophylactic prevention of acute pouchitis (37). In this study only 10% of patients receiving probiotics recorded an acute episode in the 12 month follow-up compared to 40% in the placebo group (37). Very favourable outcomes have been demonstrated for probiotic treatment of pouchitis but it is still inconclusive as a treatment option for colitis.

While the use of probiotics in the treatment or maintenance of colitis is not common in North America it is becoming increasingly discussed in clinical settings (27). This may be in part due to the increased popularity of probiotics in the commercial health product industry (38). While there have been no double blinded clinical trials examining the effectiveness of VSL#3 or any other probiotics for treating UC, several open labelled trials have been conducted with promising results. Venturi and associates first demonstrated a role for VSL#3 in the maintenance of ulcerative colitis in an open label trial which saw 75% of patients maintaining remission after 12 months of probiotic therapy (39). Then, Bibiloni and associates, in another open label trial, demonstrated the effectiveness of VSL#3 to induce remission in active ulcerative colitis. They reported 77% of the patients in the study responding to probiotic therapy when previous attempts using conventional methods to obtain remission failed. VSL#3 has also been shown to be effective as a co-treatment for diverticular colitis when used with the glucocorticoid, beclomethasone (40). The same authors showed the capacity of VSL#3 combined with balsalazide (pro-drug version of 5-ASA which is activated in the colon by resident bacteria) to achieve remission faster (4 days vs. 7.5 and 13 days) than through drug treatment alone (balsalazide or mesalazine) (41).

Milk products fermented using lactic acid bacteria, like bifidobacteria, have been reported to help induce remission of colitis when used in combination with pharmacological means. Ishikawa and associates showed the effectiveness of a bifidobacteria-fermented milk product to improve disease outcomes in colitis patients in combination with salazosulfapyridine, mesalazine and steroids (42). The authors reported only 27% of those receiving the milk product had worsening of the colitis while 90% of those receiving only pharmacological treatment had worsened disease indicators (42). Similar findings were reported by Kato and associates who also examined the use of a bifidobacteria fermented milk product in conjunction with standard 5-ASA treatment to improve disease activity. They reported higher reductions in disease activity when both fermented milk products and 5-ASA were used in combination versus pharmacological treatment only (43). Unfortunately, there have not been any randomized placebo controlled trials of probiotic treatment in ulcerative colitis. However, there is mounting experimental data which points to a possible role for probiotics in reducing the proinflammatory immune response during colitis.

Probiotics and Experimental Models of Colitis

The most common experimental models of IBD are chemically induced colitis (dextran sodium sulphate or DS, 2,4,6-trinitrobenzene sulfonic acid or TNBS) and the IL-10 knockout mouse model of colitis (44-46). While the histological presentation of these models is similar to colitis they each elicit an immune response different from human IBD. Despite this fact they are each very robust models of colitis and testing potential treatments in these models clarifies the mechanism of action of potential treatments.

Responses to probiotic treatment in experimental models of colitis are variable. Shibolet and associates studied the effect of 2 different types of probiotic treatment in two models of colitis in rats, dinitrobenzene sulfonic acid (DNBS) and iodoacetamide induced colitis. They found both types of probiotics (VSL#3 and Lactobacillus GG) were effective for reducing all the parameters of disease activity for the iodoacetamide induced colitis but had no effect in the DNBS model (47). These findings point toward a specific action associated with the beneficial properties of probiotic administration during colitis. By

characterizing the damage associated with each model understanding the specific mechanism of action of probiotics becomes clearer.

Probiotics have been reported to mediate improvements of the gut barrier integrity and reduce bacterial translocation in rodents without colitis but under psychological stress (48). Zareie and collegues exploited the water avoidance behaviour of rats to induce chronic psychological stress. Chronic psychological stress has been shown to lead to some loss of mucosal integrity and increase susceptibility to bacterial pathogenesis (49). By supplementing with 2 species of lactic acid bacteria, *Lactobacillus rhamnosus* and *Lactobacillus helveticus*, the authors were able to demonstrate reduced bacterial adherence to the mucosa and prevented bacterial translocation to the mesenteric lymph nodes. This outcome would be quite favorable in colitis if these properties of the probiotics were achievable during colitis.

Madsen and colleagues suggested certain species of bacteria in the VSL#3 mixture protect the mucosal barrier via expression of a particular protein which alters the intestinal mucosa (50). Their study, using IL-10 deficient mice and human T_{84} intestinal epithelial cell, demonstrated capacity of VSL#3 to reduce TNF-α concentrations in both ileum and colon, improve epithelium function and histological parameters. Similarly, Osman and associates demonstrated a protective role for individual lactic acid species (51). This study, in a rodent DS-colitis model, showed improved disease activity scores and reduced bacterial translocation in rats receiving *L. plantarum, Bifidobacterium* sp. 3B1 and *B. infantis,* which are components of the VSL#3 mixture. *Lactobacillus casei* was also shown to be protective in rats inoculated with this probiotic prior to TNBS induced colitis (52). The authors reported a reduction in the percentage of mucosal area damaged, reduced myleoperoxidase activity and less bacterial translocation to the mesenteric lymph nodes, liver and spleen in *L. casei* treated rats compared to controls. A recent study by the same group identified part of the possible mechanism for the *L. casei* capacity to reduce damage indicators in TNBS-colits (53). Pre-treating rats with *L. casei* lead to decreased expression of ICAM-1, a molecule required for leukocyte acute adhesion in inflamed tissue. Interfering with the immune cell recruitment and adhesion

would be an effective characteristic for this probiotic in reducing the inflammatory damage associated with colitis.

A study by Gaudier and associates have reported contradictory results as to the capacity of VSL#3 to reduce disease severity or enhance barrier function. The authors demonstrated, using a rodent DS-induced colitis model, the capacity of VSL#3 to survive the gastrointestinal tract and colonize the colon, however they saw no reduction in the severity of the colitis (54). The authors also assessed the capacity of probiotics to increase the expression of mucin in the colon, as this would be a possible mechanism by which probiotics may indeed protect the mucosa. No changes in the expression of colonic mucins, *Muc1-4*, were attributed to VSL#3 and was there was no difference in mucus wall thickness due to treatment.

There is also evidence to suggest the probiotic bacteria need not be viable or colonize the colon microenvironment. Rachmilewitz and associates examined the effect of providing live versus nonviable, irradiated VSL#3 to mice with DS-induced colitis (55). The nonviable bacteria were just as effective as the viable bacteria for reducing disease severity and MPO activity compared to control mice. The mice receiving the nonviable bacteria also demonstrated better histological scores than both the controls and those receiving viable VSL#3. The authors went on to suggest oral ingestion is not required and that toll-like receptors, especially TLR-9, may be the key mediator of this action. Using mice with TNBS-induced colitis and subcutaneous injections of probiotic DNA, the authors were able to demonstrate reduced disease activity score, lower colon MPO activity and lower histological damages scores versus controls. In summary, the authors demonstrated that the presence of components of the probiotics bacteria versus the re-colonization of the colonic microflora was the key component of the probiotic anti-inflammatory properties.

The anti-inflammatory role of VSL#3 was also confirmed by Di Giacinto and colleagues, studying how probiotics affect recurrent TNBS-colitis in mice. In this study VSL#3 probiotics stimulated the increased secretion of IL-10 from lamina propria mononuclear

cells (LPMC) (56). The authors were also able to demonstrate the role IL-10 played in the reduced severity of the recurrent colitis by blocking the effect of IL-10 using anti-IL-10 receptor antibodies. The VSL#3 ameliorated the recurrent TNBS-induced colitis but this protective effect was blocked when anti-IL-10R antibodies were administered during VSL#3 treatment.

The anti-inflammatory role of probiotics has also been researched for treating liver disease. Loguercio and associates studied the effect of providing VSL#3 to patients with various forms of liver disease (57). VSL#3, administered for 90 and 120 days in patients with alcohol induced cirrhosis, lead to decreases in circulating liver enzymes, increased plasma total protein and albumin concentrations while lowering bilirubin and decreasing TNF-α and IL-6 concentration while increasing IL-10 concentrations (57). These findings and the reductions in circulating lipid peroxidation products indicate VSL#3 is able to modulate immune function beyond the gastrointestinal tract. While these data are in line with the data generated from studies of VSL#3 in gastrointestinal diseases they need confirmation through via a double blinded clinical trial.

Similarly, Li and colleagues using a mouse model of non-alcoholic fatty liver disease (NAFLD), demonstrated VSL#3 reduced circulating liver enzymes and lead to anti-inflammatory changes in mice receiving the probiotics versus controls (58). The study also used anti-TNF-α antibodies as a third treatment group. The authors reported similar patterns for both treatment groups compared to controls for the anti-inflammatory changes which occurred. Both treatment groups had reduced JNK activity (TNF-α activated stress kinase) compared to NAFLD control mice but higher TNF-α mRNA levels than healthy mice suggesting probiotics interfere with TNF-α function versus reducing expression.

Overall, probiotics, especially VSL#3, are emerging as a possible immuno-modulating treatment for gastrointestinal and hepatic diseases. Early indication as to mechanism action appeared to be related to direct protection of the mucosa and changes in intestinal microflora but research focus is shifting to investigate the metabolic changes probiotics

cause in tissues and mapping the specific signalling pathways involved (59). The other properties which have been attributed to probiotic administration need further study. Specifically, whether probiotics affect nutritional outcomes, both in healthy and inflamed situations, requires investigation (31). Other metabolic functions of probiotics need to be described and well controlled double blinded clinical trial for effectiveness of ameliorating inflammation in humans is needed.

NUTRITIONAL STATUS AND IBD

Anorexia and Growth Failure

Individuals with IBD, treated and untreated, usually become mildly anorexic due to either deliberate food avoidance or cytokine induced anorexia. There are a number of aspects of the pathology of IBD including protein-energy malnutrition, micronutrient deficiencies, treatment measures, or the heightened/altered immune response, which can lead to a sustained catabolic state (60, 61).

Weight loss is a common outcome in active IBD; 65 – 75% CD patients and 18 – 62% UC patients with 33 – 85% prevalence in paediatric cases of UC and CD (19, 61). Nutritional assessment of CD and UC will usually detect mild PEM if present, yet most micronutrient deficiencies are sub-clinical in mild to moderate IBD and therefore untreated. Weight loss during an active flare of IBD is generally associated with decreased energy intake, but as discussed previously the condition may be a result of the complex metabolic response occurring during active IBD.

Deficiencies of both macro and micronutrients during critical periods of growth can have long-lasting impacts for a growing child, including brain and nervous system impairments (psychomotor and cognitive delays) and growth failure (decrease stature, poor musculature and bone development) (62). Losses of lean body mass and changes in protein synthesis are of particular concern in paediatric cases of CD and UC. Growth failure has been an established aspect of paediatric IBD since the mid seventies and has generally been attributed to the mild anorexia which almost always accompanies active CD and to a lesser extent UC (63-65). Impaired synthesis of hormones and growth

factors in growing children maybe involved in the growth stunting which is seen in this population. The decrease seen in insulin like growth factor 1 (IGF-1) in children with IBD has been linked to the malnutrition rather then the acute phase response. The capacity to increase IGF-1 through re-feeding is a promising discovery in identifying what factors cause growth failure through the mild malnutrition seen in active CD and UC (66).

Decreased energy intake as an underlying cause for growth failure is supported by 85% CD and 65% UC patients having significant lost weight at the time of diagnosis of the disease and that enterally feeding paediatric patients with active disease promotes growth catch-up (19, 67, 68). Growth failure is a negative deviation from an established growth curve path resulting in a decrease in height velocity. If this fall is greater then 1 standard deviation or more compared to the normal population it constitutes a growth failure. Growth failures are usually corrected by nutritional interventions. While the clinician may not always detect a growth failure, the set-backs are most evident when linear growth is tracked over time on individuals (66). McCartney and Ballinger (1999) suggest that the growth failure observed in paediatric IBD cases has actually three components: a) decreased energy intake, b) increased energy requirements and c) excessive loss of nutrients from the gut (69). While this model has merit it excludes other complexities of the disease, which impact nutritional status and growth. The more comprehensive model of growth failure which includes the anabolic response of the mucosa (inflammation and repair) and liver (production of acute phase proteins) coupled with the cytokine/glucocorticoid-mediated catabolism of muscle protein and the nutritional component is better suited for investigation of growth and development during IBD (70).

Energy Requirements during IBD

Increased energy requirements are a consequence of acute phase response (catabolic state), repair of damaged tissues, increased metabolic requirements as a result of steroidal therapies or combination of all these factors (27, 71). Standard IBD corticosteroid treatment itself can causes energy deficits via the unmatched increase metabolic/energy requirements, a common avenue of treatment for the disease along with 5-aminosalicylic

acid. A conservative estimate of the increase resting energy expenditure (REE) in IBD is +19% for colitis patients and would be expected to be higher in CD patients depending on severity of attack (72). Decreased energy intake is usually present in active cases of both CD and UC due to either abdominal discomfort or appetite suppression from elevated cytokine production (73). The mild to moderate malnutrition seen in active CD and UC is not comparable to common PEM, which occurs by decreased energy and protein intakes. In voluntary or disease free restriction of energy and protein (i.e. anorexia nervosa) the body conserves both energy and protein by reducing energy expenditures and protein synthesis, respectively (71). This decrease in REE is not seen in UC or CD patients (71, 72). Weight loss is therefore very common in those with acute attacks; it also tends to explain the high percentage of those newly diagnosed suffering from significant weight loss (65-75% in CD and 20-60% in UC) (74).

Other Contributing Factors to IBD Progression

Nutritional deficiencies are common to both CD and UC and occur with higher frequency and severity in CD (61, 75). Along with the pathological reasons for the decrease in energy intake are the social aspects to the disease such as depressed mood, fear of eating/associated pain and reduced intake on medical advice (76). Each of these psychosocial aspects of the disease could have an appreciable effect on energy consumed but are difficult to measure in a population as to how much they contribute to malnutrition.

PROTEIN METABOLISM IN IBD

Protein Turnover

Unlike other macronutrients, protein and/or amino acids are not primary sources of fuel for the body; rather they serve as functional and structural components throughout the body. The body uses and stores amino acids in quite a different fashion than lipids and carbohydrates. While lipids and carbohydrates are used as precursors for components in many different body systems the main role of these nutrients is for energy and the storage of these nutrients is efficient for quick liberation and metabolism. Usually, the body obtains amino acids from the diet and under normal healthy conditions this source is

adequate to maintain nitrogen balance. In situations were metabolic demand exceeds dietary amino acid supply the deficient is balanced by liberating amino acids from muscle protein or degradation of circulating plasma proteins.

Key indicators derivations from normal protein status include growth failure in paediatric subjects, significant weight loss and abnormal plasma protein (i.e. albumin) levels (77). While dietary protein deficiency will cause these symptoms there is a corresponding change in metabolic rate to compensate and eventually the body can adapt to the reduced protein intake. Not all protein deficits are caused by decreased intake. Response to various insults to body systems (i.e. infection, autoimmune disease, cancer) usually involves increases in protein synthesis to control the condition. This normal response is referred to as an acute phase response (APR) and is characterized by increased production of inflammatory and immune proteins, selected transport proteins, and coagulation/fibrinolytic proteins (78). The APR is also accompanied by metabolic changes which put the body in a catabolic state through decreases in anabolic hormones and factors (i.e. IGF-1) and increases in catabolic hormones and factors (i.e. cortisol) (78). How then does the body respond to both reduced protein intake and APR occurring simultaneously, as is the case during active IBD?

Acute Phase Response in IBD

It is well known that inflammation will increase muscle wasting and elicits an acute phase response in plasma protein synthesis (79). Protein synthesis rates are increased in inflammatory states, but are usually accompanied by reduced protein intakes resulting in negative nitrogen balance (80-82). It is difficult to obtain a clear picture of the complex process of protein turnover given the different rates at which specific pools turnover (i.e. human $ApoB_{100}$ has an FSR of $600\%\cdot day^{-1}$ while muscle protein has a fractional synthesis rate (FSR) of $1.5\%\cdot day^{-1}$) when most experiments using stable isotopes are carried out over hours versus days (83). It is therefore difficult to estimate the impact of sustained alterations of protein synthesis in these slowing turning pools. Chronic low protein intakes, without inflammation usually results in decreases in tissue (liver, intestinal tissue, skeletal proteins) protein synthesis, but during inflammation synthesis rates in these tissues are increased at the expense of muscle protein (84, 85). Secretory proteins

in the gastrointestinal tract seem to be affected by IBD in animal models as well. Despite significant damage to goblet cells in the colon (DS treated rats) there is no change in the FSR of mucin, while small intestine synthesis of mucin was decreased (no lesions) (86). The current understanding of protein/amino acid kinetics during active flares of either UC or CD is limited by a lack of research into protein turnover during these episodes.

Acute Phase Response and Catabolic State

When examining whole body protein turnover in individuals with active IBD it becomes important to determine why a specific shift in protein synthesis is occurring. Both treatment and active disease will increase protein requirements for these patients. The infiltration of activated leukocytes in the inflamed regions of the GI tract will usually lead to increased release of pro-inflammatory cytokines (including IL-6, IL-1β, TNF-α) which promote acute phase protein production. Acute phase proteins (APP) are those plasma proteins which either increase (positive APP) or decrease (negative APP) by 25% during periods of inflammation or trauma (78). These changes are not uniform, positive APP out number the negative APP, usually leading to increased protein requirements and negative nitrogen balance in sustained inflammation.

In IBD, the active inflammation is usually accompanied by a catabolic shift related to the acute phase reactants and changes in cytokine profile. This catabolic state is characterized by increases in gluconeogenesis and insulin resistance and catabolism of tissue protein to provide the necessary substrates (87). Since food restriction is also a common feature of active IBD, the individual is presented with an increased catabolic stress on the tissue proteins in favour of the ongoing inflammation. It has been reported that 75% of CD patients suffer from significant intestinal protein losses and 69% suffer from negative nitrogen balance (61). This rate of protein loss in IBD is astounding and reducing this loss is understandably critical in relation to controlling the active flare of the disease and preventing growth failure in paediatric IBD patients.

Hypoalbuminaemia in IBD

While negative energy balance in IBD is generally associated with decreased food consumption, negative nitrogen balance appears to be a more complex scenario. Common to both CD and UC patients is low serum albumin, which may be a result of increased catabolism and micro-vascular losses at the site of inflammation opposed to malnutrition (88-91). Hypoalbuminaemia has been described as a marker for poor prognosis in a clinical setting but is too dependant on fluid dynamics to be used as a clinical outcome measure (92). As a negative APP albumin decreases in response to inflammation, however it has been shown that the decrease is not related to a reduction is albumin synthesis but rather losses and catabolism (89, 93, 94).

Unfortunately, albumin concentrations are neither specific nor sensitive to short term changes in malnourished or inflammatory states. However, Ballmer has reported the degree of decline in serum albumin is usually related to disease states; serum albumin between 23 – 30g/L is indicative of acute phase reactions and/or protein-energy malnutrition while < 20g/L is associated with some form of sepsis or protein losing gastroenteropathy (88). Nevertheless, since both malnutrition and inflammation occur concurrently in IBD, the contribution of each to the decrease of plasma albumin is indiscernible.

It is becoming clearer that using a kinetic approach to explore albumin's role in disease progression may shed light on the complex nature of albumin metabolism. We have shown previously how albumin synthesis increases during DS-induced colitis in both well-nourished and malnourished piglets (89). However the increases in synthesis, approximately 50% above controls, did not translate into increases in plasma concentrations. Using ^{131}I-albumin, Steinfeld and colleagues demonstrated that during colitis albumin is lost into the gastrointestinal lumen, possibly through trans-capillary route (91). The authors also reported the presence of free and albumin bound ^{131}I, suggesting catabolism of albumin. Unfortunately, the study could not identify the cause of albumin hydrolysis, host or micro-organism. Further study of albumin kinetics is need to unravel the complexities of this plasma protein as it relates to IBD.

TRACE ELEMENT STATUS IN IBD

Copper, iron and zinc status are commonly compromised in inflammatory bowel diseases (IBD), however these deficiencies are not only related to reduction in intake or impaired absorption (60, 61, 95-97). Endogenous losses make a substantial contribution to the decline in mineral status independent of dietary intake in both clinical and experimental IBD (98, 99). Impaired copper, iron and zinc status with concurrent acute or chronic inflammation, even at the sub-clinical level, can deleteriously influence immune and repair functions (100-103). In paediatric IBD or consecutive long term chronic flare-ups in adult patients the combined loss of homeostasis of these trace elements can also limit erythropoiesis, linear bone growth and lean body mass accretion (96, 104-111).

Copper Homeostasis in IBD

While deficiencies in each trace element affect different systems, the negative effects usually overlap thereby worsening the disease progression. For example, copper is a key component of several enzymes including the antioxidant enzyme copper/zinc superoxide dismutase and the respiratory enzyme cytochrome c oxidase. Reduction in the activity of these enzymes would impact antioxidant protection in an oxidatively stressful disease and potentially impact cellular respiration.

Copper status is also linked directly to the immune response in acute phase situations. An increase in plasma copper concentration, via increasing hepatic synthesis of ceruloplasmin, is stimulated by IL-1 and IL -6. These cytokines are increased during acute phase responses and help coordinate the host immune response to invading pathogens. The increase in plasma copper, through increased circulating ceruloplasmin, directly stimulates an increased IL-2 secretion in leukocytes involved in the acute phase response. Increased IL-2 secretion by these cells directly stimulates the acquired immune system by increasing T cell proliferation (112-115). Therefore, copper status is tightly linked to immune responses and perturbations in copper status may negatively impact immune responses.

Iron Homeostasis in IBD

Similarly, iron deficiency in IBD, caused by a combination of reduced food intake, impaired absorption and intestinal occult blood losses, also impacts the progression of inflammatory diseases. Aside from the impact on oxygen carrying capacity impaired iron status also affects immune function. Both macrophages and neutrophils have decreased pathogen eradicating capacity during iron deficiency, caused by a decreased hydroxyl radical formation and myleoperoxidase activity, respectively (116-119). Macrophages are also know to be iron scavengers/storage sites during microbial invasion, limiting available iron to both host and invader (120). Collectively, the impact of iron deficiency during IBD affects a wide range of systems at both the whole body and cellular level.

There is evidence that maintaining iron stores during active IBD may improve the quality of life for individuals suffering from chronic inflammation (111). Unfortunately, there are drawbacks to providing iron therapy to individuals with active IBD. Supplemental iron has been shown to increase bleeding, histological damage and oxidative stress in DS-induced colitis models (15, 121, 122). Therefore, it would be favourable to provide the required iron either in low doses through the diet or via an intravenous route.

Zinc Homeostasis in IBD

The ubiquitous nature of zinc has far reaching contributions to normal cellular and antioxidant functions. The human body contains between 1.5 - 2.5 grams of zinc, which is distributed throughout both structural and functional tissues (123). Approximately 90% of the body's zinc is found in slowly turning over pools such as muscle, collagen and bone while the rest is distributed among all 6 classes of enzymes either as functional sites or structural components as well as being or associated with plasma proteins such as albumin for transport (123-125). Zinc is required for connective tissue metabolism , gene expression, cell proliferation and differentiation , normal immune function , protein synthesis and antioxidant protection via copper-zinc superoxide dismutase (126-129). Each of these metabolic processes requires normal zinc homeostasis and active IBD can negatively affect each of these processes. Given the wide distribution of zinc and its

actions in human metabolism and physiology it should not be surprising that deficiencies in the mineral will lead to complex changes within the body.

Zinc homeostasis is controlled primarily in the small intestine, through changes in absorption efficiency and re-absorption of endogenous zinc secreted during the digestive processes (123, 130). The majority of zinc absorption occurs between the duodenum and jejunum by a number of processes which have not been completely derived (130). Nevertheless, several zinc transporters have been characterized (ZnT-1, ZnT-2, ZnT-4 and DCT-1) and proposed to be mediators of zinc absorption (131-133). However zinc may also be absorbed by the enterocytes bound to peptides and other compounds found in the digestive muddle (134). Metallothionein (MT), a protein which sequesters absorbed zinc (and copper) in the enterocyte similar to that ferritin which binds iron, plays a key role in controlling the movement of zinc from the enterocyte to the liver (eventually into circulation) after absorption. Synthesis of intestinal MT is stimulated by increased dietary zinc, an apparent preventive measure to zinc over-absorption, storing the mineral in the absorptive cells until either transferred to transport proteins in the blood or lost in the absorptive cell turnover of the gut (135). A similar increase in MT synthesis has been observed in the absorptive cells during infection/inflammation (positive acute phase response protein) which points to the key role of zinc in immune responses (136). Other major losses of zinc are from pancreatic and gastrointestinal secretions as well as seminal fluids in males. The majority of zinc losses, under normal physiological/disease free conditions, therefore come from fecal losses of both dietary and secreted zinc and to a lesser extent (< 10% of total fecal losses) from seminal, urinary and skin/hair losses (134). It has also been demonstrated in rats, but not yet in humans that some degree of zinc absorption occurs in the colon (95). If the colon plays a role in zinc homeostasis those suffering from UC may be especially susceptible to a deficiency of the mineral.

Consequences of Zinc Deficiency

Zinc deficiency has been directly linked to reduced tissue/muscle protein synthesis as well as reduced collagen turnover, which also contributes to growth retardation in paediatric cases (104, 108, 109, 137). Zinc deficiencies also effect affect growth on a

cellular level through complex actions on gene transcription, RNA synthesis, signal transduction and mitogenic hormones together reducing cell division, protein synthesis and growth (100, 137-139). Immune function is also compromised during zinc deficiency, resulting in reduced monocyte function, decreasing neutrophil phagocytosis and cytotoxicity of natural killer cells (101, 140). The integral role of zinc in gene transcription, translation and growth result increases the complexity of targeting key side effects of deficiency reduced dietary zinc has a variety of manifestations in human physiology. In terms of paediatric zinc status zinc is very important to normal growth and development. Zinc status has been directly linked to linear growth of young boys not displaying signs of overt clinical deficiency of zinc (105, 141).

During zinc deficiency a number of changes occur to control zinc tissue and plasma concentrations. Both absorption efficiency increases and endogenous losses decrease to limit losses of zinc in the GI tract (142, 143). In doing so the plasma concentration of zinc is maintained within a narrow range despite changes in dietary intakes. Zinc absorption may also be decreased by excess iron in the diet, which likely reduces zinc absorption by competition for metal transport proteins and association with other dietary constituents (144). The tight control of zinc under normal conditions depends on proper function of the gastrointestinal tract, action of zinc and other metal ion transporters and MT production. These processes are compromised under conditions of inflammation or gastrointestinal infection, both of which are trademarks of inflammatory bowel disease.

Maintaining Zinc Homeostasis Maybe Important in Controlling IBD Symptoms

As described earlier, the active UC and CD disease involves unabated inflammation of the gastrointestinal tract, affecting the cells in a variety of regions of the GI tract and in with varying degrees of severity. Zinc deficiency is well known to be a problem in IBD. Crohn's disease has been directly linked to acrodermatitis enteropathica-like skin lesions which is usually only observed in individuals with a genetic disorder of zinc transporter expression (103, 145). Zinc status can be compromised to this extent by the decrease in food intake or reduced absorption efficiency due to cellular damage in regions of zinc absorption. However, as a result of the inflammation, increases in MT production reduce

also the amount of zinc transported from the enterocytes to circulation and increase endogenous losses (increased intestinal secretions, reduced re-absorption) (78). Leaky gut (reduced tight junctions) and infections (bacterial overgrowth) which may also accompany active IBD can be prolonged by the compromised immune response during zinc deficiency, reduced cell division and altered protein synthesis (146). Poor zinc status has been linked to growth stunting in paediatric IBD patients but with conflicting comparisons to healthy controls (96). While not linked directly, poor zinc status may also be related to the decreased bone mineral density seen in both UC and CD (60, 147, 148).

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Chapter 3

Pilot Studies and Supplemental Methods

PILOT STUDY #1

Synopsis

The objective of pilot study #1 was to determine the feasibility of implanting 4 catheters (jugular and femoral veins, stomach and bladder) during pre-study surgery and if the commercial meal replacement Ensure®,by Ross Canada is a division of Abbott Laboratories Canada, supplemented with whey protein concentrate would make a suitable liquid diet for the pre-weaned piglets.

Main Findings

The placement of 4 catheters at surgery is manageable for 2 researchers in approximately 2 hours. The use of larger catheters for both the stomach and bladder is recommended to maximize either diet delivery or urine collection and to avoid blockages. The use of Ensure® with added whey protein appeared to cause osmotic diarrhoea within 48 hours post surgery. A custom formulated diet consisting of complex carbohydrates and proteins would appear to be a more useful dietary protocol to use in these enterally fed piglets.

PILOT STUDY #2

Synopsis

The objective of pilot study #2 was to determine the most appropriate tracer dose for the measurement of tissue and liver derived protein synthesis using L-[ring-$^{2}H_5$]phenylalanine (35 μmol/(kg•hr)). This pilot study was also used to determine the feasibility of using a double tracer solution to simultaneously measure glutathione synthesis and concentration. Using [^{15}N,1,2-$^{13}C_2$]glycine (60 μmol/(kg•hr)) as the tracer we combined both tracers into the same infusion solution of sterile physiological saline and conducted a 6-hour phenylalanine infusion and glycine tracer study.

Main Findings

Based on low enrichment of the L-[ring-$^{2}H_5$]phenylalanine tracer in muscle tissue and the failure to achieve plateau enrichment of plasma L-[ring-$^{2}H_5$]phenylalanine by 3 hours of infusion it was decided to increase the priming dose of L-[ring-$^{2}H_5$]phenylalanine to the

equivalent of 2 hours of infusion versus 1 hour. Similarly, the glycine tracer enrichment was determined to be too low but the priming dose caused a spike in plasma enrichment above steady state plateau. Consequently, the priming dose of L-[ring-$^{2}H_5$]phenylalanine was increased to the equivalent of 1.5 hours of tracer while the infusion concentrations was kept at 35 μmol/(kg•hr) for subsequent studies. The priming dose of [^{15}N,1,2-$^{13}C_2$]glycine was reduced to the equivalent of 1 hour of infusion while the actual tracer dose was increased to 75 μmol/(kg•hr).

PILOT STUDY #3

Synopsis

The objective of the third pilot study was to both train another Master of Science student in the surgical techniques and to confirm all surgical and isotope infusion aspects of the study as well as the feasibility of the sample collection procedures to be used prior to beginning the complete animal study.

Main Findings

The refined tracer doses were adequate to assess both protein synthesis rates and the synthesis rate of glutathione as determined by GCMS and tandem LCMS analysis, respectively. The sample collection protocols were feasible for the collection of trace element, oxidative stress and protein synthesis data. The final study protocol is diagrammed in Figure 3.1.

DIET FORMULATION

Introduction

All piglets received a liquid diet designed to meet the requirements of all nutrients for a healthy growing piglet (1). Diets were custom-formulated in our laboratory (modified from (2); Table 3.1 and 3.2) using spray dried egg white and low-lactose whey protein concentrate as protein sources (Table 3.3), maltodextrin as the carbohydrate source and a blend of soybean, coconut and flaxseed oils as lipid source (Table 3.6). Vitamin and mineral mixes were be added to the diet to provide > 120% of the NRC requirement for each micronutrient (Tables 3.4 and 3.5). Excess biotin (10 times requirement) was

provided to overcome binding/inactivity of the vitamin due to the high avidin content of egg albumin. The diets were infused over a 16 hour period (Compat Enteral Feeding Pump; Novartis Nutrition) via the gastric catheter to achieve a metabolizable energy intake of 925 kJ/(kg•day) based on each piglet's daily weight.

Study Specific Diets

Study 1

Both groups of well nourished piglets received a diet that supplied all indispensable amino acids in excess of requirement (Table 3.3) (1). Protein, carbohydrate and lipid were supplied as 25%, 56% and 19% of energy, respectively. The minimum daily requirement of zinc for young growing piglets on corn/soybean feed is 50 ppm of diet which translates to an intake of approximately 3 mg/(kg•day) for a weaned growing piglet (1). This high requirement is also supported by Dr. Ian Griffin's pilot data on zinc absorption presented in this chapter. However, most commercial feeds provide zinc at greater than 100 ppm (> 6 mg/(kg•day)) to promote growth and overcome the reduced bioavailability of zinc due to the high phytate content of the diet components (3). Zinc present in and added to phytate-free diets is more highly bioavailable, therefore the requirement is much lower (6 – 20 ppm) (4, 5) which gives way to the current recommendation for casein/glucose-based diets of 15 ppm of diet or 30% of the requirement of diets high in phytates (1). Therefore, for our adequate diet, we used 15 ppm as the zinc requirement and apply the same factor (2X) that is applied to commercial feeds to ensure healthy zinc intake in our piglets. This resulted in a diet containing 30 ppm and a daily intake of 1.8 mg/(kg•day).

Study 2

Both groups of malnourished piglets received a diet that supplied 50% of the macronutrients that were supplied by the diet in study 1 while all micronutrients were supplied at 120% of the NRC requirement by adjusting the micronutrient mixes added to the diet. This diet was intended to simulate the food avoidance behavior often seen during active flares of IBD. As in part 1 of the study the diet was administered over a 16

hour period via the gastric catheter to achieve a metabolizable energy intake of 461 kJ/(kg•day).

GLUTATHIONE SYNTHESIS

Introduction to Importance of Glutathione in IBD

Glutathione (GSH) is a tripeptide of glutamate, cysteine and glycine. As the major intracellular thiol, where its concentration is three orders of magnitude higher than in plasma, it has many functions including oxidant defense (6). The colon is particularly reliant on glutathione for oxidant defense where, in contrast to the liver, GSH peroxidase activity is much higher than either catalase or superoxide dismutase (7). Furthermore, glutathione concentration is low both in ileum (8) and in colon (9) of patients with CD. Concentrations are lower in both healthy and inflamed tissue of malnourished but not well-nourished IBD patients. As with plasma proteins, changes in glutathione concentration are the result of changes in synthesis rate relative to catabolism or removal. The influences on GSH metabolism in IBD are complex.

Malnutrition would be expected to decrease synthesis due to limited substrate availability, whereas inflammation would be expected to increase consumption. In our piglet model of systemic inflammation and malnutrition, the combined stresses decreased jejunal mucosal glutathione concentrations and synthesis rate by 70% (10). In fact, the gut was the organ most severely affected; while liver remained unaffected. In our piglet colitis model, synthesis rate and concentration of glutathione were compromised in colon and muscle but not in liver (unpublished). This suggests that synthesis could not be increased to compensate for increased consumption of glutathione, and therefore antioxidant defense was compromised in the colon and in peripheral tissues not directly affected my inflammation. Cysteine, the rate limiting amino acid in GSH synthesis, is high in metallothionein and albumin, two other proteins upregulated by the acute phase response. This suggests that therapies to decrease mucosal damage may also decrease the demand for cysteine, and increase its availability for protein synthesis.

GSH Stable Isotope Tracer Infusion Study Methodology

On study day 13 the tracer, [^{15}N,1,2-$^{13}C_2$]glycine (Cambridge Isotope Laboratories, Cambridge, MA), was dissolved in sterile 0.9% saline (75 μmol/L) and filtered through a 0.22 μm filter. The tracer was then infused at 75 μmol/(kg•hr), by syringe pump, following a priming dose of tracer equal to 75 μmol/kg. To accommodate the rapid synthesis rate of GSH (10, 11), the glycine tracer was infused for 5 hours opposed to a longer infusion which is common to other stable isotope tracer studies of protein synthesis.

Sampling Protocol

Blood samples (0.5 mL) were collected at baseline and then hourly from hours 3-5. To determine GSH concentration and enrichment, samples were derivatized immediately (as detailed in the following section). To determine enrichment of free glycine in erythrocytes (the precursor pool for erythrocyte GSH synthesis), a second blood sample was taken, plasma was removed and packed red cells were washed 3 times by adding an equal amount of isotonic saline, inverted several times and then centrifuged at 2500 x g for 10 minutes. Trichloroacetic acid (10% w/v) was added to the washed, packed erythrocytes to precipitate proteins. After centrifugation, the supernatant containing free intracellular glycine was frozen in liquid nitrogen. Whole blood was collected at hour 1 and hour 4 in heparinized capillary tubes for hematocrit (Hct). Tissue samples (colon, ileum, liver, masseter, and longissimus dorsi) were taken, and the GSH derivatized immediately as follows.

GSH Isotopic Enrichment by Tandem Liquid Chromatography Mass Spectrometry (LCMSMS)

Glutathione Sample Preparation

GSH in erythrocytes and tissue samples were derivatized immediately as each sample was taken. Blood samples were centrifuged, and plasma removed from packed erythrocytes. Immediately, 10μl of γ-glutamyl-leucine, 10mM, (GL; internal standard) and 200μL of N-ethylmaleimide, 100mM, (NEM; derivatizing agent) were added, vortexed and left standing at 25°C for 15 minutes to derivatize GSH and all other free

sulfhydryls. After derivatization, cells were lysed and proteins precipitated with 50 µl $ZnSO_4$, 0.4 M, and 1 ml ice cold methanol then centrifuged at 10,000x g. The supernatant was removed and stored at – 80°C until analysis. Similarly, tissues were homogenized with 3.0 ml of NEM and 50 µl of GL immediately after samples are taken. After derivatizing for 15 minutes at 25°C, proteins were precipitated with 5 ml of ice-cold methanol, centrifuged at 1500 x g and the supernatants stored at -80°C.

Isotopic Enrichment of GSH by Tandem LCMS

Isotopic enrichment and concentration of GSH was analyzed on a Sciex API 4000 Turbo IonSpray liquid chromatograph triple quadrupole mass spectrometer (Sciex, Thornton, ON). The LC (Agilent 1100 Series) was fitted with a C18 precolumn filter (Waters) and an Xterra™ Intelligent Speed™ C18 column (2.5 um x 2.1 mm x 20 mm, Waters). The instrument was operated in MRM mode: monitoring single ions produced in the first quadrupole and then their daughter ions in the third quadrupole. We developed this method primarily to determine GSH concentration and synthesis, and chose the internal standard GL because it has a similar fragmentation pattern to GSH showing constant neutral loss of 129 m/z as the gamma-glutamyl group. The following analytes were determined from the sample injection: NEM derivatives of GSH, cysteine and homocysteine, as well as the underivatized disulfides GSSG and cystine. To determine concentration, peak areas of each analyte (integrated with Analyst v1.2 software, Sciex) was measured against the internal standard, and compared to an external standard curve for each analyte run on that day.

Isotopic Enrichment of Intracellular Glycine by GCMS

Intracellular erythrocytes and tissues glycine were determined as the corresponding n-propyl heptafluorobutyramide derivative in single ion monitoring mode for negative chemical ionization GCMS, using methane as the ionizing gas. The [M-FH]- ions with m/z ratio 293 and 296, which correspond with unlabelled and labeled ions respectively, were monitored.

Glutathione Concentration

$$\text{Eq.1} \quad \text{GSH (mmol/L)} = \frac{\text{GSH peak area x GL added to sample (mmol)}}{\text{GL peak area x [sample volume (L) x Hct] x RF}}$$

Where Hct used for erythrocytes but omitted for tissues, and RF is the response factor calculated as the slope of the line in the standard curve of response ratio (peak area of unknown over peak area of internal standard) plotted against amount ratio (moles of unknown over moles of internal standard). GSSG, cystine and cysteine will be calculated similarly.

GSH Synthesis

Fractional synthesis rate (FSR) of tissue GSH was calculated as for mixed tissue protein FSR except using a 5-h infusion time. FSR of erythrocyte GSH was calculated as for plasma protein FSR except (t2-t1) refer to the increase in enrichment between hours 2 and 5.

Absolute synthesis rate (ASR) erythrocyte GSH was calculated as:

$$\text{Eq.2} \quad \text{ASR (mmol·kg/day)} = \frac{\text{FSR x conc (mmol/L) x erythrocyte volume (mL/kg)}}{\text{100\% x 1000}}$$

Where conc is GSH concentration in erythrocytes, and packed erythrocyte volume is calculated using Hct and an average blood volume of 80 mL/kg body weight (wt) (12).

DETERMINATION OF INTESTINAL INTEGRITY

Introduction to the Importance of Determining Intestinal Integrity in IBD

Paracellular permeability in intestinal mucosa is regulated primarily by tight junctions between enterocytes. Most studies have found that intestinal permeability is increased in patients with IBD (13-15). Increased permeability is not solely due to inflammation, since increased permeability is also associated with malnutrition (16) and with parenteral nutrition (17), which can be viewed as selective malnutrition of the gut. Administration of probiotics (VSL#3) in IL-10 deficient mice resulted in normalization of mannitol flux, an in vivo measure of paracellular permeability (18). Soluble factors from the same probiotic preparation were effective in improving epithelial barrier function in

enterocytes *in vitro* (18). We propose that decreased paracellular permeability is one primary mechanism by which probiotics will affect protein and trace element metabolism.

Gut Integrity Methodology

Administration of Permeability Markers

On study day 12, intestinal permeability was determined following an intragastric bolus of 3 permeability markers. Lactulose and polyethylene glycol are absorbed by the para-cellular route so their urinary excretion assesses increased gut permeability; whereas urinary excretion of mannitol which is absorbed by the trans-cellular route will act as a positive control to confirm normal absorptive function. After emptying the bladder catheter at time 0, each piglet received an intragastric bolus (10 mL/kg) of the 3 permeability markers dissolved in sterile water: mannitol (50 mg/kg), lactulose (500 mg/kg, and polyethylene glycol 4000 (3 g/kg). Urine was collected through the bladder catheter in a container on ice over the following 24 hours to assess fraction of the dose of each marker excreted.

Analysis of Permeability Markers

The fraction of the markers excreted in urine was determined by analyzing their concentration in urine by HPLC as described by Kansagra et al. (17). An aliquot of centrifuged urine was injected onto an Aminex HPX 87C 300 x 7.8-mm id cation-exchange column (Bio-Rad Laboratories, Hercules, CA) protected with a Carbo-C guard column (30 x 4 mm id, Bio-Rad). The column was maintained at 80*C, and sugars eluted using degassed de-ionized water as a mobile phase at a flow rate of 0.6 mL/min. Peaks were detected by differential refractometer, identified using commercially available reference standards of PEG 4000, lactulose, and mannitol (Sigma Chemical, St Louis, MO) for calibration, and integrated to determine the fraction of each sugar dose recovered in urine.

Conclusions on Determining Gut Integrity in Growing Piglets.

The appearance of lactulose and PEG in urine is proportional to the degree of permeability of the gut for molecules in the approximate molecular weight range of the sugars used. Neither recovery of lactulose nor PEG in urine differed between REF and colitis groups (Table 3.7). A possible confounding aspect of this analysis was the fact the piglets were not of weaning age and the custom formulated experimental diet itself may have impacted the normal paracellular permeability of the gut in all the piglets.

ZINC ABSORPTION AND ENDOGENOUS LOSSES EXPERIMENT

Introduction Importance of Zinc in IBD

For this thesis we initially planned to complete a dual isotope infusion as part of the original thesis proposal. We did conduct the experimental procedures listed below. We did not produce any usable data from this analysis to include in any of the results sections. The nature of the problems is still not clear but was narrowed down to either an isotope enrichment error, error in isotope administration or technical issue with the TIMS mass spectrometry analysis. The following information has been included for the benefit of the reader to understand the rationale of the final manuscript in the thesis.

Zinc Homeostasis in IBD

Zinc deficiency induces changes in gene transcription, RNA synthesis, signal transduction, protein synthesis and immune function, all of which affect metabolism on many fundamental levels (19-22). The critical role of zinc in immune function and growth has been demonstrated in zinc supplementation studies, which show prevention of childhood pneumonia and diarrhea, two of the most serious childhood killers in developing countries (23), as well as improved linear growth in “at-risk” populations (24, 25). Even mild zinc deficiency without overt clinical signs has been reported to reduce linear growth in young boys living in Canada (26). Unfortunately, marginal zinc deficiency is quite common and difficult to assess because of the non-specific nature of the symptoms (20, 27). Plasma zinc concentrations are relatively maintained during mild deficiency and thus is a poor indicator of zinc status (20, 27). Therefore, there is a need

for more specific and functional measures of zinc status including kinetic assessment of zinc homeostasis in states of malnutrition and inflammation, particularly IBD.

Zinc homeostasis is controlled primarily in the gastrointestinal tract, where approximately 20% of dietary zinc is absorbed (20, 27). Most absorption of dietary zinc occurs in the duodenum and jejunum (21). It has been demonstrated in the rat that some degree of absorption occurs in the colon in the rat (28). The recovery mechanism for zinc by the large intestine is probably from endogenous zinc secretions as part of the normal digestion process. Several zinc transporters have been characterized and proposed to be mediators of absorption (29-31). This saturable carrier-mediated process is stimulated by low zinc (32). Mutations in the gene SLC39A4, which encodes for hZIP4, a zinc/iron-regulated transporter protein normally expressed in all regions of the GI tract, are responsible for acrodermatitis enteropathica (33). The observation that this condition responds to high dose zinc supplementation may be because at high intakes zinc may also be absorbed by paracellular transport or abound bound to cyst(e)ine, histidine and small peptides (34). This nonsaturable mechanism is independent of zinc status; but seems to be linked to amino acid and protein nutrition. Zinc status is also related to protein in that once absorbed, zinc is always found bound to a protein ligand.

Metallothionein (MT) is a 6 – 7 kD protein, found in several isoforms, all rich in cysteine residues which confer its capacity to bind cations (7 Zn2+ or Cd2+ ions and 12 Cu+ ions) (35), and it has a role in zinc homeostasis, sequestration of potentially harmful metal ions, and redox control. Intestinal MT plays a key role in controlling the movement of zinc from the enterocyte into the circulation. In the enterocyte, it binds zinc until it is either transferred to albumin in the portal blood or is lost in the normal cell turnover of the gut (36). In an apparent measure to prevent over-absorption of zinc in the well-nourished state, synthesis of intestinal MT is stimulated by increased dietary zinc to decrease fractional absorption. Conversely, the reduction in intestinal MT during zinc deficiency (37) increases fractional absorption.

Changes in absorption efficiency of dietary zinc provide 'coarse control" of zinc homeostasis, whereas changes in endogenous zinc secretion in bile and pancreatic fluid provide "fine control" to achieve zinc balance (19, 20). Development of a dual stable isotope tracer technique (38) has been instrumental is providing information on how the two processes interact to modulate zinc homeostasis. Absorption of dietary zinc is measured by the ratio of tracers in plasma following simultaneous administration of ^{67}Zn orally and ^{70}Zn intravenously. Fecal excretion of endogenous zinc is measured from the cumulative urinary and fecal excretion of the intravenously administered tracer. And finally, zinc balance is calculated by multiplying fractional absorption by total zinc intake and subtracting endogenous fecal excretion and urinary losses over the 6-d study. It is clear from this model that urine is neither a major route of loss of zinc nor a major control point in maintaining zinc homeostasis.

Clearly the gut is the major organ regulating zinc status. This dual stable isotope model has been applied to study adaptations to low dietary zinc intake. Adults maintain zinc homeostasis at low intakes by increasing fractional zinc absorption and decreasing endogenous fecal zinc excretion to limit losses of zinc in the GI tract (39, 40). However, children are at greater risk of zinc deficiency because of their higher requirement for growth (34). In a randomized crossover design, Griffin et al 2004 studied premenarcheal girls at high (150% of their RDA) and low (50% of RDA) intakes of zinc (41). At the low intake level, the girls were in negative zinc balance; however neither plasma zinc concentration nor urinary zinc excretion were changed. The decrease in endogenous fecal losses without an increase in zinc absorption was inadequate to restore zinc balance. It is not clear why zinc absorption did not increase at the low intake, perhaps it was because absorption in these adolescents was already quite high at 30%. Nonetheless, it is clear that major changes take place in the gut during dietary deficiency as an adaptation to maintain zinc homeostasis.

Griffin has recently studied graded zinc restriction in the growing piglet using the dual stable isotope tracer model (unpublished with analysis ongoing). Using a corn/soybean meal diet which contained 10 ppm intrinsic zinc as the deficient diet, he added 50 ppm

for the zinc-adequate diet, and produced graded restriction by adding 5, 15, or 25 ppm to the deficient diet. Preliminary data show zinc absorption increases from 35% to 79% in response to moderate zinc restriction (25 ppm added zinc) and remains high with severe restriction. Analysis of fecal zinc for calculation of endogenous fecal losses is not yet complete. However, based on a study showing decreased zinc content of biliary and pancreatic secretions in zinc deficient pigs (42), we expect a reduction in endogenous fecal losses to be important in adapting to low zinc intake. Weight gain is shown in Table 3.8. Severe (0 or 5 ppm added zinc) but not moderate restriction (15 or 25 ppm) causes a severe decrease in growth rate. Taken together with the absorption data and anticipated endogenous excretion data, we expect that the adaptations at the level of the gut can maintain zinc homeostasis during moderate restriction in the growing piglet. Interestingly, all animals in the 0 and 5 ppm groups developed anorexia, and all animals in the 0 ppm and 2 of the 3 animals in the 5 ppm group developed parakeratosis, clearly showing that increased absorption was not sufficient to maintain homeostasis during severe deficiency.

This piglet study by Griffin provides additional support for the role of absorption and endogenous losses in regulating zinc status, and for the applicability of the pig as a model to study zinc metabolism in growing children. It provided preliminary data for the study zinc metabolism in our piglet model of colitis, where piglets have the dual stresses of reduced dietary intake and inflammation. Clinically, zinc deficiency is relatively common in children with IBD (43-46). Zinc status is even low in children before diagnosis with UC (47). Because zinc is widespread in foods, zinc intake is generally not particularly low unless food intake is restricted. This suggests secondary zinc deficiency due to a failure of adaptation at the level of the gut.

Previously, absorption of zinc has been shown to be either low (48, 49) or normal in IBD (50). However, absorption has not been studied in conjunction with endogenous fecal losses using the dual isotope model until recently. Griffin et al (45) studied adolescents with stable Crohn's disease with an elemental zinc intake of 150% of their RDA. Zinc absorption was decreased by 53% compared with healthy age-, gender-, and ethnicity-

matched controls. In contrast to healthy pre-adolescents who adapt to low zinc intake by showing a substantial reduction in endogenous losses (41); these adolescents with Crohn's were unable to compensate for low dietary intake by decreasing endogenous fecal excretion to maintain normal zinc balance or plasma zinc concentration. This study suggests that zinc requirement is higher in IBD and that some level of zinc supplementation may be required to compensate for dysregulation of both absorption and fecal loss at the level of the gut. The nature of that dysregulation is complex, depending in many ways on the interaction of malnutrition and inflammation, as well as interactions between protein and zinc itself. Several factors appear to interact to compromise zinc absorption in IBD, including:

a) decreased food intake
b) reduced absorption efficiency of dietary zinc due to intestinal mucosal damage in the upper regions of the GI tract responsible for absorption of dietary zinc
c) stimulation of intestinal MT mediated by the acute phase response (glucocorticoids and Th1 cytokines particularly IL-6) and oxidative stress (51)
d) decreased fractional zinc absorption even when dietary intake is low because it binds zinc so strongly it limits transfer across the enterocyte
e) hypoalbuminemia, which may reduce the capacity for zinc transport as zinc is transferred across the basolateral membrane of the enterocyte

Several factors also appear to interact to increase endogenous zinc losses in IBD, including:

a) mucosal damage and increased intestinal permeability, which may increase endogenous zinc losses across the gut particularly of zinc bound to albumin
b) mucosal damage and increased intestinal permeability, which may also decrease
c) recovery of endogenous zinc which was secreted into the small intestine from bile and pancreas

Therefore, we specifically targeted zinc for isotopic study due to its tight links to normal whole body protein synthesis and the relationship between gut integrity and whole bosy zinc homeostasis.

Isotope Preparation

Zinc isotopes, ^{67}Zn and ^{70}Zn (88% and 95% enriched by mass, respectively), were purchased from Cambridge Isotopes Laboratories (Andover, MA) as ZnO. The ZnO was converted to $ZnCl_2$ by dissolving with several drops of concentrated HCl (12 M) and brought to the desired final volume using sterile saline (0.45% w/v) and adjusted to pH 5.5 with NaOH. Zinc solutions were finally filtered to ensure sterility using 0.20µm filter (Fisher Scientific Canada, Ottawa, ON) into sterile 30ml vials and stored at 4°C until administered.

Plasma and urine samples for zinc absorption analysis were digested in concentrated HNO_3 overnight, dried and reconstituted in 1M HCL and purified by anion exchange chromatography. Samples were then loaded onto rhenium filaments for thermal ionization magnetic sector mass spectrometry (Finnigan MAT 261, Thermo Finnagan, Bremen, Germany) determination of isotope ratios (52). Values of ^{67}Zn and ^{70}Zn were expressed according to the non-administered ^{64}Zn isotope and corrected for the temperature specific effect of fractionation (52). The resulting values were then converted to tracer:tracee ratios for determination of absorption.

Zinc Absorption

Zinc absorption was determined by adapting the previously described dual stable isotope method of Friel *et al* (38) to the piglet model and diagramed in Figure 3.1. Briefly, zinc isotopes were administered on day-8 of study or when colitis was confirmed by occult blood in feces with all piglets receiving 0.5 mg ^{70}Zn intravenous and 1.0 mg ^{67}Zn orally as described previously. Plasma samples were taken daily through out the collection and a spot urine sample was taken 72 hours after isotope administration. Absorption was calculated using the tracer:tracee ratios of the urine or plasma samples collected as per equation 1.

Eq.1: $$\text{Zinc Absorption} = \frac{\text{TTR p.o. } ^{67}\text{Zn tracer/dose of p.o. } ^{67}\text{Zn tracer}}{\text{TTR i.v. } ^{70}\text{Zn tracer/dose of i.v. } ^{70}\text{Zn tracer}}$$

Zinc Enrichment by Thermal Ionization Mass Spectrometry

Samples (plasma, feces, urine) are dried (feces and urine), ashed (feces: dry; urine: wet) and purified by anion exchange chromatography (AG 1-X8 resin; Bio-Rad Laboratories, Hercules, CA) and collected in 6 mL ultra pure water. Then 10 µL of 0.7 mol phosphoric acid was added, and the sample was dried on a hot plate overnight in a teflon vial before being re-suspended in 40 µL double-distilled water. Finally, 10–20 µL of this solution, 2 µL of 0.7 mol phosphoric acid, and 6 µL silica suspensions were loaded onto rhenium filaments.

Isotope enrichments were measured by magnetic sector thermal ionization mass spectrometer (Finnigan MAT 261; Bremen, Germany). Isotope ratios were expressed with respect to the nonadministered isotope, ^{66}Zn, and were corrected for temperature- and mass-specific differences in fractionation, using the ratio of ^{64}Zn to ^{66}Zn. Ten scans per block were preformed, and replicate blocks repeated until the desired degree of precision (<0.2%) are obtained. Isotope ratios were converted to tracer:tracee ratios as described previously (53).

Trace Element Balance

Trace element balance was approximated by diet, urine and fecal concentrations, ignoring losses from hair and shedding of skin cells. The balance of each trace element (TE) was calculated using equation 2, fractional zinc absorption was calculated from DITR technique while copper and iron fractional absorption was obtained for growing piglets from literature values (38, 54, 55).

Eq.2: Zn Balance = (intake x fractional Zn absorption) – (Zn fecal excretion + Zn urine excretion)

Table 3.1 Dry weight composition of custom formulated piglet diet for enteral feeding.

Diet Composition	Weight (g)	CHO (%)	Protein (%)	Fat (%)	Ash (%)	H_2O (%)	ME[a]/kg	Pro (g)	CHO (g)	Fat (g)
Egg white, dried	210	8%	81%	0%	5%	6%	3107	170	16	0
Whey protein	175	49%	35%	3%	8%	3%	2633	60	85	6
Maltodextrin (DE10[b])	441	94%	0%	0%	1%	6%	6860	0	412	0
Soybean oil	57	0%	0%	100%	0%	0%	2134	0	0	57
Coconut oil	14.5	0%	0%	100%	0%	0%	545	0	0	15
Flax Seed oil	2	0%	0%	100%	0%	0%	75	0	0	2
Vitamin mix	0.05	0%	0%	0%	0%	0%	0	0	0	0
Mineral mix	25	0%	0%	0%	0%	0%	0	0	0	0
Total	**925**						**15355**	**231**	**514**	**80**

[a] Calculated metabolizable energy, kilojoules.

[b] Dextrose equivalent of 10.

Table 3.2 Macronutrient and energy breakdown of complete liquid diet and nutrient density as fed.

Diluted Formula (kg/5L)[a]		**Study 1[b]**	**Study 2[b]**
Energy	3070 kj/L	925 kj/kg	461 kj/kg
Carbohydrate	102.8 g/L	31 g/kg	15 g/kg
Protein	46.1 g/L	14 g/kg	7 g/kg
Lipid	15.8 g/L	4.7 g/kg	2 g/kg

[a] Enteral diet was prepared by mixing 1 kg of dry diet premix in 5L water.

[b] Kj or g per kg body weight of each piglet, body weights measured each day and feed adjusted accordingly.

Table 3.3 Amino acid requirement and amount provided in liquid diet as fed.

REQUIREMENTS[(1)*]				CUSTOM DIET	
Amino Acids	**Amt/day**	**BW**		**DIET**	**NRC**
		g/(kg•d)		**g/(kg•d)**	**% Req't**
Arginine	1.00 g	0.33		0.68	204%
Histidine	0.80 g	0.27		0.30	112%
Isoleucine	1.37 g	0.46		0.79	173%
Leucine	2.54 g	0.85		1.22	144%
Lysine	2.51 g	0.84		1.01	121%
Methionine	0.68 g	0.23		0.42	186%
Methionine + Cystine	1.44 g	0.48		0.80	166%
Phenylalanine	1.50 g	0.50		0.71	143%
Phenylalanine + Tyrosine	2.36 g	0.79		1.21	153%
Threonine	1.58 g	0.53		0.74	140%
Tryptophan	0.46 g	0.15		0.20	130%
Valine	1.71 g	0.57		0.85	150%

* Based on mean piglet body weight throughout the 14 days of the study of 5kg.

Table 3.4 Vitamin requirement and amount provided in liquid diet as fed.

REQUIREMENTS[(1)*]				CUSTOM DIET	
Vitamins	**Amt/day**		**wt/(kg•d)**	**DIET**	**NRC**
				wt/(kg•d)	**% Req't**
Vitamin A	460.0	IU	153	177	115%
Vitamin D3	1.09	µg	0.363	0.42	116%
Vitamin E	2.25	µg	0.750	0.87	116%
Vitamin K	0.09	mg	0.030	0.03	116%
Vitamin B6	0.40	mg	0.133	0.15	115%
Vitamin B12	3.72	µg	1.240	1.43	115%
Biotin	0.01	mg	0.003	0.03	921%
Choline	0.13	g	0.043	0.05	116%
Folicin	0.05	mg	0.017	0.02	118%
Niacin	3.93	mg	1.310	1.52	116%
Pantothenic Acid	2.30	mg	0.767	0.88	115%
Riboflavin	0.78	mg	0.260	0.84	322%
Thiamin	0.27	mg	0.090	0.10	115%

* Based on mean piglet body weight throughout the 14 days of the study of 5kg.

Table 3.5 Mineral requirement and amount provided in liquid diet as fed.

REQUIREMENTS[(1)*]				CUSTOM DIET	
Minerals	**Amt/day**	**wt/(kg•d)**		**DIET**	**NRC**
				wt/(kg•d)	**% Req't**
Calcium	1.68 g	0.560		0.65	115%
Phosphorus	1.29 g	0.430		0.49	115%
Sodium	0.56 g	0.187		0.39	208%
Chlorine	0.58 g	0.193		0.55	285%
Magnesium	0.07 g	0.023		0.14	588%
Potassium	0.54 g	0.180		0.63	352%
Copper	1.14 mg	0.380		0.44	115%
Iron[a]	9.01 mg	3.003		3.46	115%
Manganese	0.87 mg	0.290		0.33	115%
Selenium	0.06 mg	0.020		0.02	123%
Zinc[b]	2.7 mg	0.900		1.03	115%

* Based on mean piglet body weight throughout the 14 days of the study of 5kg.

[a] Iron requirements are those of piglets receiving phytate free formula type liquid diet.

[b] Zinc requirements are those of piglets receiving phytate free formula type liquid diet.

Table 3.6 Lipid and fatty acid profile of custom formulated liquid diet.

Fatty Acids	**Coconut Oil**	**Soybean Oil**	**Flax Seed Oil**	**Test Diet Lipids**
C6	*0.5%*	*0.0%*	*0.0%*	**0.1%**
C8	*7.1%*	*0.0%*	*0.0%*	**1.3%**
C10	*6.0%*	*0.0%*	*0.0%*	**1.1%**
C12	*47.1%*	*0.0%*	*0.0%*	**9.2%**
C14	*18.5%*	*0.1%*	*0.0%*	**3.7%**
C16	*9.1%*	*10.6%*	*5.0%*	**10.1%**
C16:1	*0.0%*	*0.1%*	*0.0%*	**0.1%**
C18	*2.8%*	*4.0%*	*4.0%*	**3.8%**
C18:1	*6.8%*	*23.2%*	*19.0%*	**19.8%**
C18:2	*1.9%*	*53.7%*	*14.0%*	**42.0%**
C18:3	*0.1%*	*7.6%*	*58.0%*	**8.2%**
C20	*0.1%*	*0.3%*	*0.0%*	**0.2%**
C22	*0.0%*	*0.3%*	*0.0%*	**0.2%**

Test Diet n-6:n-3 Ratio

Lipid	**Amount (g)**	**Amt n-6 (g)**	**Amt n-3 (g)**	**n-6:n-3 (g/g)**
Soybean	57	43.83	4.39	10.0
Coconut	14	1.22	0.01	87
Flaxseed	3	0.99	1.74	0.57
Total	**74**	**46.0**	**6.14**	**7.49**

Table 3.7 Gut integrity analysis by 6-hour post administration urinary recovery of paracellular absorption of non-digestible and non-metabolizable carbohydrates – lactulose and polyethylene glycol

	Study 1		Study 2			Pooled
	REF	**WNC**	**MR**	**MRP**	**MR-C**	SEM
Urinary Mannitol Recovery						
Total (mg)	94	55	38	43	41	7
%	48	31	25	29	27	3
Urinary Lactulose Recovery						
Total	3.7	2.1	1.4	1.4	1.4	0.4
%	0.20	0.12	0.09	0.10	0.09	0.02
Urinary PEG Recovery						
Total	6.7	5.5	2.0	4.2	3.2	0.7
%	0.06	0.05	0.02	0.05	0.03	0.005
Lac:Man	0.42	0.39	0.38	0.31	0.34	0.05
PEG:Man	0.13	0.13	0.09	0.17	0.13	0.01

Table 3.8 Preliminary weight gain data on zinc deficiency in growing piglets[a]

Dietary Zinc (ppm diet)	Weight Gain g/(kg•d)
0	6.7 ± 8.3
5	17 ± 12
15	30 ± 12
25	33 ± 10
50	32 ± 11

[a] Data was collected and provided through a personal communication with Dr. Ian Griffin, Dept of Pediatrics, Baylor College of Medicine, Houston, Texas (reproduced with permission).

Figure 3.1 Conceptual overview of animal and experimental protocol for protein synthesis and gastrointestinal pathophysiology in a piglet model of colitis.

Figure 3.2 Zinc absorption/endogenous loss and trace element mass balance protocol.

Figure 3.1

Conceptual Model of Animal and Study Protocol

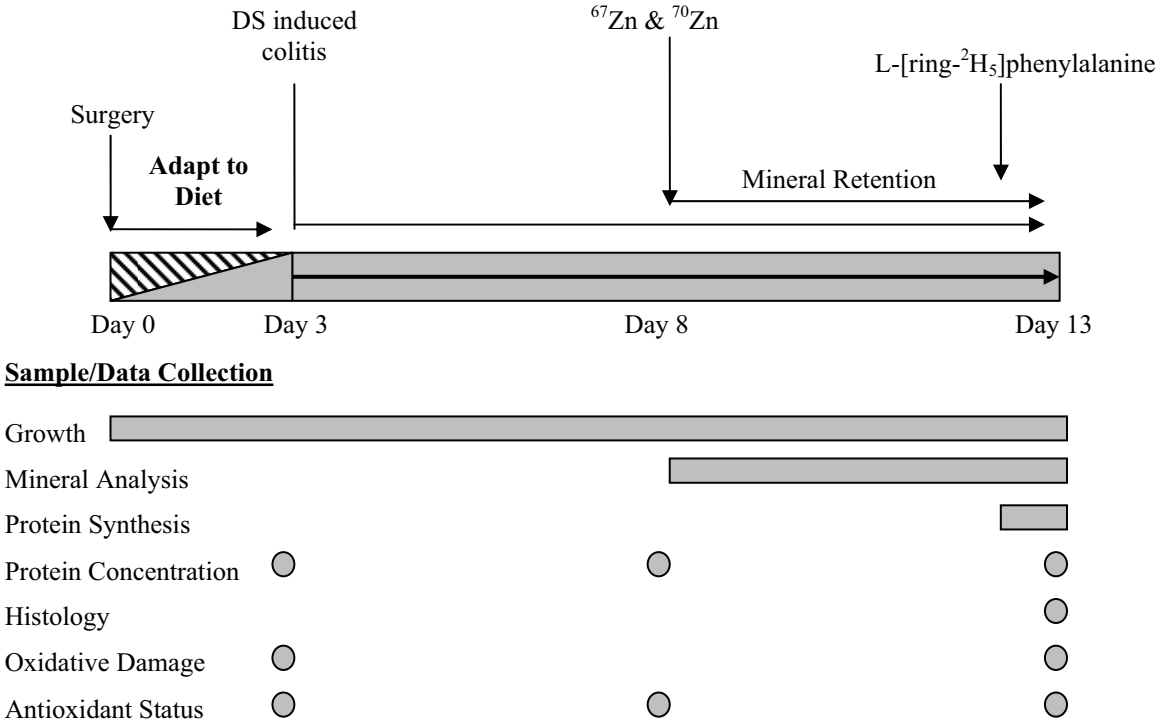
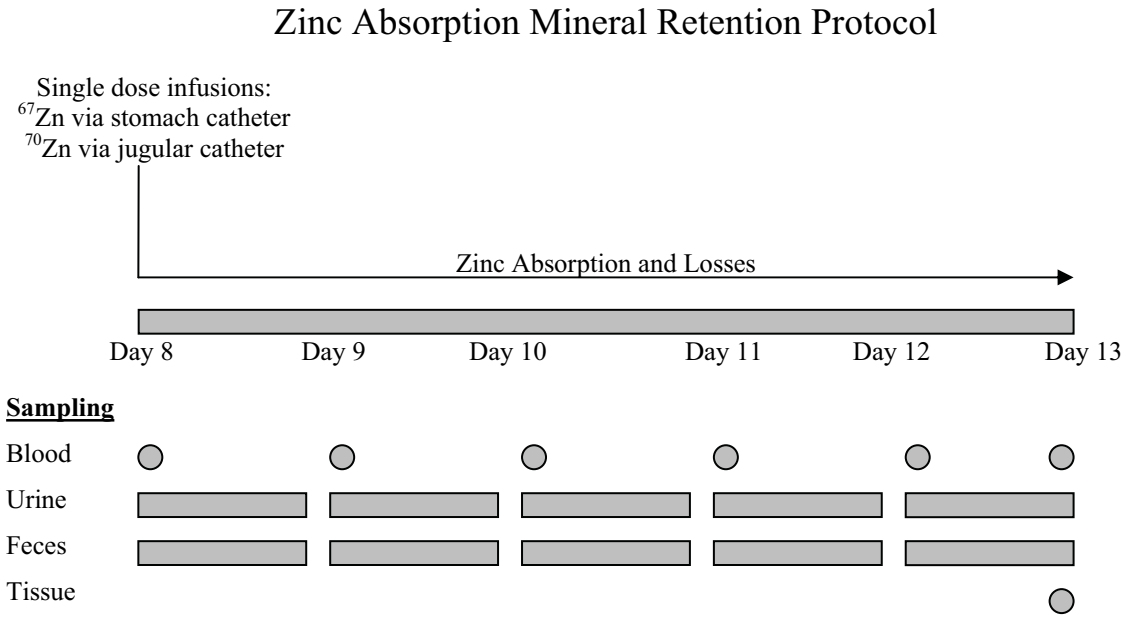


Figure 3.2


Zinc Absorption Mineral Retention Protocol
Single dose infusions:
67Zn via stomach catheter
70Zn via jugular catheter
Zinc Absorption and Losses
Day 8
Day 9
Day 10
Day 11
Day 12
Day 13
Sampling
Blood
Urine
Feces
Tissue

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from formula with additives of calcium, phosphorus, zinc, copper and iron in young piglets. *J Nutr* 123:1586-1593.

Chapter 4

Manuscript 1

Probiotics stimulate liver and plasma protein synthesis in piglets with dextran sulphate colitis and macronutrient restriction.

PROBIOTICS STIMULATE LIVER AND PLASMA PROTEIN SYNTHESIS IN PIGLETS WITH DEXTRAN SULPHATE COLITIS AND MACRONUTRIENT RESTRICTION.

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Running Title: Malnutrition and Probiotics in DS-induced Colitis

ABSTRACT

Adequate nutrition and probiotics have both been shown to reduce the severity of colitis but their impact on hepatic and gastrointestinal protein metabolism has not been studied. Our objective was to determine both how probiotic administration in the malnourished colitis state and maintained adequate nutrition with colitis affected protein synthesis rates of ileal mucosa, colon and liver (including select liver-derived plasma proteins) compared to malnourished controls with colitis. Piglets (n=8) were randomized to four treatment groups, 3 receiving dextran sulphate induced colitis and 1 histological reference group (well-nourished colitis, WNC; macronutrient restricted colitis, MR; macronutrient restricted colitis with probiotics, MRP; health reference group, REF). A primed constant infusion of the tracer L-[ring-$^{2}H_{5}$]phenylalanine was performed (WNC, MR, MRP) to determine the protein synthesis in small intestinal mucosa, colon, liver and of plasma proteins (total protein, fibrinogen, albumin). Histological and inflammatory assessments were also performed. Both adequate nutrition and probiotics lead to higher protein synthesis rates in liver and plasma protein pools. However, only well-nourished piglets had normal colon protein synthesis rates and demonstrated decreased colitis severity. While probiotics did not stimulate gut protein synthesis or reduce colitis severity, a signaling mechanism between the gut and liver would appear to be responsible for the probiotic increases in liver protein synthesis. A strategy for correcting compromised nutrition appears to be more beneficial for reducing damage during colitis accompanied by mild malnutrition in the growing piglet, than using probiotics only.

INTRODUCTION

The current use of probiotics in gastrointestinal health ranges from treating diarrhea to severe inflammatory bowel disease (1, 2), supported primarily by studies reporting favorable clinical outcomes. Various strains of probiotic bacteria have been suggested to have beneficial clinical outcomes but the most consistent evidence has been reported for lactic acid producing bacteria. While the clinical evidence for the use of probiotics in gastrointestinal diseases is strong there is a gap in the information available on how probiotic use affects tissues and metabolic processes downstream of the gastrointestinal tract.

The commercial probiotic preparation VSL#3 has been highlighted recently as a candidate for use in the treatment of IBD. Two insightful clinical studies conducted by Gionchetti and colleagues on the use of VSL#3 in pouchitis clearly demonstrated improvement in disease management for the individuals receiving the probiotics (3, 4). Since then others have shown similar clinical improvements in IBD patients, including the induction of remission in ulcerative colitis patients (5, 6). Nonetheless, speculation as to how probiotics act in the gut to reduce disease severity their mechanism of action is still largely unknown.

Several studies have focused on improved gut integrity as a possible mechanism but the results from these animal models is speculative (7-9). An alternate hypothesis, by Dotan and Rachmilewitz, suggests a complex series of molecular events involving the stimulation of toll-like receptors (10), reduced production of pro-inflammatory cytokines, interference with bacterial adherence in the colon and a possible role for NF-κB and heat shock protein cell signaling events (11) are responsible for the actions of probiotics in ameliorating IBD symptoms. The most interesting aspect of the Dotan and Rachmilewitz hypothesis is how these cellular and metabolic changes can be brought about even when non-viable probiotics are administered. It appears the current collection of studies into the mechanism of action of probiotics in treating IBD only scratches the surface of their possible underlying metabolic effects.

A chronic inflammatory state, like that of IBD, has been shown to lead to cytokine-induced catabolism of tissues in order to supply the immune system with required amino acids, micronutrients and energy (12). In IBD, this immune-driven catabolism is usually coupled with reduced food intakes resulting from the pain and discomfort associated with active inflammation in the gut (13, 14). Fasting for several days without inflammation can itself reduce gut integrity and mucosal cell metabolism and would be expected to worsen during GI inflammation (15). Furthermore, chronic protein deficiency decreases gut protein synthesis (16), and when inflammation is superimposed glutathione synthesis is also decreased (16, 17). Despite the possible effectiveness of enteral nutrition support for reducing the severity of colitis it is rarely used outside the pediatric population (18). Unfortunately, the difference between maintained nutritional status and mild malnutrition in chronic experimental colitis has not received much attention in either clinical or experimental models.

Therefore, our main objective of this study were two-fold; firstly, does VSL#3 administration in the malnourished colitis state affected protein synthesis rates of ileal mucosa, colon and liver (including select liver-derived plasma proteins) compared to malnourished colitis controls? Secondly, does maintaining adequate nutrition during colitis also maintain protein synthesis rates of ileal mucosa, colon and liver (including select liver-derived plasma proteins) compared to malnourished colitis controls? Previously, we demonstrated that colitis increases the rate of hepatic protein synthesis (constitutive and secretory) in both well-nourished and malnourished short term colitis (19). While this adaptation may be advantageous for controlling the inflammation in the short term, a sustained increase in the rate of protein synthesis during chronic malnutrition and inflammation may actually be deleterious. The relative duration for our previous colitis model was short, therefore in the present study we lengthened the duration of DS delivery. Our secondary objective of the study was to assess histological and oxidative stress markers in piglets with colitis receiving either VSL#3 or adequate nutrition.

MATERIAL AND METHODS

Experimental Protocol

32 piglets (5-7 days; Yorkshire x Landrace; individually housed) were randomized to 4 treatment groups based on diet and colitis status, 3 receiving DS induced colitis and 1 histological reference group. Twenty-four piglets, receiving 1g/(kg•d) DS, were randomized to receive a diet providing 100% NRC requirements for growing piglets (WNC), a 50% macronutrient restricted diet (MR), or a 50% macronutrient restricted diet with probiotics (MRP; VSL#3®, VSL Pharmaceuticals Inc.). The remaining 8 piglets were randomized into a well-nourished group without colitis (REF) for histological and oxidative stress marker comparison.

Immediately after piglets were removed from the sow (study day-1), catheters were implanted aseptically under isoflurane anesthesia into the femoral vein for infusion of tracers, jugular vein for blood sampling and the stomach for diet and DS administration (19, 20). On study day 2 probiotic supplementation was started. On study day 4, DS administration was started. Finally, 14 days after surgery, a stable isotope infusion study was conducted to determine protein synthesis rates. Blood was sampled throughout the infusion and tissues were sampled immediately after an intravenous injection of Euthansol (sodium pentobarbital; Schering Canada Inc, Pointe Claire, QC). Study protocol was approved by the McGill University Animal Care Committee in accordance to the Canadian Council on Animal Care Guidelines.

Dextran Sulphate-Induced Colitis

Our piglet model of DS-induced colitis has been described previously (19). Briefly, a DS solution (200g/L, 40,000 MW, ICN Biomedicals Inc, Aurora, OH) was administered through the gastric catheter twice daily at a dose of 1g/(kg•d). Feces were tested after 5 days for occult blood with Hemoccult® test packs (Beckman Coulter, Mississauga, ON) to confirm the presence of colitis.

Probiotics

Macronutrient-restricted piglets in the probiotics group (MRP) received 450 x 10^9 CFU of the bacterial mixture VSL#3® per day (VSL Pharmaceuticals, Gaithersburg, MA), equivalent to one VSL#3® packet/day suspended in 30 mL of liquid diet and delivered twice daily as a 15 mL bolus. The VSL#3® mixture contains three strains of *bifidobacteria: B. infantis, B. longum,* and *B. breve*; four strains of *lactobacilli: L.acidofilus, L.plantarum, L. delbrueckii subspecies Bulgaricus* and *L. casei*; and one strain of *Streptococcus: S. salivarius* subspecies *thermophilus*.

Diet

All piglets received a liquid diet designed to meet the following requirements. Piglets in both MR and MRP groups were supplied with a diet providing 50% of the required macronutrients for a growing piglet, while piglets in the WNC group received a nutritionally adequate diet for growing piglets (21, 22). Diets were custom-formulated in our laboratory using spray dried egg albumin (Harlan Teklad, Madison, WI) and low-lactose whey protein concentrate (Glanbia Nutritionals, Monroe, WI) as protein sources, maltodextrin (Harlan Teklad, Madison, WI) as the carbohydrate source and a blend of soybean, coconut (Harlan Teklad, Madison, WI) and flaxseed oils (MP Biomedicals, Irvine, CA) as lipid source (n-6:n-3 ratio 7.5:1) (Table 1). Vitamin and mineral mixes were added to the diet to provide adequate micronutrients for all groups. Diets were infused over a 16-hour period (Compat Enteral Feeding Pump; Novartis Nutrition) via the gastric catheter to achieve the metabolizable energy intake of 925 kJ/(kg•d) for WNC piglets and 461 kJ/(kg•d) for both macronutrient-restricted piglets, based on each piglet's daily weight.

Stable Isotope Infusion Protocol

The tracer solution was prepared by dissolving L-[ring-$^2H_5$]phenylalanine (Cambridge Isotope Laboratories, Cambridge, MA) in sterile 0.9% saline for a final concentration of 35 μmol/ml, and filtering through a 0.22 μm filter. The tracer was infused through the femoral catheter at a constant rate of 35 μmol/(kg•h) for 6 hours, following a priming dose equivalent to a 1.5-h of infusion. Venous blood was sampled at baseline and hourly

thereafter, separated and frozen in liquid nitrogen. After the 6-h sample, the piglets were euthanized and tissue samples (liver, colon and ileal mucosa) were frozen immediately in liquid nitrogen and stored at -80°C.

Protein Analysis

Total protein and albumin concentrations were determined by automated biochemistry analyzer (Hitachi 911, Hitachi America, Ltd). Plasma fibrinogen concentration was measured using a functional assay which measured turbidity of undiluted pig plasma against an equine standard at the clinical laboratory of McGill University Health Centre Royal Victoria Hospital.

Total protein in plasma was isolated by precipitation with ice-cold 0.6 mol/L trichloroacetic acid (TCA) and processed as reported previously (19). To isolate fibrinogen, an ethanol/saline (1:8) solution was added to plasma (4:1) and placed in an ice bath for 15 – 30 minutes. Samples were then centrifuged at 4°C for 10 minutes. The resulting fibrinogen pellet was dissolved in SDS PAGE sample buffer without β-2-mecaptoethanol. To isolate albumin proteins were precipitated from the fibrinogen-free supernatant with TCA and centrifuged. Albumin was re-solubilized from the pellet using 95% ethanol and agitation. The supernatant containing the dissolved albumin was mixed 1:1 with SDS PAGE reducing sample buffer. Both fibrinogen and albumin were resolved separately by SDS PAGE on a MINI-PROTEAN II System (Bio-Rad Laboratories, Hercules, CA). Fibrinogen and albumin samples along with pure standards and molecular weight markers were loaded onto the gel and run at 200V for 120 and 45 minutes, respectively. The appropriate protein bands were excised from the gel and hydrolyzed.

Tissue Free and Protein-Bound Amino Acid Isolation: n-propyl ester heptafluorobutyramide derivatives, as previously described.(19) Tracer:tracee ratios were determined using raw ion abundances and analysis of the tracer and natural abundance of phenylalanine. Isotopic steady state was confirmed between hours 3 and 6.

FSR for tissues and plasma proteins and approximate ASR for plasma proteins was calculated using the equations previously reported.(19)

Oxidative Stress Markers

Myeloperoxidase (EC# 1.11.1.7) activity (MPO) activity is commonly used as an indicator of neutrophil infiltration and oxidative stress in animals models of IBD and was assayed using the method described by Bradley *et al* (23).

Ferric reducing ability of plasma (FRAP) is a measure to total reducing or antioxidant capacity of the sample of interest. The assay was carried out according to the procedure described by Friel et al (24), which was first described by Benzie and Strain (25), with modification for use of microplate spectrophotometer.

Urinary 15-isoprostane F2t concentrations were measured by competitive ELISA assay (Oxford Biomedical Research, Oxford, MI) specific to 15-isoprostane F_{2t} (also known as 8-epi-$PGF_{2\alpha}$ or 8-iso-$PGF_{2\alpha}$). Concentrations were expressed per mg creatinine (crt), which was determined spectrophotometrically by creatinine assay kit (Oxford Biomedical Research, Oxford, MI).

Histology and Immunohistology

Segments of colon were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin using standard slide preparation techniques. Each section was then examined by 2 blinded investigators (SVH and KGF) and graded for histological damage using the scale previously published by Geboes et al (26). KI-67 expression, a measure of cell proliferation, was assessed using the KI-67 antigen kit from Vector Laboratories (Vector Laboratories, Burlington, ON, Canada; Cat #: VP-453) and expressed as % of cells, which stained positive for KI-67 in crypt and surface epithelial cells. Cellular apoptosis was quantified using the *in situ* death detection kit from Roche (Roche Life Science, USA, Cat #: 1684817).

Plasma Copper and Zinc Concentrations

Copper and zinc concentrations were determined after appropriate dilution with 0.1 M HNO_3 by atomic absorption spectrophotometry (AAS; Perkin-Elmer 3100 AAS; Perkin-

Elmer Life Sciences, Woodbridge, ON) using the method described by Makino and Takahara.(27)

Statistical Analysis

Protein kinetics parameters and oxidative stress markers were analyzed by GLM univariate ANOVA with confidence interval adjustments by Bonferroni correction and independent student t test. Histological damage scores were reported as mean damage score but assessed using the Kruskal-Wallis H non-parametric test with pair-wise determinations performed using Mann-Whitney U test. Crosstabs and Chi-square procedures were used to analyze histological grading proportions. Exact P values are reported for GLM univariate ANOVA and pairwise differences identified when post hoc comparisons were $P \leq 0.05$ with the exact post hoc P value reported in text. Values in the text are mean ± SEM. Statistical analysis was carried out using SPSS version 11.0 (SPSS Inc, USA).

RESULTS

Tissue Protein Synthesis

While we observed no difference in the FSR of the ileal mucosa between groups; however the FSR of the colon (mucosa and underlying tissue) was approximately 50% higher in well-nourished piglets compared to both macronutrient restricted groups (Table 2). Well-nourished piglets and macronutrient-restricted piglets receiving probiotics tended to have 2-fold higher FSR of the liver compared to macronutrient-restricted controls. While the GLM ANOVA met the significance criteria of $P \leq 0.05$ the post hoc comparisons did not (WNC vs. MR, $P = 0.08$; MRP vs. MR, $P = 0.06$).

Total Plasma Protein

The rate of incorporation of phenylalanine tracer into the intravascular pools of total plasma proteins, albumin and fibrinogen was linear (Figure 1). The FSR of plasma total protein was higher in the MRP group compared to the MR controls (Table 3, $P = 0.05$). This trend was also observed in the ASR. Interestingly, these increases in fractional and

absolute synthesis did not translate into an increase in plasma concentration. FSR and ASR for WNC were marginally but insignificantly higher than MR controls (Table 3).

Albumin

Both WNC (P = 0.008) and MRP (P = 0.06) piglets tended to have higher albumin FSR than the macronutrient-restricted controls (Table 3). Similarly, approximate ASR was higher for both WNC (P = 0.04) and MRP (P = 0.05) compared to MR piglets. This increase in the rate of protein synthesis translated into an increase in plasma albumin concentration for the MRP group (P = 0.05).

Fibrinogen

The positive acute phase protein fibrinogen demonstrated a similar, but insignificant, pattern for FSR to that of the total plasma protein pool however (Table 3). Nonetheless, ASR was higher for the MRP (P = 0.03) group compared to MR control piglets, but for WNC piglets the ASR was intermediate.

Myeloperoxidase Activity

MPO activity for all piglets with DS-induced colitis (combined groups; n = 24) was higher than the healthy reference piglets (Table 4; P = 0.03). Neither adequate nutrition nor VSL#3 administration resulted in a decrease in MPO activity compared to the MR controls.

Histological Presentation of the Model

The various degrees of damage according to treatment groups and diet are illustrated in the representative histographs in Figure 2. Panel A (Figure 2) is a representative section from a well-nourished and healthy piglet. No neutrophil infiltration is evident in the surface or crypt epithelial cells and crypt structure is normal. Panels B – D (Figure 2) illustrate the various degrees of damage to the colon mucosa induced by the administration of dextran sulphate.

Histological Damage

Histological damage scores were higher for all groups with DS-induced colitis (combined colitis groups, n = 24) compared to the healthy reference piglets (Table 4, P = 0.0001). Providing adequate nutrition decreased damage scores by approximately 50% (WNC vs combined MR and MRP, P = 0.005) compared to the combined macronutrient restricted groups. Interestingly, 2 of the 8 piglets from the MRP group (scores < 2) had histological scores similar to the REF group while none from the MR group had lower than 2.2. This observation was not determined to be statistically different when assessed by χ^2 analysis.

Crypt Cell Characteristics

Cell numbers followed a reciprocal pattern to that of the damage scores where the healthy reference group had a higher number of cells per intact crypt than the piglets with DS-induced colitis (Table 4; P < 0.001). Despite having a lower number of cells per intact crypt than the REF piglets, WNC piglets had crypts with higher numbers of cells than either MR (P = 0.01) or MRP (P = 0.05). No difference observed among the groups for percentage of crypt cells which were goblet cells.

Apoptosis and Cell Proliferation

Apoptosis rates were significantly higher for both MR (P = 0.01) and MRP (P = 0.006) compared to healthy reference piglets while WNC was not different from either group (Table 4). Rates of cell proliferation for WNC group (Table 4, P = 0.04) was lower than the combined macronutrient restricted colitis groups.

Oxidative Stress Indicators

Mean day-14 plasma FRAP values and mean urinary F_2-isoprostane concentrations were similar for all groups. However, plasma copper:zinc ratio was higher in the MR controls compared to the WNC (P = 0.005) and REF piglets (P = 0.006), indicating higher systemic oxidative stress. Interestingly, the copper:zinc ratio for MRP was intermediate and not different from any other group.

DISCUSSION

Avoiding malnutrition during colitis and other gastrointestinal diseases is desirable but receives low priority compared to effective and fast-acting steroidal and anti-inflammatory treatments (28). Our findings suggest the role of maintaining adequate nutrition in colitis may complement other treatments in reducing disease severity, in terms of both histological damage and nutritional status. While nutritional therapy is commonly used for adults in Japan (29), it is usually reserved for pediatric cases of IBD in North America (28, 30). Unfortunately, there are few studies exploring nutritional support or nutritional status in IBD.

The concept of correcting malnutrition as a viable strategy for reducing the inflammatory response in IBD was demonstrated a study of Crohn's disease patients, in which a positive correlation existed between malnutrition and pro-inflammatory cytokines, most strongly TNF-α (31). The connection between active IBD, an individual's nutritional status and pro-inflammatory cytokine levels is now accepted as commonplace but is seldom acted on from the point of nutritional status. Furthermore, it has been well established, in studies of total parenteral nutrition, that food restriction eventually leads to gut atrophy and other functional changes of the intestine (32), as well as increased intestinal cell apoptosis coupled with decreasing cell proliferation (33). We have demonstrated, in our piglet model of colitis both previously and in the current study, that preserving nutritional status helps to maintain normal growth, weight gain and rates of protein synthesis (data not shown) while reducing histological damage and oxidative stress. Nutritional support during active IBD should receive more attention when combining other standard treatments for the disease.

Probiotic administration did not clearly reduce disease severity in our study, however most damage outcomes were intermediate and not different from either the MR controls or the WNC piglets. While the number of studies examining the effectiveness of probiotics in treating IBD is few, the consensus seems to support reduced MPO activity and histological damage. Rachmilewitz and colleagues demonstrated VSL#3 reduced both MPO activity and histological damage in mice with DS-induced colitis (10).

Rachmilewitz and colleagues also demonstrated the effectiveness of non-viable probiotics to reduce the same parameters. Furthermore, Di Giacinto and associates have demonstrated the effectiveness of VSL#3 in ameliorating reoccurring histological damage in rats with trinitrobenzenesulfonic acid (TNBS)-induced colitis (34). Likewise, Madsen and colleagues (IL-10 deficient mouse colitis) have shown a decrease in histological damage in mice administered VSL#3 (8). We did not see a reduction in MPO activity or histological damage scores in our piglets treated with VSL#3. Variations in the models, severity of colitis and problems estimating optimal probiotic dose might explain these findings.

Model-specific differences in effectiveness have been demonstrated previously by Shibolet and associates (7). The authors reported VSL#3 reductions in MPO activity of rats with iodoacetamide-induced colitis while seeing no reductions in rats with dinitrobenzene sodium (DNBS)-induced colitis (7). Given the differing triggers of how each model initiates colitis and the possibility of species-specific responses to these initiating factors, thus caution should be taken when comparing between studies. Additionally, MPO activity is a very good indicator of neutrophil infiltration of the colon, confirming the presence of colitis. However, MPO may not be a good measure for comparing between studies which use different colitis inducing compounds or species because of possible differences in MPO activity. Histological damage assessment is very useful in determining the extent of damage to the mucosal barrier and the extent of neutrophil infiltration but again comparing across models and species coupled with the subjective nature of the scores makes it difficult to draw conclusions as to effectiveness.

The mechanism of how probiotics affect metabolic aspects of the acute phase response during colitis has not been investigated, to our knowledge. We have previously shown how muscle protein synthesis is reduced in favor of hepatic protein synthesis in macronutrient restricted piglets with colitis (19). Also, chronic protein malnutrition in the absence of inflammation reduces protein synthesis rates in most tissues but increases in plasma protein synthesis, possibly compromising muscle protein synthesis and growth when inflammation is superimposed (16). Protein synthesis rates in small intestine and

colon in piglets with colitis receiving probiotics did not increase as we expected, rather they were similar to MR controls. Similar results have been reported by Gaudier and associates, who observed no stimulatory effect of VSL#3 on mucin production during DS-induced colitis in the mouse (9). In contrast, another study demonstrated that direct adherence of probiotic bacteria to intestinal epithelial cells increased the expression of mucin RNA and prevented adherence of enteropathogenic bacteria (35). Whether probiotics stimulate the synthesis of any specific proteins in the gut is still unresolved.

The stimulation of liver and liver-derived plasma proteins by probiotics however suggests probiotic-specific metabolic changes in the acute phase response of these piglets compared to MR piglets with colitis not receiving probiotics. While there are reports in colitis and other inflammatory conditions of increased hepatic proteins synthesis, in humans and animal models, there are no studies on whether probiotics stimulation of protein synthesis in colitis or other conditions by probiotics (19, 36, 37). Loguercio and associates examined how probiotics (VSL#3) affect oxidative stress markers, select plasma cytokine levels and standard clinical outcomes in patients with either non-alcoholic fatty liver disease (NAFLD), alcoholic liver cirrhosis and hepatitis C virus (with and without cirrhosis) (38). Long term treatment with probiotics (90 or 120 days) decreased circulating liver enzymes by 50%, illustrating a direct and beneficial effect on liver function (38). Similar to our findings, they observed a probiotic induced increase in total plasma protein and albumin concentrations of alcohol-induced cirrhosis patients. We observed an increase in plasma albumin concentration but not total protein. The similarities between liver and liver derived protein synthesis rates in our study and increases in circulating plasma protein concentrations in the cirrhotic patients may be related to changes in circulating cytokine profiles. Loguercio and colleagues also reported that probiotic treatment decreased circulating levels of TNF-α and IL-6 while concurrently increasing circulating levels of IL-10 (38). While the authors offer no explanation as to the mechanism of the hepatic effects, their findings of a simulation of liver protein metabolism are supported by our data and confirm data from others showing reduction of pro-inflammatory markers (8, 34, 39).

Furthermore, in a non-alcoholic steatohepatitis (NASH) model in ob/ob mice, VSL#3 administration was as effective as anti-TNF-α antibodies in reducing serum liver enzymes and reducing hepatic JNK activity (TNF-α stimulated pathway) and reducing NF-κB activity (40). This study also supports our findings of liver stimulation, pointing to extra-intestinal effects of probiotic administration, specifically targeting the liver and acute phase proteins. While a mechanism has yet to be determined there appears to be a link between probiotic administration and circulating cytokines (decreased JNK activity, decreased circulating levels of TNF-α and IL-6 with increased circulating IL-10), decreased cellular pro-inflammatory response as measured by a decreased NF-κB activity and increased hepatic protein production (constitutive and secretory).

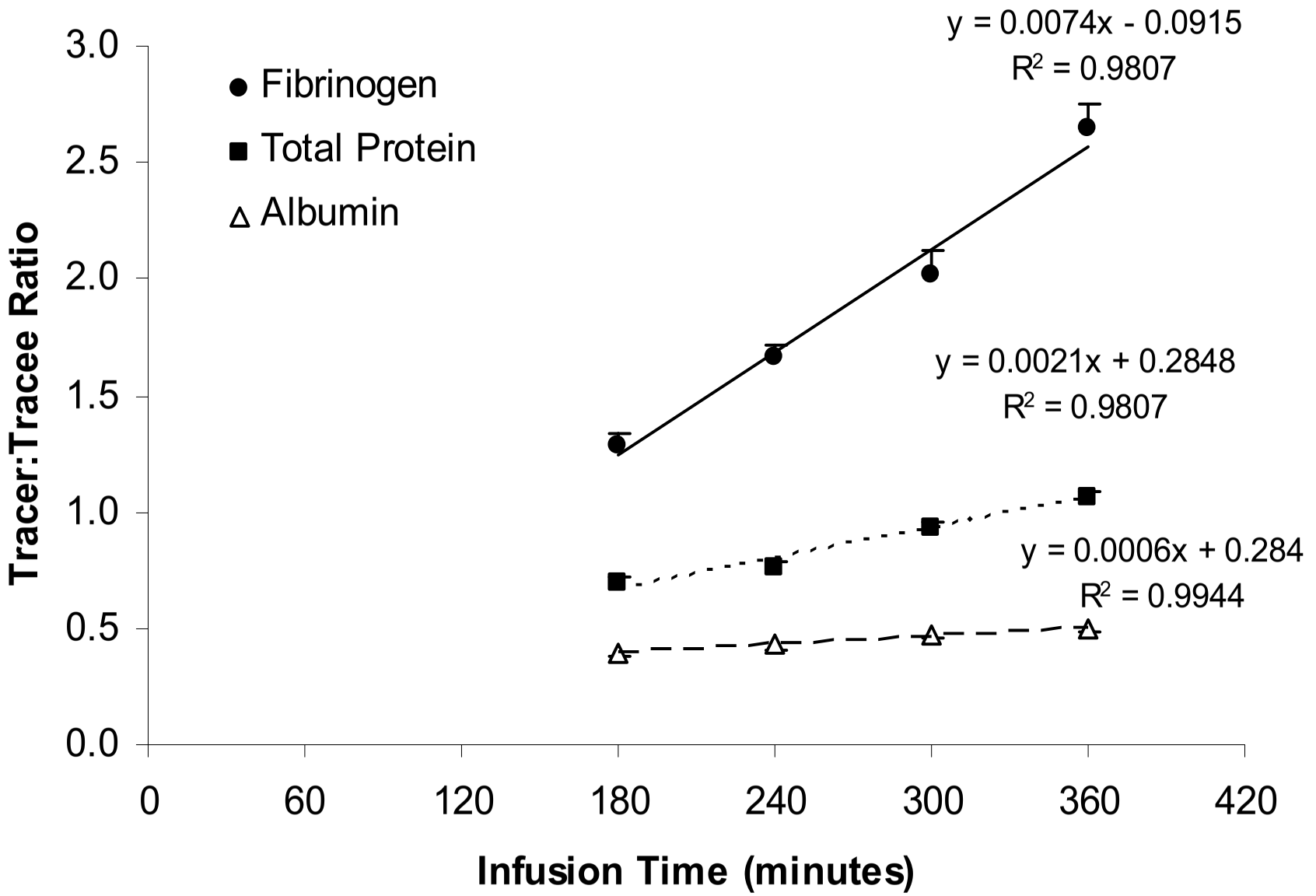
The consensus in the literature regarding VSL#3 mixture of probiotics appears to be clear in terms of the benefits of its use in both common gastrointestinal and hepatic disorders from a clinical perspective. The majority of gastrointestinal (IBD) and hepatic disorders (alcohol induced cirrhosis, NAFLD) for which VSL#3 has been shown to be beneficial have a fairly substantial pharmacological and sometimes surgical treatment component. However it is unknown whether probiotics work in concert with these treatments or if the beneficial effect of the probiotics may be blunted when used in combination with other treatments. A clearer understanding of the interaction between host's metabolic processes and probiotics is therefore fundamental to designing appropriate treatment strategies that include both traditional pharmacological and complimentary probiotic therapy.

In conclusion, an aggressive approach avoiding malnutrition in colitis may be affective as a strategy to use clinically for reducing histological damage and systemic oxidative stress. We have also shown that when macronutrients are restricted, probiotics selectively stimulate synthesis of liver and plasma proteins while having no effect on protein synthesis in the gut itself. Additional studies are needed to clearly identify the metabolic and nutritional effects of probiotic administration in diseases like colitis.

Figure 4.1 Histological micrographs (H&E; original magnification 20X) demonstrating range of damage from DS-induced colitis. Panel A is a section from a healthy control piglet, histological damage score 0.3. Panel B is a section from a macronutrient-restricted piglet with colitis, histological damage score 3.1. Panel C is also a section from a macronutrient-restricted piglet with colitis, histological damage score 4.3. Panel D is a macronutrient-restricted piglet with colitis receiving probiotics, histological damage score 5.3.

Figure 4.2 Typical pattern of L-[ring-$^{2}H_{5}$]phenylalanine tracer incorporation into newly synthesized liver derived plasma proteins. Mean tracer:tracee ratio (±SEM) of L-[ring-$^{2}H_{5}$]phenylalanine in WNC group (n=8) total plasma protein, albumin and fibrinogen.

Figure 4.1


y = 0.0074x - 0.0915
R2 = 0.9807
Fibrinogen
Total Protein
Albumin
y = 0.0021x + 0.2848
R2 = 0.9807
y = 0.0006x + 0.284
R2 = 0.9944
Tracer:Tracee Ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
60
120
180
240
300
360
420
Infusion Time (minutes)

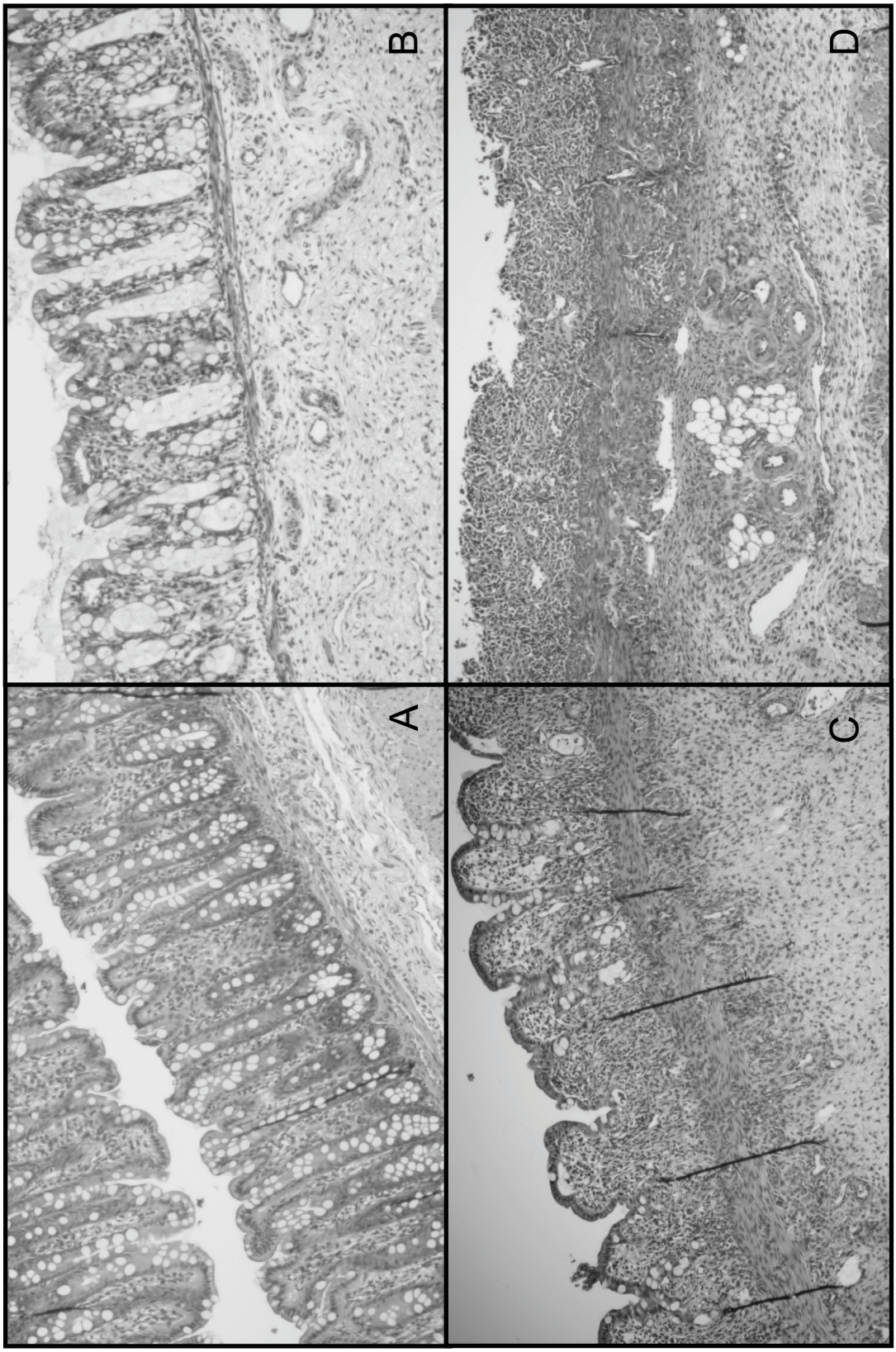


Figure 4.2

Table 4.1 Composition of liquid diets (as fed).

Diet Composition	**Well-nourished**	**Nutrient Restricted**
Energy (ME)	3085 kJ/L	1542 kJ/L
Carbohydrate	103 g/L	51 g/L
Protein	46 g/L	23 g/L
Lipid	16 g/L	8 g/L
Diet Components		
Egg Albumin	42 g/L	21 g/L
Whey	35 g/L	17.5 g/L
Maltodextrin	88.2 g/L	44.1 g/L
Soybean Oil	11.4 g/L	5.7 g/L
Coconut Oil	2.9 g/L	1.45 g/L
Flax Seed Oil	400 mg/L	200 mg/L
Vitamin Mix*	50 mg/L	50 mg/L
Mineral Mix**	5 g/L	5 g/L
Total	**185 g/L**	**95 g/L**

*Vitamin Mix (amount/L): Vitamin A Palmitate (500,000 IU/gm) – 1.2mg; Vitamin D3 (cholecalciferol) – 1.4µg; Vitamin E Acetate (335 ug/gm) – 8.7µg; Menadione Sodium Bisulfite (62.5% Menadione) - 190µg; Biotin - 70µg; Choline Chloride – 45mg; Cyancocobalamin, 0.1% - 3.8mg; Folic Acid - 32µg; Nicotinic Acid – 4.1mg; Pantothenic Acid - 120µg; Thiamin - 124µg; Pyridoxine-HCl - 350µg.

**Mineral Mix (amount/L): Calcium Phosphate, Dibasic (29.5% Ca, 22.8% P) – 4.8g; Sodium Chloride - 0.1g; Cupric Carbonate (57.5% Cu) – 2.3mg; Ferrous Citrate (22.8% Fe) – 37mg; Manganous Sulphate (36.4% Mn) – 2.3mg; Sodium Selenite (45.7% Se) - 180µg; Zinc Sulphate (22.4% Zn) – 14mg.

Table 4.2 Tissue protein fractional synthesis rates in piglets with DS-induced colitis.

FSR (%/day)	WNC	MR	MRP	SEM	GLM ANOVA P Value
Ileal Mucosa	91.6[a]	83.5[a]	78.5[a]	12.6	0.76
Colon	34.1[a]	21.6[b]	22.0[b]	3.2	0.02
Liver	38.4[a]	19.5[a]	39.4[a]	5.7	0.04

Mean ± pooled SEM, n = 8. Differing superscript letters indicate significant pairwise difference at $P \leq 0.05$. WNC, well-nourished with colitis; MR, macronutrient restricted with colitis; MRP, macronutrient with colitis and probiotics.

Table 4.3 Plasma protein fractional synthesis rates and concentrations in piglets with DS-induced colitis.

	WNC	MR	MRP	SEM	GLM ANOVA P Value
Total Protein					
FSR (%/d)	42[ab]	26[a]	53[b]	7	0.05
ASR (g/(kg•d))	944[ab]	556[a]	1376[b]	222	0.05
Concentration (g/L)	34[a]	33[a]	38[a]	2	0.14
Albumin					
FSR (%/d)	22[a]	11[b]	19[ab]	2	0.01
ASR (g/(kg•d))	276[a]	121[b]	268[a]	40	0.02
Concentration (g/L)	18[ab]	17[a]	21[b]	1	0.05
Fibrinogen					
FSR (%/d)	85[a]	53[a]	89[a]	12	0.07
ASR (g/(kg•d))	77[ab]	43[a]	96[b]	13	0.03
Concentration (g/L)	1.4[a]	1.3[a]	1.6[a]	0.2	0.52

Mean ± pooled SEM, n = 8. Differing superscript letters indicate significant pairwise difference at $P \leq 0.05$. WNC, well-nourished with colitis; MR, macronutrient restricted with colitis; MRP, macronutrient with colitis and probiotics.

Table 4.4 Characterization of disease severity, histological impact of colitis and systemic oxidative stress.

Colitis Severity	REF	WNC	MR	MRP	SEM
Colon MPO (units/mg tissue)[1]	0.1[c]	0.3	0.6	0.5	0.4
Histological Damage Score (0-5)[2]	0.4[c]	1.9[a b]	3.9	4.0	na
Crypt Index (cells/crypt)[2]	136[c]	117[b]	89	86	5.2
Goblet Cell Index (%)[2]	21	20	20	26	0.9
Cell Death and Proliferation					
Apoptosis Index (%/crypt)[2]	0.2[c]	0.6	0.9	1.2	0.6
KI-67 Index (%/crypt)[2]	5.9	4.5[b]	6.8	6.8	2.5
Oxidative Stress Markers					
Plasma [Cu]:[Zn][1]	1.6[a]	1.5[a b]	3.2	2.5	1.2
Urine F_2-Isoprostanes (ng/mg crt)[1]	31	26	28	20	13
Plasma FRAP (mmol Eq)[1]	123	110	120	117	5

Mean ± pooled SEM. [a]$P \leq 0.05$ vs. MR; [b]$P \leq 0.05$ vs. Malnutrition (combined MR and MRP); [c]$P \leq 0.05$ vs. Colitis (combined MR, MRP and WNC). [1]GLM univariate ANOVA with Bonferroni correction for multiple comparisons, independent student t test and [2]Kruskal-Wallis H and/or Mann-Whitney U tests. REF, well-nourished without colitis; WNC, well-nourished with colitis; MR, macronutrient restricted with colitis; MRP, macronutrient with colitis and probiotics.

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THESIS LINKING STATEMENT

In the previous manuscript we illustrated how modulation of hepatic protein synthesis rates could be achieved through the administration of probiotics during acute persistent colitis. This link between liver, both constitutive and secreted, protein synthesis has been shown previously in models of liver disease but this is the first such link shown in experimental colitis (1, 2). This finding, while unique, points to a complex mechanism of action for the favourable outcomes attributed to probiotic use in IBD (3, 4). Unfortunately, our study design does not allow us to further explore this link, the findings do provide a starting point for future study of this stimulation.

Interestingly, administering probiotics did not affect protein synthesis in colon or small intestinal mucosa. If in fact probiotics do enhance mucosal barrier function then an increase in protein synthesis of the colon would have been expected (5). The previous study also demonstrated that the administration of probiotics does not necessarily ensure mucosal protection during colitis. While the histological analysis was variable there was no concrete evidence to suggest there was any protective effect for the GI tract afforded to the piglets receiving the probiotics.

Maintaining adequate nutrition was show to positively maintain protein synthesis rates for the GI tract and for hepatic proteins. Reduced gastrointestinal damage was also shown to be a beneficial aspect of maintaining adequate nutrition during active GI inflammation. Collectively, these data suggest more attention should be paid in the nutritional maintenance of IBD patients, doing so may improve recovery time or assist pharmacological interventions in achieving this end.

The following manuscript is a set of studies designed to determine how colitis impacts whole body nutritional status, with specific attention to whole body protein turnover, muscle protein synthesis rates and trace element balance. Study 1 in the following manuscript explores how colitis affects whole body protein turnover and trace element balance in well nourished piglets with and without colitis. The second study, explores any positive effect attributable to administering probiotics in malnourished piglets with

colitis. It has also been proposed that probiotics can affect specific nutrient availability and this may be a component of their mechanism of action (4). Finally, we will then compare the effect of dietary macronutrient, but not micronutrient, deficiencies affect piglet with colitis.

Preventing changes in body composition and growth is also a goal in clinical treatment of IBD (6, 7). The following study will illustrate the independent roles colitis, nutrition and probiotics play in whole body protein turnover and trace element nutritional status.

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Chapter 5

Manuscript 2

Growth and trace element nutritional status in growing piglets: Implications of colitis and probiotic supplementation.

GROWTH AND TRACE ELEMENT NUTRITIONAL STATUS IN GROWING PIGLETS: IMPLICATIONS OF COLITIS AND PROBIOTIC SUPPLEMENTATION.

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Running Title: Malnutrition and Probiotics in DS-induced Colitis[2]

ABSTRACT

Nutritional deficiencies and growth failure are common aspects of inflammatory bowel disease (IBD) however the contribution specific nutrient perturbations make to the management of IBD has only recently gained attention. The present manuscript consists of 2 studies, in the first of which we examine the effect acute persistent colitis has on muscle protein synthesis and trace element status in well nourished growing piglets. In the second study, we examine the effect of probiotic supplementation on muscle protein synthesis rate and trace element status in malnourished piglets with colitis and how probiotics compare to maintaining adequate nutrition. Piglets (N=32), receiving 1g•kg^{-1}/day dextran sulphate, were randomized to receive a 50% macronutrient restricted diet, a 50% macronutrient restricted diet with probiotics or a diet providing 100% NRC requirements for growing piglets receiving phytate-free liquid diet and the additional 8 piglets were randomized into a well-nourished group without colitis. Linear growth velocity was the only parameter of growth and protein synthesis affected by colitis in well-nourished piglet. However, malnourished piglets demonstrated decreased weight gain and growth as well as reduced protein synthesis rates, regardless of presence of probiotics. Plasma iron declined to a greater extent in all piglets with colitis compared to those without, regardless of nutritional status. Zinc losses were higher in malnourished piglets versus well-nourished piglets. In light of our findings we suggest the use of dual isotope techniques to determine the role endogenous zinc loss and absorption rates affect status during acute inflammation. In conclusion, maintaining adequate nutrition appears to be essential for maintaining normal growth, weight gain and muscle protein synthesis during acute persistent colitis, while providing probiotics did not affect these outcomes.

INTRODUCTION

Decreased growth velocity is frequently evident in children at the time of diagnosis of both ulcerative colitis (UC) and Crohn's disease (CD) (1, 2), and may be associated with delayed menarche and ultimately lead to permanent stunting (3). Decreased dietary intake due to abdominal pain or appetite suppression from elevated cytokine production is a contributing factor (4). A more comprehensive view of growth failure includes the inflammatory injury and repair responses of the intestinal mucosa, and increased synthesis of acute phase proteins in the liver, coupled with the cytokine/glucocorticoid-mediated catabolism of muscle protein (5). Both trace elements and protein are intimately involved in cell division, protein synthesis, immune function and deficiencies of each have long been associated with growth failure. Clinically, deficiencies of both nutrients are relatively common in children and adults with IBD (6).

Maintenance of muscle mass is dependent on a balance between muscle protein synthesis and proteolysis, a balance which must be positive if growth is to occur. Results from our piglet model (7) support a critical role for compromised protein synthesis in the muscle wasting of IBD; however one must also consider the impact of inflammation on muscle proteolysis. Rather than showing a reduction in proteolysis corresponding to decreased synthesis, there is evidence that breakdown of muscle proteins is increased during cachectic and inflammatory conditions (8, 9). As Reeds et al. (10) suggested, skeletal muscle can be viewed as an amino acid reservoir that can supply amino acids when dietary protein is not adequate to meet elevated metabolic demands. Our previous study provided evidence that maintaining protein and energy intake over the short term prevents the decrease in muscle protein synthesis and growth associated with inflammation. Furthermore, improved weight gain and normalization of body composition appears to play an important role in controlling IBD disease activity (11, 12). Therefore improving nutritional support both in terms of protein quantity and quality has an important role in supporting the acute phase response while maximizing protein synthesis and minimizing amino acid loss.

Malnutrition and muscle wasting are well known complications of chronic gastrointestinal (GI) inflammation, as seen in individuals with IBD (13-15). In children, the diagnosis of IBD is frequently preceded by indicators of compromised nutritional status including decreased height velocity and body weight, with severe growth failure occurring in approximately one third of these children (16, 17). Decreased food intake and cytokine induced anorexia are largely to blame for disease-associated wasting, which is reversible when nutritional demands are met (18, 19). Despite evidence to suggest that refeeding may play an important role in inducing remission and/or improving clinical outcomes, the focus of many investigations often neglect the role of nutrition in managing GI inflammation (11, 12).

Our group has previously investigated the impact of decreased macronutrient intake and acute GI inflammation on protein metabolism in our piglet model of DS-induced colitis (7). We showed that colitis had no effect on protein metabolism or growth when piglets were well nourished (7). More interesting was the effect of macronutrient restriction during colitis, which decreased whole body protein turnover, muscle protein synthesis and growth, while hepatic synthesis of acute phase proteins was increased. Colitis induced a doubling of albumin synthesis regardless of nutritional status (7). During infection and/or injury, immune function is compromised by malnutrition, this creates a vicious cycle which negatively affects the acute phase response, as well as muscle protein synthesis and growth (7, 20-22). Interventions which may disrupt this cycle either through decreased disease severity and/or improved immune function may play an important role in controlling GI inflammation and thereby indirectly improve muscle protein synthesis and growth.

Along with perturbations in protein nutrition, copper, iron and zinc status are commonly compromised in IBD however these deficiencies are not only related to reduction in intake or impaired absorption (23-27). Endogenous losses make a substantial contribution to the decline in mineral status independent of dietary intake in both clinical and experimental IBD (28, 29). Impaired copper, iron and zinc status with concurrent acute or chronic inflammation, even at the sub-clinical level, can deleteriously influence

immune and repair functions (30-33). In paediatric IBD or repeated long term chronic flare-ups in adult patients the combined loss of homeostasis for these trace elements can limit erythropoiesis, linear bone growth and lean body mass accretion (26, 34-41).

While a deficiency in each trace element affects different systems, the negative effects usually overlap thereby worsening as the disease progressed. For example, copper is a key component of several enzymes including the antioxidant enzyme copper/zinc superoxide dismutase and the respiratory enzyme cytochrome c oxidase. Reduction in the activity of these enzymes would impact antioxidant protection in an oxidatively stressful disease and potentially impact cellular respiration. Copper status is liked directly to the immune function in acute phase response situations. An increase in plasma copper concentration, via increasing hepatic synthesis of ceruloplasmin, is stimulated by IL-1 and IL -6 (42). These cytokines (IL-1, IL-6, TNF-α) are increased during acute phase responses and help coordinate the host immune response to invading pathogens. The increase in plasma copper, through increased circulating ceruloplasmin, directly stimulates an increased IL-2 secretion in leukocytes involved in the acute phase response. Increased IL-2 secretion by these cells directly stimulates the acquired immune system by increasing T cell proliferation (43-46). Therefore, copper status is tightly linked to immune responses and perturbations in copper status may negatively impact immune responses.

Similarly, iron deficiency in IBD, caused by a combination of reduced food intake, impaired absorption and intestinal occult blood losses, also impacts the progression of inflammatory diseases. Aside from the impact on oxygen carrying capacity, impaired iron status also affects immune function. Both macrophages and neutrophils have decreased pathogen eradicating ability during iron deficiency, caused by a decreased hydroxyl radical formation and myleoperoxidase activity, respectively (47-50). Macrophages are also know to be iron scavengers/storage sites during microbial invasion, limiting available iron to both host and invader (51). Collectively, the impact of iron deficiency during IBD affects a wide range of systems at both the whole body and cellular level.

Furthermore, zinc deficiency also influences outcomes in IBD. The ubiquitous nature of zinc has far reaching contributions to normal cellular and antioxidant functions. Zinc is required for connective tissue metabolism (52), gene expression, cell proliferation and differentiation (53), normal immune function (54), protein synthesis (55) and antioxidant protection via copper-zinc superoxide dismutase. Each of these metabolic processes requires normal zinc homeostasis and active IBD can negatively affect each of these processes.

A better understanding of the functional impact and status of these nutrients during active IBD is required. We have developed a model of colitis in the growing piglet which simulates colitis under anabolic conditions (56). Using this model we are investigating the interactions of colitis, nutrition and trace element metabolism through 2 studies. The objective of study 1 was to determine how colitis affects muscle protein synthesis and trace element balance in well nourished piglets. The objective of study 2 was to investigate the impact of probiotic supplementation and mild macronutrient restriction in piglets with colitis.

Our focus on probiotics, specifically the VSL#3 mixture, stems from the positive clinical data in colitis patients (57, 58). Recent studies have shown that probiotic supplementation may prove beneficial in controlling inflammation in individuals with IBD, as well as in treating and/or preventing experimental colitis (59-65). However, treatments with the potential to augment the resident host microflora might also affect amino acid utilization and the absorption and endogenous losses of trace elements. Furthermore, there is evidence to suggest that the effect of probiotics may not be limited to the GI tract, as supplementation has also been shown to improve recovery from malnutrition and increase feed efficiency after short term starvation (66, 67). Moreover, the mechanism by which probiotics decreases GI inflammation may be through a direct effect on nutrition. More specifically, it is not yet known if probiotics affect specific nutrient metabolism during colitis which may in turn improve growth and muscle protein synthesis. Therefore, the present set of experiments were designed to determine the

specific effect of colitis and the combined effects of malnutrition and probiotic supplementation have on protein and trace element status in the growing piglets acute persistent gastrointestinal inflammation.

METHODS AND MATERIALS

Animals and Study Protocol

32 piglets (5-7 days; 8 piglets/group; Yorkshire x Landrace) were purchased from the Macdonald Campus Farm Swine Complex of McGill University and taken immediately to the Large Animal Research Units and randomized into 1 of 2 studies. Study 1 consisted of 2 groups of well nourished piglets, 1 group receiving dextran sulphate (DS) treatment in order to see the effect of colitis on plasma trace element concentrations and retention compared to a healthy reference group of piglets (REF: healthy, well-nourished; WNC: colitis, well-nourished). In Study 2, we studied the effect probiotics on trace element concentrations and retention in two groups of piglets with DS induced colitis receiving a macronutrient restricted diet (50% NRC recommendations) with 1 group receiving VSL #3® probiotic supplementation (MR: colitis, malnourished; MRP: colitis, malnourished, probiotics).

Each piglet underwent aseptic surgery under isoflurane anaesthesia to implant a central venous catheter (external jugular vein, 1.02mm ID x 2.16mm OD) as well as gastric and bladder catheters (1.57mm ID x 3.18mm OD). Piglets were then maintained individually at the Small Animal Research Unit in specially designed piglet metabolic cages. The piglets recovered and were adapted to the enteral diet during the following 3 days post-surgery. Probiotic supplementation was started on study day 1. On study day 3, twice daily intragastric administration of dextran sulphate to induce mild-moderate colitis. Blood samples (for analysis of plasma trace element concentration) were collected on days 3 and 13. A separate 5-day collection of urine and feces was started on Day 8 once colitis piglets tested positive for occult blood in feces.

The study protocol was approved by the McGill University Animal Care Committee according to the Canadian Council on Animal Care Guidelines.

Dextran Sulphate Induced Colitis

Our piglet model of DS induced-colitis has been described (56). We had previously established a piglet colitis model in our laboratory, which we adapted and modified from the rodent model (56). A DS solution (200g/L, 40,000 MW, ICN Biomedicals Inc, Aurora, OH) was administered through the gastric catheter in 2 daily doses for a daily total of 1g DS•kg^{-1}/day for 11 days. Feces were tested for occult blood with Hemoccult® test packs (Beckman Coulter) to confirm the presence of colitis. While the previous model we used simulated acute colitis over a short period, the modification reported here, along with the changes in diet formulation and delivery, allow for better control of severity of colitis and diet delivery. This model is very well suited to study nutritional outcomes during colitis.

Probiotics

Macronutrient restricted piglets in the probiotics group (MRP) received 450 x 10^9 CFU of the bacterial mixture VSL#3® per day (VSL Pharmaceuticals, Gaithersburg, MA), equivalent to one VSL#3® packet/day suspended in 30 mL of liquid diet. The VSL#3® mixture contains three strains of *bifidobacteria: B. infantis, B. longum,* and *B. breve*; four strains of *lactobacilli: L.acidofilus, L.plantarum, L. delbrueckii* subspecies *Bulgaricus* and *L. casei*; and one strain of *Streptococcus: S. salivarius* subspecies *thermophilus*. The dose was calculated based on previous studies using VSL#3® in rodents and humans, using $body\ weight^{0.73}$ to scale between metabolic body sizes (68-70). The bacterial suspension was mixed with diet, divided equally and delivered twice daily as 15 mL boluses, infused directly into the gastric catheter.

Diet

All piglets received a liquid diet custom designed to meet the following requirements. Piglets in study 1 were supplied with a diet which provided adequate nutrient intakes according to National Research Council guidelines for growing piglets (Table 3.1) (71). Piglets in study 2 were supplied with a diet which provided 50% of the macronutrient requirement but adequate levels of all micronutrients (Table 4.1). Diets were custom-

formulated in our laboratory using spray dried egg albumin (Harlan Teklad, Madison, WI) and low-lactose whey protein concentrate (Glanbia Nutritionals, Monroe, WI) as protein sources, maltodextrin (Harlan Teklad, Madison, WI) as the carbohydrate source and a blend of soybean, coconut (Harlan Teklad, Madison, WI) and flaxseed oils (MP Biomedicals, Irvine, CA) as lipid source (n-6:n-3 ratio 7.5:1). Vitamin and mineral mixes were added to the diet to provide > 120% of the NRC requirement for each micronutrient (Table 3.1). Excess biotin (10 times requirement) was provided to overcome binding/inactivity of the vitamin due to the high avidin content of egg albumin.

The minimum daily requirement of zinc for young growing piglets on corn/soybean feed is 50 ppm of diet which translates to an intake of approximately 3 (mg•kg)/day for a weaned growing piglet (71). However, most commercial feeds provide zinc at greater than 100 ppm (> 6 mg•kg/day) to promote growth and overcome the reduced bioavailability of zinc due to the high phytate content of the diet components (72). Zinc present in and added to phytate-free diets is more highly bioavailable, therefore the requirement is much lower (6 – 20 ppm) (73, 74) which is the rationale for the current recommendation in casein/glucose-based diets of 15 ppm of diet or 30% of the requirement of diets high in phytates (71). Therefore, for our diet, used 15 ppm as the zinc requirement and apply the same factor (2X) that is applied to commercial feeds to ensure adequate zinc intake in all our piglets. This resulted in a diet containing 30 ppm and yields a daily intake of 1.8 $mg \cdot kg^{-1}$/day.

Diets were infused over a 16-hour period (Compat Enteral Feeding Pump; Novartis Nutrition) via the gastric catheter to achieve the metabolizable energy intake of 925 $kJ \cdot kg^{-1}$/day (Table 3.1) for study 1 and 461 $kJ \cdot kg^{-1}$/day based on each piglet's daily weight.

Growth and Weight Gain

Snout to rump length and chest circumference were measured at the beginning of the study (preoperatively) and again after 14 days of treatment after the animal had been euthanized. Weight was measured daily.

Plasma Urea and Cortisol

Plasma urea and cortisol concentrations was measured using an automated clinical biochemistry analyzer (model Hitachi 911; Hitachi) at the Animal Resource Center, Faculty of Medicine, McGill University.

Amino Acid Tracer Analysis

Isotopic steady state in plasma free phenylalanine was defined as the mean tracer:tracee ratio of phenylalanine above baseline between hours 3 and 6 of the infusion.

Total phenylalanine flux (Q) was calculated as:

$$\text{Eq 1.}\quad Q_{Phe}\,[\mu mol/(kg \bullet hr)] = i\,[(E_i / E_p) - 1]$$

Where Q_{Phe} is the total turnover rate of phenylalanine in $\mu mol \bullet kg^{-1}$/hour; i is the rate at which the tracer was infused [μmol/(kg•hr)]; E_i is the enrichment of the L-[ring-$^2H_5$]phenylalanine infusate; and E_p is the tracer:tracee ratio of plasma phenylalanine between hours 3 and 6 of the infusion (7, 75). Whole body protein turnover was calculated from phenylalanine flux based on the phenylalanine content of body protein in piglets (3.7 g/100g) (76).

Tissue Free and Protein-Bound Amino Acid Isolation: Aliquots of frozen tissue were homogenized in ice-cold 0.6 mol/L TCA and centrifuged at 1500 x g and 4°C for 10 minutes. The supernatant containing the free amino acids was removed for analysis as tissue free amino acids, and the protein pellet washed twice with deionised water and hydrolysed.

All purified protein samples were hydrolysed under nitrogen for 16 hours in 4 mol/L HCl at 110°C. The resulting amino acids were isolated by ion exchange chromatography (Dowex 50WX8, Bio-Rad, Mississauga, ON), dried and converted to n-propyl ester heptafluorobutyramide derivatives, as previously described.(56)

Phenylalanine enrichment was analyzed by methane negative chemical ionization gas chromatography-mass spectrometry (Hewlett Packard, Model 5988A, Palo Alto, CA), by

monitoring the [M-FH]- ions at mass to charge ratio 383 and 388, corresponding to unlabelled and labelled ions, respectively. Tracer:tracee ratios were determined using raw ion abundances and analysis of the tracer and natural abundance of phenylalanine. Isotopic steady state, was confirmed between hours 3 and 6.

Protein Synthesis Calculations

Fractional Synthesis Rate (FSR) of mixed proteins in each tissue was calculated as:

$$\text{Eq. 2} \qquad \text{FSR (\%/day)} = \frac{\Delta E_{bound} \times 24 \times 100}{E_{free} \times 6}$$

Where ΔE_{bound} is the net tracer:tracee ratio of protein-bound phenylalanine above baseline in the tissue at hour 6, E_{free} is the net tracer:tracee ratio of tissue free phenylalanine at steady state.

Sample Preparation and Analysis

Full 5-day fecal collections (study days 8-13) were dry ashed in a muffler furnace at 550°C overnight, the resulting ash was accurately weighed with a sub-sample taken and dissolved in 1 M HNO_3. Fecal copper, iron and zinc concentrations were determined after appropriate dilution with 0.1 M HNO_3 by atomic absorption spectrophotometry (AAS; Perkin-Elmer 3100 AAS). Plasma and urine samples were digested in concentrated HNO_3 overnight, dried and reconstituted in 1 M HNO_3. Copper, iron and zinc concentrations were then determined, following appropriate dilution with 0.1 M HNO_3, using the method described by Makino and Takahara.(77)

Statistical Analysis

All data was analyzed using SPSS version 11.0 (SPSS Inc, USA). Growth and protein synthesis outcomes were analyzed by univariate analysis of variance. Weight gain, plasma urea and masseter FSR were log transformed for statistical testing due to heterogeneous variance as determined by Levene's Test. Repeated measures analysis of variance (2 factors) was used to determine any time, group and time-by-group differences in plasma trace element concentrations. Time effects for each comparison are reported and further analyzed using paired T test. In the case of significant time-by-group

interactions both between and with-in group T tests were performed and adjusted (Bonferroni correction). Trace element mass balance was assessed using univariate analysis of variance. Frequency of positive trace element balance in each group was assessed using crosstabs and X^2 test. All data are reported as mean ± SEM and differences considered significant if $P < 0.05$. Statistical power is >80% for all parameters with $P < 0.05$.

RESULTS

Weight Gain and Growth

In study 1, colitis alone did not significantly affect weight gain or chest circumference but there was a reduction in snout-to-rump length increase for the WNC piglets (Table 5.1, $P < 0.05$). In study 2, probiotics had no effect on weight gain, chest circumference or snout-to-rump length. The affect of mild malnutrition with superimposed colitis was evident when both groups of malnourished were combined (MR-C) and compared to the WNC piglets. Both weight gain and chest circumference were reduced in the malnourished piglets (Table 5.1, $P < 0.05$) while there was no difference in snout-to-rump length.

Muscle Protein Synthesis and Whole Body Protein Turnover

The affect of colitis alone in the well nourished piglet did not affect the fractional protein synthesis rate of muscle tissue nor did it affect whole body protein turnover or phenylalanine flux (Table 5.2, $P > 0.05$). In study 2, probiotics did not affect FSR or whole body protein turnover (Table 5.2, $P > 0.05$). The comparison between the MR-C piglets and WNC indicate approximately a 30% decrease in estimated FSR of muscle tissue, whole body protein turnover and phenylalanine flux. Plasma urea concentrations are also higher in the MR-C piglets (Table 5.2, $P < 0.05$), indicating the increased use of amino acids as energy substrates.

Plasma Trace Element Concentrations

In study 1 neither time nor colitis affected on plasma copper concentrations (Figure 5.1). Plasma iron concentrations for all piglets in study 1 decreased ($P = 0.03$) however when

groups were analyzed separately using paired t tests the colitis groups tended to have the greater decline in concentration (REF P = 0.38; WNC P = 0.06). No effect of colitis was detected between groups for plasma zinc concentrations, the WNC piglets did demonstrated a decline over time (P = 0.009).

Similarly in study 2, plasma copper concentrations did not change over time or as a function of probiotic administration (Figure 5.2). In contrast, there was a decline over time in plasma iron concentrations for the probiotics group when analyzed by paired t test; however plasma iron concentrations did not differ between groups in this study. Plasma zinc concentrations declined over time for all piglets in study 2 regardless of treatment group (combined, P < 0.0001; MR, P <0.001; MRP, P = 0.001). Interestingly, piglets receiving probiotics had consistently higher plasma zinc levels that MR piglets (between subject analysis, P = 0.03).

Finally, to compare the effect of macronutrient restriction during colitis on plasma trace element concentrations we combined the MR and MRP piglets and compared them to the WNC piglets (Figure 5.3). Again, plasma copper concentrations were not different between groups, either over time or as a function of diet. Collectively colitis caused a decrease in plasma iron concentrations (P = 0.004) but when analyzed by paired t test the decline was only significant for the combined macronutrient restricted piglets (MR-C, P < 0.05; WNC, P = 0.06). Similarly, both groups had declining plasma zinc concentrations over time (all piglets p < 0.0001; MR-C, P < 0.0001; WNC, P = 0.009). A significant time-by-diet interaction was also observed indicating macronutrient restriction led to a larger decline in plasma zinc concentration (time-by-diet interaction, P = 0.04).

Trace Element Balance

Mean copper balance was slightly positive for well fed piglets with colitis while healthy reference piglets were for the most part in balance (Table 6.1; 1/5 in positive balance). Mean absolute iron retention was not different between healthy piglets and those with colitis but the percentage of piglets in positive balance was significantly lower in the REF

group compared to WNC piglets (2/5 vs 6/6; X^2, P = 0.04). However, zinc balance was similar between both healthy and colitis piglets.

Probiotics did not have any effect on balance of copper, iron or zinc. However, comparing the combined results from the macronutrient restricted piglets to the balance data from WNC piglets showed interesting parallels between well nourished and malnourished colitis for copper and iron. Surprisingly, the majority of the malnourished piglets were not in positive balance as was the case in study 1. Fecal losses in the combined malnourished groups were higher compared to WNC piglets (P < 0.05) which translated into a trend toward lower apparent absorption (P = 0.06) and retention (P = 0.07) for zinc.

DISCUSSION

Weight Gain and Growth

Ensuring adequate nutrient intakes during active colitis maintained normal weight gain and chest circumference growth rates in the piglets of study 1. The linear growth rate of the WNC piglets was less than that of the healthy control piglets but this finding is similar to what has been observed in clinical paediatic cases of IBD (1). There was no appreciable affect on weight gain or growth parameters by providing probiotics in the malnourished piglet with colitis; these outcomes were identical in both groups. However the impact of mild malnutrition was evident when these 2 groups of piglets were combined and compared to the WNC piglets. Weight gain and chest circumference growth rate were reduced by approximately 50% and 30%, respectively, while no difference was observed in linear growth rate. The importance of maintaining adequate nutrition during severe inflammation is evident in this comparison. Reduction in growth rates of this magnitude are over a 14 day period, as was the case in our study, would have serious implications for stunting in a human paediatric population.

Protein Synthesis and Whole Body Protein Turnover

The growth failure, muscle wasting and hypoalbuminemia so typical of IBD are related to the interactive effects of protein nutrition and the acute phase response. Rates whole body

protein synthesis and breakdown, which represent a weighted average of protein kinetics in the whole body, may be increased in adults (78) and children (79) with IBD, yet may be accompanied by negative nitrogen balance, growth failure and muscle wasting. While protein synthesis in the affected organ is increased (80), protein synthesis in other organs may be decreased especially when dietary intake is low. The results of study 1 demonstrated that in situations where adequate nutrient intakes are maintained, muscle tissue protein synthesis is not compromised or reduced. Moreover, as with the comparison of growth rates in study 2 probiotic supplementation did not affect the protein synthesis rate of muscle tissue not of whole body protein turnover or phenylalanine flux. However, the comparison of the combined macronutrient restricted piglets and the WNC piglets demonstrated the impact of limiting macronutrient intake, approximately a 30% reduction in FSR.

Clinically, both cytokine-induced anorexia and food avoidance contribute to the frequent finding of low intake in children and adults with IBD. Infiltration of activated leukocytes in inflamed regions of the GI tract lead to release of pro-inflammatory Th1 cytokines (i.e. IL-6, IL-1β, TNF-α), which stimulate the acute phase response. The acute phase response includes increased production of inflammatory and immune proteins, selected transport proteins, and coagulation/fibrinolytic proteins (81), and is accompanied by metabolic changes which put the body in a catabolic state via decreases in anabolic hormones and factors (i.e. IGF-1) and increases in catabolic hormones (i.e. cortisol) (81). Therefore, increased metabolic demands imposed by immune activation, tissue injury and repair of the inflammatory response, including the synthesis of acute phase proteins, are complicated by the restriction of macronutrient intake.

Our group has previously described the impact of nutrition on protein metabolism in a 7 day model of acute DS-induced colitis (7). In this previous study we found that when macronutrient intake was restricted, there was an adaptation to acute inflammation which involved redistribution of metabolic processes away from growth and muscle protein synthesis to support the more urgent demands of the acute phase response (7). The present study and the findings of the previous study show that provision of adequate

nutrition is essential to preventing growth impairment and maintaining muscle protein synthesis during active GI inflammation (7). Finally, the use of probiotics in the malnourished state does not appear to have any affect on growth rate or muscle protein synthesis rate.

Trace Element Balance and Status

Trace element status in individuals with IBD is not routinely assessed unless there are functional complications such as anaemia or skin lesions (33, 82). Considering the impact trace elements have on immune and acute phase responses, maintaining optimal levels may help control the extensive inflammatory damage caused by IBD. The two experimental conditions we have assessed in the present study illustrate how trace element metabolism is affected by colitis.

In the well-nourished state, colitis has minimal impact on trace element status. Plasma concentrations of copper, iron and zinc for the most part were similar between the all colitis and healthy reference groups. Our findings for plasma copper and zinc concentrations were comparable to those reported by other researchers, in piglets of similar age (83). Plasma iron concentrations declined in both groups, as is expected in any rapidly growing animal with an increasing red cell mass. Despite the clear indication of active colitis, from the presence of occult blood in the feces of the WNC piglets, plasma iron was not different from the healthy reference group.

However, the iron balance data suggests colitis increases dietary iron requirement. All WNC piglets had positive iron balances, which indicates an increased requirement of iron for these piglets. While mean retention was not statistically different from the controls the number of piglets in positive balance was higher in the colitis group. While the level of dietary iron was adequate over the duration of this study, this might not be the cases of long term or recurrent episodes of inflammation. This finding is important clinically due to the controversy regarding the provision of oral iron supplements during gastrointestinal inflammation. Giving oral iron supplements has been shown, in

experimental models of colitis, to increase oxidative damage (84, 85). Therefore, careful management of oral iron supplementation or intravenous administration is necessary.

Increases in serum copper, and to a lesser extent serum zinc, are normal during inflammatory conditions; however we observed a decrease in the plasma zinc concentrations of all colitis piglets. Dalekos and associates, in a study of circulating zinc, copper and immune markers in colitis patients, reported increasing serum copper and zinc levels in well-nourished patients with active colitis compared to controls (86). Likewise, a study investigating serum trace elements in IBD also reported higher serum copper and zinc concentrations in patients with colitis and consistently higher copper in all forms if IBD studied when compared to healthy controls (87). In contrast, Sturniolo and associates reported no difference in plasma copper and zinc but a decline in plasma iron in patients with active colitis (27). Similarly, Al-Awadi and associates showed no difference in serum concentrations of copper or zinc in rats with TNBS-induced colitis (28). Since plasma zinc tends to remain relatively constant during inflammatory conditions it is difficult to attribute the decline we observed exclusively to the inflammation and damage versus a function of growth and expanding blood volume with superimposed inflammation.

Furthermore, a study by Lih-Brody et al demonstrated how biopsy collected, mucosal tissue concentrations of copper and zinc decrease while iron increases in IBD compared to healthy controls (88). This study illustrates 2 key points regarding trace element status in IBD, 1) despite normal plasma levels for copper and zinc functional levels in affected tissues may not be adequate and 2) the decline in plasma iron concentrations is more complex than can be explained simply by losses due to inflammation and damage. These contradictions in the literature make serum levels of trace elements an unreliable biomarker for trace element status in well-nourished colitis.

Plasma copper and iron concentrations in macronutrient restricted piglets responded similarly to those of well-nourished piglets. Plasma zinc concentrations declined in both groups of macronutrient restricted piglets over time, with a sharper decline in the piglets

receiving VSL#3. However, the plasma zinc concentrations were different at baseline and declined at virtually the same rate. The difference between plasma zinc concentrations was the only change related to the probiotic administration given that all other trace element data were virtually identical for both these groups. We are at a loss to explain how probiotics may have lead to a decrease in only plasma zinc concentrations versus the other trace elements assessed.

An interesting comparison was then made between the well nourished piglets with colitis from study 1 and the combined groups of macronutrient restricted piglets. The lower plasma iron concentrations in the WNC group is most likely related to the expanding blood volume in the more rapidly growing piglets since there was no diet interaction in this comparison (89). This is a common feature of blood iron levels observed in growing subjects. Plasma zinc concentrations however declined at a higher rate in the macronutrient restricted piglets, as indicated by a time-by-diet interaction. These piglets also demonstrated higher fecal zinc losses and negative zinc retention while the well-nourished colitis piglets were in positive balance. These findings point to a specific perturbation in zinc metabolism and not iron and copper metabolism only in the macronutrient restricted piglets with colitis. There is a possibility that these increased losses may be due to increase endogenous losses of zinc as a function of the damage caused by the underlying colitis (29).

In summary, the provision of adequate macronutrient intakes before and during an active insult of colitis appear to maintain normal weight gain, growth rates and muscle protein synthesis rates. However, despite adequate micro and macronutrient intakes linear growth appears to decrease during active inflammation. Probiotics do not have any appreciable influence on growth or protein status parameters despite a promising clinical record for treating colitis (58). Trace element status, as measured by mass balance, points to zinc homeostasis being somewhat affected in well-nourished colitis and a specific alteration in zinc status in macronutrient restricted colitis. Colon crypt damage and cell proliferation maybe related to the negative effects observed given the close relation between zinc, immune function and cell proliferation (90). Preliminary data

from this study as to histological damage and cell proliferation indicate that such a relationship may exist (91). Furthermore, Griffin et al have already demonstrated excessive zinc loss in adolescents with Crohn's disease is multi-factorial; pointing to both changes in absorption and loss as the mechanism responsible (29). Due to the lack of suitable biomarkers for copper and zinc status, stable isotope studies, which simultaneously examine both zinc absorption and endogenous losses, would help clarify the relationship between nutrition, colitis and zinc homeostasis. Finally, while no clear benefit for probiotics was show for either protein nutrition or trace element status, nether was there any detriment to their administration.

Figure 5.1 Mean (±SEM) plasma copper, iron and zinc concentrations at day 3 and day 13 for healthy reference piglets (REF, n = 9) and well nourished piglets with colitis (WNC, n = 8). * indicates significant change over time for all piglets regardless of treatment ($p = 0.031$). † indicates decrease over time by paired T test ($p = 0.009$). REF, well-nourished without colitis; WNC, well-nourished with colitis; MR, macronutrient restricted with colitis; MRP, macronutrient with colitis and probiotics; MR-C, combined macronutrient restricted with colitis (MR and MRP).

Figure 5.2 Mean (±SEM) plasma copper, iron and zinc concentrations at day 3 and day 13 for macronutrient restricted piglets (MR, n = 6) and well nourished piglets with colitis (MRP, n = 8). * indicates significant change over time for all piglets regardless of treatment ($p < 0.0001$). ‡ indicates significant difference in concentration due to probiotic administration (main effect; $p = 0.028$). REF, well-nourished without colitis; WNC, well-nourished with colitis; MR, macronutrient restricted with colitis; MRP, macronutrient with colitis and probiotics; MR-C, combined macronutrient restricted with colitis (MR and MRP).

Figure 5.3 Mean (±SEM) plasma copper, iron and zinc concentrations at day 3 and day 13 for well-nourished piglets with colitis (WNC, n = 8) and combined macronutrient restricted piglets groups with colitis (MR-combined, n = 14). † indicates decrease over time by paired T test (plasma iron WNC, $p = 0.047$; plasma zinc WNC, $p = 0.009$; MR-C, $p < 0.0001$). ** indicates significant time-by-diet interaction and larger decline over time for MR-C plasma zinc concentration ($p = 0.042$). REF, well-nourished without colitis; WNC, well-nourished with colitis; MR, macronutrient restricted with colitis; MRP, macronutrient with colitis and probiotics; MR-C, combined macronutrient restricted with colitis (MR and MRP).

Table 5.1 Growth outcomes for piglets with various inflammatory and nutritional states .

	Study 1		Study 2			Pooled SEM
	REF	WNC	MR	MRP	MR-C	
Weight Gain (g/(kg•day))	125	98.9	46.5	47.8	47.1*	7.2
Chest Circumference (%/day)	22.8	11.8	4.0	3.0	3.5*	2.1
Length (%/day)	27.6*	21.6	19.6	19.3	19.4	1.0

Values are means ± pooled SEM. * indicates mean difference at $P \leq 0.05$ compared to WNC piglets. REF, well-nourished without colitis; WNC, well-nourished with colitis; MR, macronutrient restricted with colitis; MRP, macronutrient with colitis and probiotics; MR-C, combined macronutrient restricted with colitis (MR and MRP).

Table 5.2 Muscle protein fractional synthesis rates, whole body protein turnover, phenylalanine flux, plasma cortisol and urea concentrations for piglets with various inflammatory and nutritional states.

	Study 1		Study 2			Pooled SEM
	REF	WNC	MR	MRP	MR-C	
L. Dorsi FSR (%/day)	11.3	12.1	8.89	8.00	8.44*	0.72
Masseter FSR (%/day)	10.6	13.8	8.13	8.38	8.25*	0.68
Phenylalanine Flux (µmol/(kg•day))	4169	4063	2571	2547	2558*	14.2
Protein Turnover (g/(kg•day))	313	305	193	191	192*	189
Plasma Cortisol	50.2	47.0	75.5	38.1	56.8	8.8
Plasma Urea (µmol/L)	1.48	0.97	2.99	2.23	2.61*	0.3

Values are means ± pooled SEM. * indicates mean difference at $P \leq 0.05$ compared to WNC piglets. REF, well-nourished without colitis; WNC, well-nourished with colitis; MR, macronutrient restricted with colitis; MRP, macronutrient with colitis and probiotics; MR-C, combined macronutrient restricted with colitis (MR and MRP).

Figure 5.1

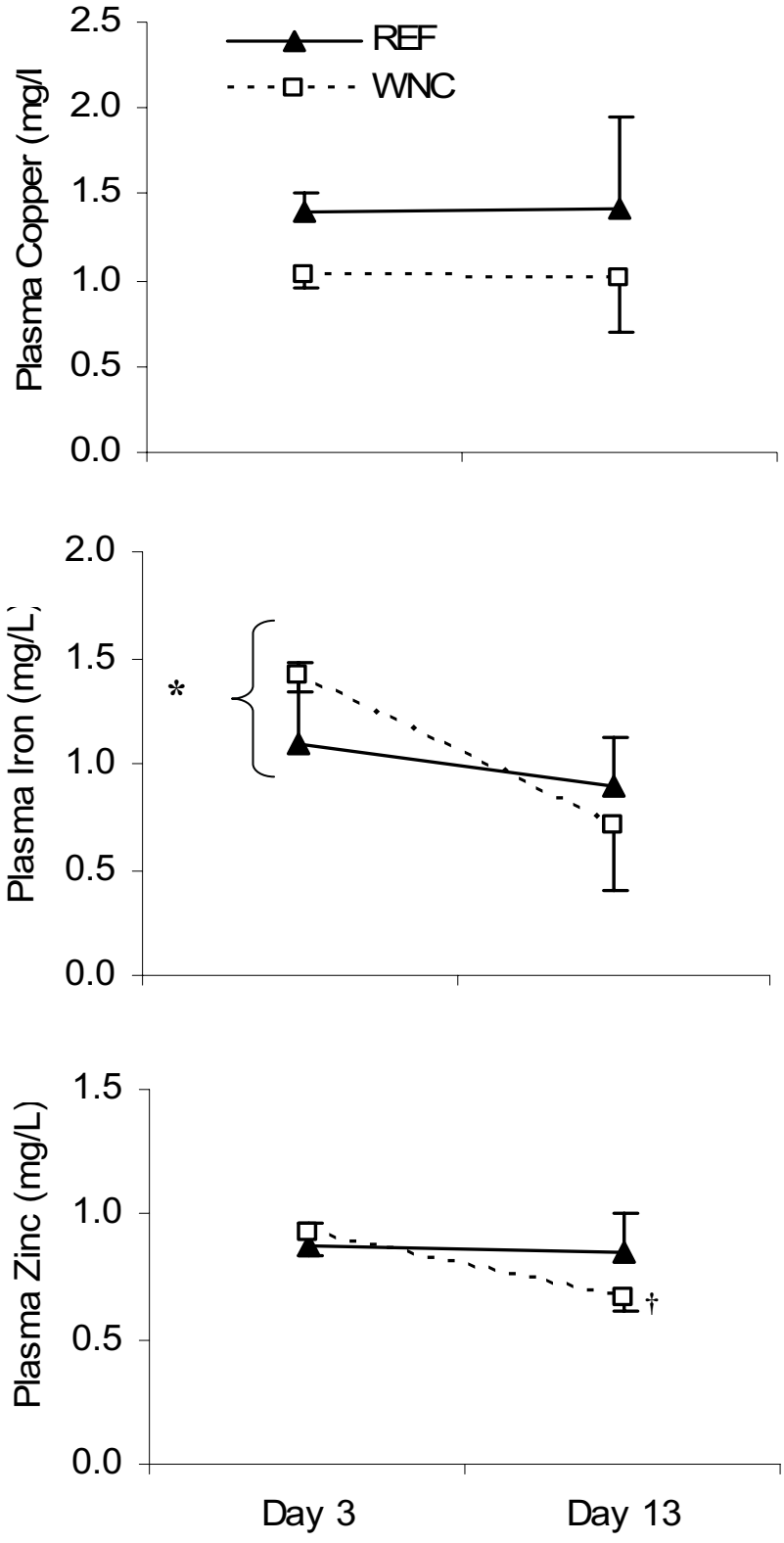
Plasma Copper (mg/l
2.5
2.0
1.5
1.0
0.5
0.0
REF
WNC
Plasma Iron (mg/L)
2.0
1.5
1.0
0.5
0.0
*
Plasma Zinc (mg/L)
1.5
1.0
0.5
0.0
†
Day 3
Day 13

Figure 5.2

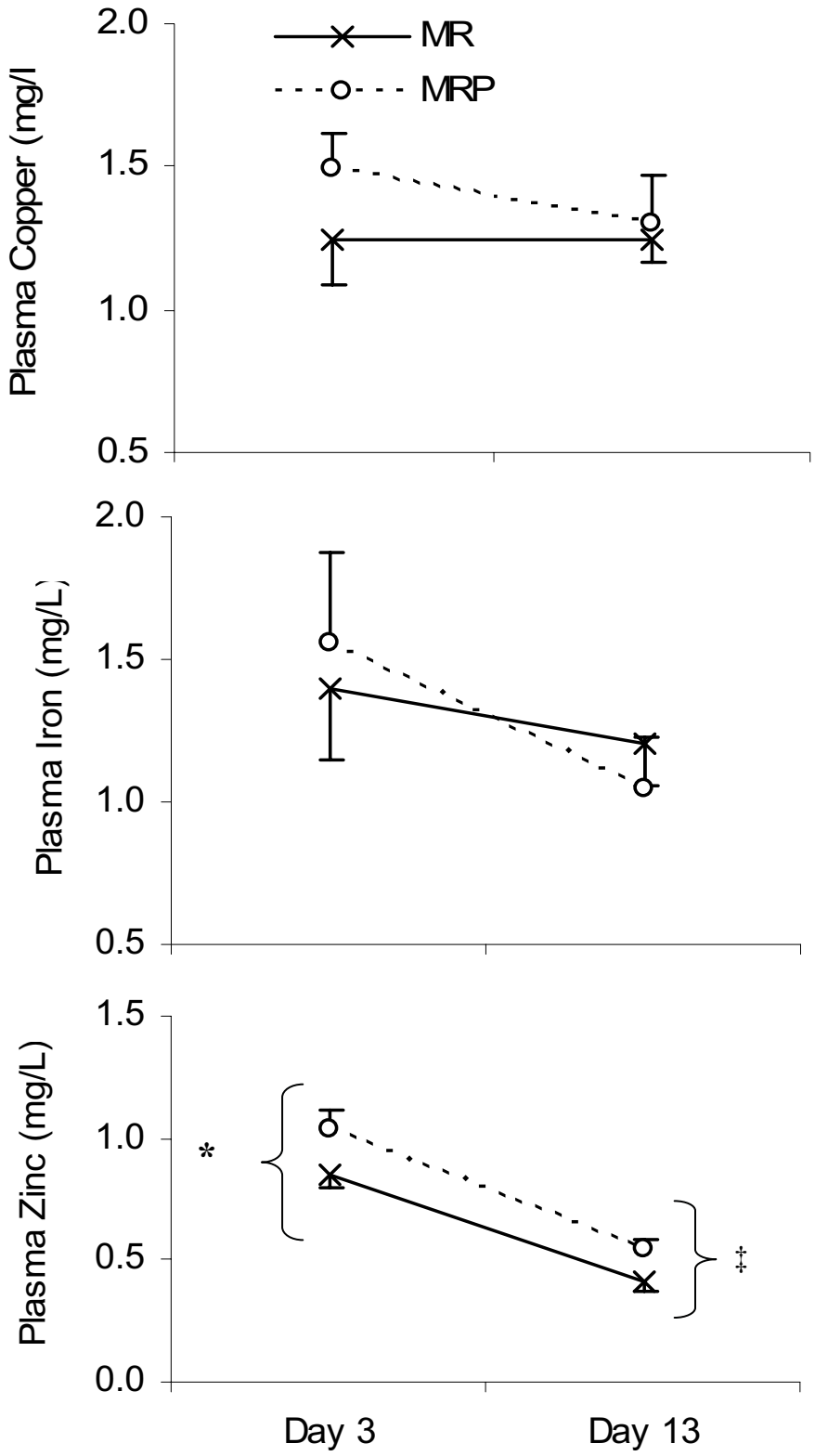
MR
MRP
Plasma Copper (mg/l
2.0
1.5
1.0
0.5
Plasma Iron (mg/L)
2.0
1.5
1.0
0.5
Plasma Zinc (mg/L)
1.5
1.0
0.5
0.0
*
‡
Day 3
Day 13

Figure 5.3

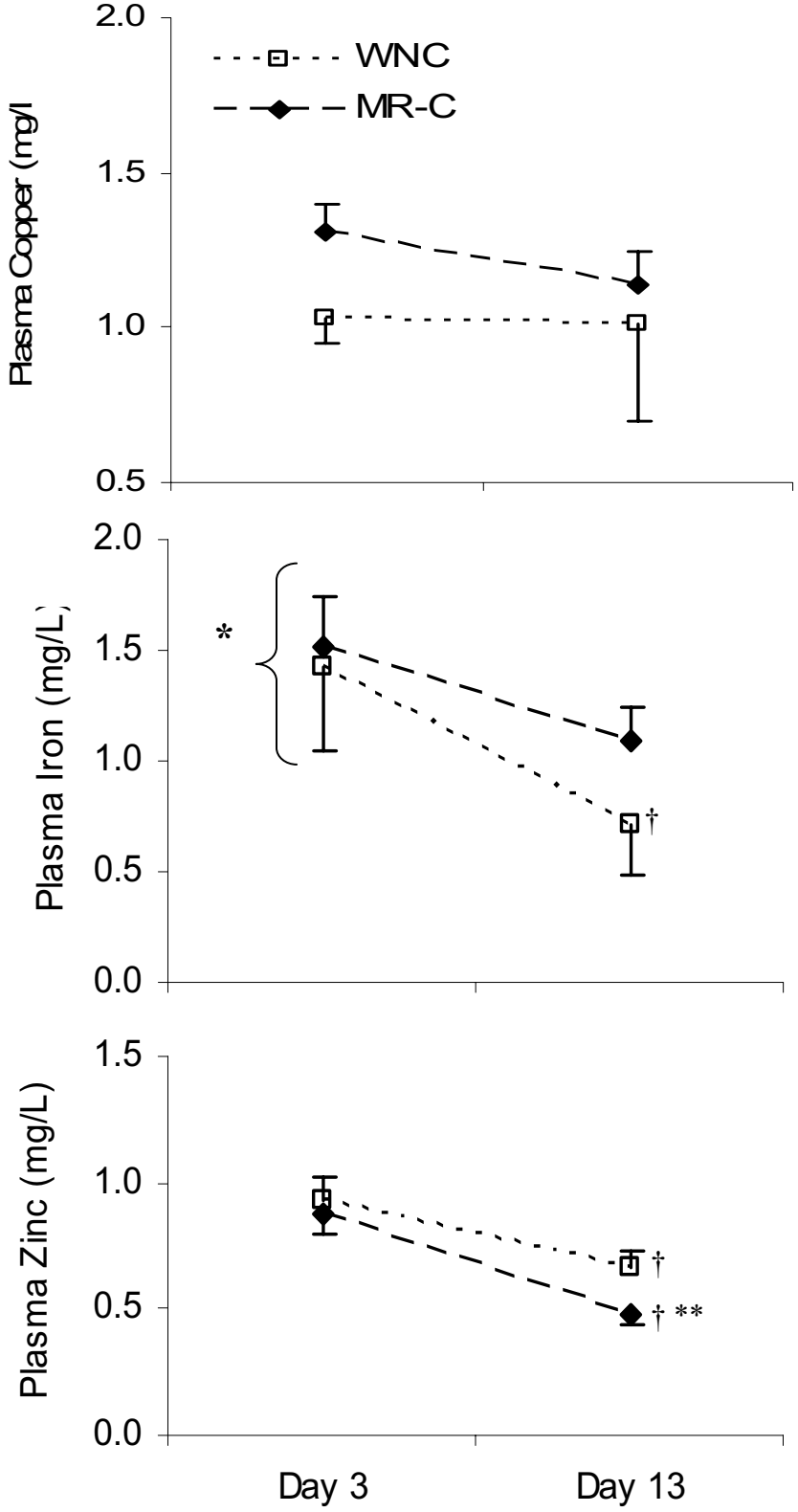
2.0
1.5
1.0
0.5
WNC
MR-C
Plasma Copper (mg/l
2.0
1.5
1.0
0.5
0.0
*
†
Plasma Iron (mg/L)
1.5
1.0
0.5
0.0
† †
† **
Plasma Zinc (mg/L)
Day 3
Day 13

Table 5.3 Copper, Iron and Zinc Mass Balance.

	Study 1		Study 2		
Copper	**Ref**	**WNC**	**MR**	**MRP**	**MR-C**
Intake (mg/(kg•day))[a]	0.38 (0.03)	0.43 (0.01)	0.43 (0.01)	0.46 (0.01)	0.44 (0.01)
Feces (mg/(kg•day))	0.57 (0.17)	0.40 (0.10)	0.34 (0.09)	0.34 (0.09)	0.35 (0.07)
Urine (µg/(kg•day))	2.3 (0.2)	2.6 (1.0)	BD[b]	BD	BD
Absorbed (mg/(kg•day))[c]	-0.19 (0.16)	0.03 (0.10)	0.09 (0.09)	0.12 (0.09)	0.10 (0.06)
Retained (mg/(kg•day))[d]	-0.19 (0.16)	0.03 (0.10)	0.09 (0.09)	0.12 (0.09)	0.09 (0.07)
# in Positive Balance[e]	1/5	3/6	3/6	4/5	7/11
Iron					
Intake (mg/(kg•day))	3.01 (0.21)	3.37 (0.08)	3.43 (0.10)	3.61 (0.09)	3.51 (0.08)
Feces (mg/(kg•day))	3.02 (0.85)	2.30 (0.69)	2.04 (0.40)	1.87 (0.47)	2.01 (0.31)
Urine (µg/(kg•day))	5.0 (1.0)	22.1 (17.0)	9.2 (1.3)	7.6 (2.2)	8.2 (1.2)
Absorbed (mg/(kg•day))	-0.01 (0.72)	1.07 (0.69)	1.40 (0.35)	1.74 (0.45)	1.55 (0.27)
Retained (mg/(kg•day))	-0.01 (0.72)	1.05 (0.68)	1.39 (0.35)	1.73 (0.46)	1.49 (0.29)
# in Positive Balance	2/5*	6/6	5/6	5/5	10/11
Zinc					
Intake (mg/(kg•day))	0.89 (0.06)	1.00 (0.02)	1.02 (0.03)	1.08 (0.03)	1.05 (0.02)
Feces (mg/(kg•day))	0.55 (0.17)	0.77 (0.19)	1.08 (0.14)	1.32 (0.13)	1.22 (0.11)*
Urine (µg/(kg•day))	7.4 (1.0)	8.7 (1.0)	11.8 (2.8)	12.9 (5.8)	12.5 (3.2)
Absorbed (mg/(kg•day))	0.35 (0.18)	0.24 (0.18)	-0.06 (0.14)	-0.24 (0.13)	-0.14 (0.10)
Retained (mg/(kg•day))	0.34 (0.18)	0.23 (0.18)	-0.07 (0.15)	-0.25 (0.13)	-0.19 (0.10)*
# in Positive Balance	4/5	3/6	2/6	1/5	3/11

Values are mean (± SEM).

[a] Intake on an as fed basis

[b] BD = below the limit of detection, negligible loss.

[c] Apparent Absorption = intake – fecal loss

[d] Retained = intake – (fecal + urinary losses)

[e] # positive balance = number of piglets with trace element balance > 0.

* $p \leq 0.05$ vs. WNC piglets

REF, well-nourished without colitis; WNC, well-nourished with colitis; MR, macronutrient restricted with colitis; MRP, macronutrient with colitis and probiotics; MR-C, combined macronutrient restricted with colitis (MR and MRP).

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Chapter 6

Conclusion and Summary

OVERALL CONCLUSIONS

Significance

Optimizing nutritional management of children and adolescents with IBD is a vital aspect of improving their quality of life. Protein and trace elements are nutrients necessary for normal growth, protein synthesis, oxidant defence and immune function with healthy status being related from both a dietary and functional perspective. Deficiencies of both is common in IBD, however little is known about how IBD influences needs of these nutrients, the interaction between them, or how improving their status may reduce the classic nutrition-related complications of IBD (growth retardation, muscle wasting, hypoalbuminemia, skin lesions), improve oxidant defence and reduce disease severity.

Probiotics are a promising therapeutic approach in IBD, which have been shown to improve gut barrier function. However the relationships with nutrition, specifically protein and trace elements and amelioration of the common nutritional complications of IBD have not been studied. The piglet is a relevant model to study these interactions, because nutrient metabolism and gastrointestinal physiology of the piglet are quite similar to the human. This unique combination of macro and micronutrient stable isotope studies in the piglet colitis model allows precise and independent control of diet and disease to investigate mechanisms. This study will provide a strong foundation for clinical stable isotope studies and improvements in nutritional status to improve quality of life and potentially reduce dependency on drug treatment in children with IBD.

Limitations

Unfortunately, several aspects of the study and model lead to inconclusive results, especially with regard to gut integrity and endogenous losses of zinc. While the piglet model is a good model for studying gastrointestinal and nutritional diseases, the specific age of the piglets used in this study may have contributed to the analytical problems encountered. While the leaky nature of the newborn piglet gut rapidly closes during the first weeks of life when compared to other mammals, the age characteristic of the piglets in our study may have contributed to the high variability in our outcomes relating to gut integrity and damage.

Furthermore, due to the costly nature of such metabolic studies compromises were made when the present study design was developed. Ideally, the presence of several other groups – well-nourished with colitis and probiotics, well-nourished with colitis and non-viable probiotics and malnourished with colitis and non-viable probiotics – would have addressed a number of key questions that have arisen from the data presented here.

Piglet Model of Colitis

For nutritional and protein synthesis experiments the use of the growing piglet is very well characterized as a model (1). Our choice to use the piglet colitis model was also based on the anatomical and physiological resemblance of the swine gut to that of humans, and also due to the similarities in amino acid metabolism (2, 3). Our laboratory has had previous success using this model to study protein synthesis during acute colitis (4) which we have further developed here in this thesis using a longer duration of dextran sulphate-induced colitis to mimic a persistent flare-up of the disease. As with any experimental model there are drawbacks and limitations to the interpretation of the data collected from their use.

Other common models of IBD are the rodent chemical induced colitis (i.e. dextran sulphate), mouse IL-10 knockout model, infectious models (induced in germ free environments) or the various intestinal cell lines which can be manipulated to simulate IBD conditions. While all these models have their strengths they are also limited when comparing to the human disease. While our choice of model lacked commonality with the immune response of the human disease it does provided us with an exceptionally similar gut/nutrient interactions model. Similarly, the rodent and cell culture models while they have their strengths they are not adequate when drawing nutritional conclusions only. Therefore, while our model is relatively new and still has components which require development, it is very useful and applicable to the study of nutritional outcomes during large bowel inflammation.

Maintaining Adequate Nutrition

The overall goal of this research was to characterize nutritional and metabolic factors affected by either providing adequate nutrition or probiotics in piglets with DS-induced colitis. Maintaining adequate nutrition appears to contribute significantly to sustaining normal metabolic processes while helping to reduce the damage associated with DS-induced colitis. Specific conclusions from these studies regarding maintaining adequate nutrition in active colitis are:

1. Providing adequate nutrition sustains normal weight gain and growth during colitis.
2. Providing adequate nutrition maintains normal rates of protein synthesis in small intestinal mucosa, colon, liver, liver-derived plasma protein and skeletal muscle proteins.
3. Providing adequate nutrition reduces histological damage and increases the number of colonocytes per crypt compared to macronutrient-restricted piglets with colitis.
4. Providing adequate nutrition reduces some markers of systemic oxidative stress (plasma [Cu]:[Zn]).
5. Despite providing adequate nutrition (micronutrient and macronutrient) there appears to be an increased dietary iron requirement for piglets with colitis.

Our conclusions support avoiding malnutrition in active colitis but despite producing desirable outcomes, nutritional support does not receive priority in the treatment of IBD. Controlling the inflammation through the use of pharmaceutical and surgical techniques is the avenue of treatment most commonly explored while nutritional support is only used in severe or paediatric cases. If our findings are reproducible in the human population with IBD, maintaining adequate nutrition or correcting mild malnutrition may be useful in assisting the standard treatments in achieving symptom control in a more timely fashion.

Probiotic (VSL#3) Administration

Probiotic therapy, especially VSL#3, has been gaining support for treating certain forms of adult IBD (5-7). However, the metabolic and nutritional effects of providing probiotics have not been widely studied. One proposed mechanism of action is through down-regulation of the proinflammatory response associated with IBD, but has yet to be proven definitively. The metabolic and nutritional affects of providing probiotics were our primary outcome markers, in an effort to provide some insight as to the mechanism behind the action. As a secondary outcome we examined systemic indicators of inflammatory stress other than those already shown (decreased proinflammatory cytokines). Several unique findings were also identified for probiotics administration in DS-induced colitis. The key findings with regards to probiotics from these studies are:

1. Providing probiotics to macronutrient restricted piglets with colitis selectively stimulated liver and liver-derived plasma protein synthesis while not affecting small intestinal mucosa or colon protein synthesis rates.
2. Providing probiotics to macronutrient restricted piglets with colitis did not reduce histological damage or the inflammatory stress markers used in our study, contradicting he proposed mechanism of probiotics enhancing mucosal barrier function.
3. Providing probiotics to macronutrient restricted piglets with colitis had no effect on copper, iron or zinc status.

Unfortunately, our data does not answer any question as to how probiotics might help induce and maintain remission in colitis. The stimulation of liver protein synthesis does however offer a new and interesting objective for further study. Specifically, there may be a relationship between receptor mediated processes occurring in the gut as a result of providing probiotics, proposed by Dotan and Rachmilewitz, and our liver protein synthesis data (8).

Future Study

Overall, our findings now point to particular future directions for this area of study. An assessment of the combined action of both maintaining adequate nutrition and administration of probiotics is needed in both DS-induced and other models of colitis. Furthermore, study of how probiotics is able to selectively stimulate protein synthesis in the liver opposed to other tissues is also essential. Finally, characterization of the mechanism of action of probiotics in a study combining the three following concepts:

a. Reduced proinflammatory cytokine secretion via specific receptor mediated processes stimulated by probiotics, specifically VSL#3.
b. Avoiding malnutrition in colitis.
c. Stimulation of protein synthesis in tissues outside the gut by probiotics, specifically VSL#3.

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Appendix 1

Animal Care Approval

McGill University
Animal Use Protocol – Research
Guidelines for completing the form are available at
www.mcgill.ca/rgo/animal

Protocol #: 3476
Investigator #: 365
Approval End Date: MARCH 31, 2005
Facility Committee: AGR

☐ **Pilot** ☐ **New Application** ☒ **Renewal of Protocol #** 3476

Title Protein and amino acid metabolism during malnutrition and metabolic stress.
(must match the title of the funding source application)

D level

1. Investigator Data:

Principal Investigator: Linda Wykes, PhD **Office #:** 7843
Department: School Of Dietetics and Human Nutrition **Fax#:** 7739
Address: Macdonald Campus **Email:** linda.wykes@mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Linda Wykes **Work #:** 7843 **Emergency #:** 697-0603
Name: Scott Harding **Work #:** 7768 **Emergency #:** 457-2137

3. Funding Source:

External ☒
Source (s): NSERC
Peer Reviewed: ☒ YES ☐ NO**
Status : ☒ Awarded ☐ Pending
Funding period: 04/00-04/07

Internal ☐
Source (s): ____
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period: ____

ACTION | ✓ | DATE
CCG
DB | J | Jan 16 04
APPROVED

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed . e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y): ____ **or ongoing** ☒

Expected Date of Completion of Animal Use (d/m/y): ____ **or ongoing** ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: Linda Wykes **Date:** 2/3/04

Approval Signatures:

Chair, Facility Animal Care Committee:	J McSmith	**Date:** 17 March 2004
University Veterinarian:	Snatenyc	**Date:** March 17, 2004
Chair, Ethics Subcommittee(as per UACC policy):		**Date:** 4/21/04
Approved Period for Animal Use	**Beginning:** April 1, 2004	**Ending:** MARCH 31, 2005

☐ This protocol has been approved with the modifications noted in Section 13.

November 2001

19 MAR. 2004

4. Research Personnel and Qualifications: List the names of all individuals who will be in contact with animals in this study (including the Principal Investigator) and their employment classification (investigator, technician, research assistant, undergraduate/graduate student, fellow). Indicate any training received (e.g workshops, lectures, etc.). The PI certifies that all personnel listed here have suitable training and/or experience, or will be provided with the specific training which qualifies them to perform the procedures described in the protocol. Each person listed in this section must sign to indicate that s/he has read this protocol. (Space will expand as needed.)

Name	Classification	Training Information	Signature
Linda Wykes	Investigator	12 years' experience with piglet model & Basic/Adv AC Course (McGill)	
Scott Harding	PhD student	Basic/Adv AC Course & Practical Piglet Care Course (McGill & Memorial)	
Keely Fraser	MSc Student	Basic/Advanced Animal Care Course & practical Piglet Care Course (McGill)	
Sean Mark	PhD Student	Basic/Advanced Animal Care Course & Practical Piglet Care Course (McGill)	

* Enter the first name, press 'enter', then the 2nd name… complete the first column, then the 2nd, then the 3rd
** If an undergraduate student is involved , the role of the student and the supervision received must be described.

5. Summary (In language that will be understood by members of the general public)

a) RATIONALE: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge.

The relationship between malnutrition and inflammation is a vicious cycle. Children with inflammatory bowel disease typically show growth retardation, muscle wasting and hypoalbuminemia, but the causal mechanisms are not known. Both food avoidance and cytokine-induced anorexia tend to decrease dietary intake, whereas the inflammatory and acute phase responses increase demands. Previously we have developed a new model of inflammatory bowel disease in the piglet and showed that well- nourished piglets could withstand the catabolic effects of gastrointestinal inflammation while malnourished piglets showed growth retardation (correlating with the acute phase response) and decreased muscle protein synthesis. Stable isotopes are necessary to see these changes that are not evident by concentration measurements alone.

Protocol: Study will intestigate zinc absorption and losses in piglets with Dextran Sulfate (DS)-induced IBD and explore nutritional strategies to decrease inflammation and protein catabolism including growth retardation and muscle wasting in malnourished piglets with gastrointestinal inflammation. The results will be applicable to humans with IBD.

Pilot Study Rationale: A pilot study is required to adapt the model for a more chronic period required for the zine study. The total duration will still be 14 days. Previous model: 7 days of adaption and weaning (with surgery on Day 5), followed by 7 days of dextran sulphate administration. Proposed model: surgery on day 1, adaption to enterally infused liquid diet over 2 days, then 12 days of dextran sulphate administration (lower dose). This model will provided a chronic model of IBD. Chronic models of up to 6 months' duration in rodents are published. Testing of a new amino acid stable isotope for measuring glutathione synthesis will also be carried out.

Study 1: Impaired zinc absorption and increased zinc losses are commonly reported in humans with IBD. Zinc status is directly linked to growth , protein synthesis and appropriate inflammatory control. There is limited research available on how zinc is related to IBD progression. Research question: Are zinc losses increased and zinc absorption inpaired in IBD?

Study 2: Probiotic bacteria (eg Lactobaccilus GG) are a commonly used strategy in human medicine to populate the colon with bacteria that will decrease inflammation and decrease the symptoms of IBD. Research question: Will probiotic bacteria decrease GI inflammation and decrease the acute phase response and nutritional complications of IBD?

b) SPECIFIC OBJECTIVES OF THE STUDY: Summarize in point form the primary objectives of this study.

Pilot Study: Estabilsh 14 day chronic IBD model and test new amino acid stable isotope dose for measuring glutathione synthesis.
Study 1: Establish the effect IBD has on both zinc absorption/loss in the growing piglet using zinc stable isotope tracer techniques. Establish how zinc status relates to protein metabolism including whole body protein turnover, fractional and absolute synthesis rates of tissue and plasma proteins. Resting energy expenditures, oxidative stress and damage will also be determined to better understand whole body effects of acute inflammation.
Study 2: Determine the potential anti-inflammatory effect of probiotic bacteria supplemented in the diet of protein energy malnourished piglets with IBD on glutathione status in the gut, liver, muscle, and red blood cells; and on alterations in protein metabolism, including whole body protein turnover, fractional and absolute synthesis rates of tissue and plasma proteins. Resting energy expenditures, oxidative stress and damage will also be determined to better understand whole body effects of acute inflammation.

c) PROGRESS REPORT: If this is a renewal of an ongoing project, BRIEFLY summarize what was accomplished during the prior approval period and indicate if and how the current goals differ from those in the original application.

The initial study in this area was a 2x2 factorial design to test the independent and combined effects of dextran-sulphate-induced IBD on growth and protein metabolism. The importance of nutritional status in minimizing the catabolic effects of inflammation was emphasized, as well the the importance of stable isotope protein synthesis measurements in determining the mechanisms of that response. We are now focusing on nutritional consequences of chronic IBD (zinc absorption and losses study) and use of probiotic supplementation to decrease that catabolic reponse. Both studies require a more prolonged or chronic model of IBD to obtain outcome measures. The goal of this phase of studies is to determine is probiotic or zinc intake can lessen the growth retardation and muscle wasting, which is such an integral part of IBD clinically.

Over the last year we studied 2 piglets as we set up proper conditions for our indirect calorimetry system and tested our new formula based diet.

d) SUMMARY OF PROCEDURES FOR ANIMAL USE REPORT TO THE CCAC: Using KEY WORDS ONLY, list the procedures used (e.g. anaesthesia, breeding colony, injection IP, gavage, drug administration, major survival surgery, euthanasia by exsanguination, behavioural studies). Refer to Appendix 1of the Guidelines for a more complete list of suggested key words.

Major survival surgery, anaesthesia, injection (intravenous), blood sampling/testing (small volume), enteral feeding, special diet, major surgery, catheterization

6. Animals To Be Used

a) Purpose of Animal Use (Check one):

1. ☐ **Studies of a fundamental nature/basic research**
2. ☒ **Studies for medical purposes relating to human/animal diseases/disorders**
3. ☐ **Regulatory testing**
4. ☐ **Development of products/appliances for human/veterinary medicine**

b) Will the project involve breeding animals? NO☒ YES☐
Will the project involve the generation of genetically altered animals? NO☒ YES☐
Will field studies be conducted? NO☒ YES☐

c) Description of Animals

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	Pig					
Supplier/Source	Macdonald Campus farm					
Strain						
Sex	M/F					
Age/Wt	5 - 7 days/ 3-4 kg					

# To be purchased	34					
# Produced by in-house breeding						
# Other (e.g.field studies)						
#needed at one time	2 - 8					
# per cage	1					
TOTAL# /YEAR	**34**					

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals.

7. Justification of Animal Usage

a) Please justify the number of animals requested for each species described in the table 6c above, BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT. Include information on experimental and control groups, # per group, and failure rates. Also justify in terms of statistical requirements, product yield, etc. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. The numbers of animals are for one year only, not the length of funding. Use the table below when applicable (space will expand as needed).

Group sizes (n=8) are based on the initial study, and were originally calculated to provide an 80% probability of detecting a difference of 30% with a within-group coefficient of variation of 20%.

Test Agents or Procedures e.g. 2 Drugs	# of Animals and Species Per Group e.g. 6 rats	Dosage and/or Route of Administration e.g. .03, .05 mg/kg – IM, IP (4 variables)	# of endpoints e.g. 1, 7, 10 days (3 variables)	Other variables (i.e. sex, weight, genotypes,etc.) e.g. Male, Female groups (2 variables)	Total number of animals per year e.g. 2 x 6 x 4 x 3 x 2 = 288
Pilot Study:	2	1 i.g.	1	1	**2**
Study 1 - 2groups	8	1 i.g.	1	1	**16**
Study 2 - 2 groups	8	1 i.g.	1	1	**16**

*** For the above table, enter the first agent/procedure, press 'enter', then the 2nd agent... complete the first column, then the 2nd, then the 3rd...**

b) Please justify the need for live animals versus alternate methods (e.g. tissue culture, computer simulation).
Live animals are needed for metabolic "in-vivo" kinetic studies using stable isotopes to investigate absorption and losses of zinc and the relationship between malnutrition and inflammation.

c) Describe the characteristics of the animal species selected that justifies its use in the proposed study (consider characteristics such as body size, species, strain, data from previous studies or unique anatomic/physiological features)

Models of dextran-sulphate-induce colitis exist in rodents, but are not suitable for stable isotope studies of protein metabolism. The piglet is the preferred model for amino acid stable isotope kinetic studies because of its well-characterised protein and amino acid metabolism, similar pattern of amino acid requirements and amino acid composition, metabolic similarity to the human (particularly the digestive system) (Kararli, 1995), sensitivity to the amino acid profile in the diet and diet restriction, and size, which accommodates the sampling schedule (Pencharz et al., 1996; Ball et al., 1996). For the same physiological and anatomical reasons the piglet model is an applicable model of zinc absorption and loss.

8. Animal Husbandry and Care

a) **Special cages** NO ☐ YES ☒ **Specify:** Cages - Animals will be housed individually in rabbit metabolic cages adapted for piglets.- Cages are 77cm long x 65cm wide x 40cm high, with side windows, plastic-coated small-opening flooring, and fed enterally by feeding pump. Two racks of cages will be placed together to encourage visual and aural contact.- Cages will be cleaned daily.- Piglets will have access to a racquetball and squeaky toy.

Special diet NO ☐ YES ☒ **Specify:** Piglets will have gastric catheters placed during the surgery and will be fed a custom formula type diet which supplies the NRC requirements for growing swine (unless otherwise indicated by treatment group). The diet will have approximately the following macronutrient composition - 45% carbohydrate; 30% lipid and 25% protein with all approprite vitamins and minerals added. In the case of the probiotics study the calorie restricted diet the compostion will 50% of NRC requirements for these piglets.

Special handling NO ☐ YES ☒ **Specify:** Piglets will be weighed and have catheters flushed daily - Feed intake will be recorded daily.- Feces will be monitored daily.

b) **Is there any component to the proposed procedures which will result in immunosuppression or decreased immune function (e.g. stress, radiation, steroids, chemotherapeutics, genetic modification of the immune system)?**

NO ☒ YES ☐ **Specify:**

c)
Multiple institution facility housing: NO ☒ YES ☐

Indicate all facilities where animals will be housed: **Building:** Macdonald Stewart **Room No:** B50

Indicate area(s) where animal use procedures will be conducted: **Building:** Macdonald Stewart **Room No:** B51

If animal housing and animal use are in different locations, briefly describe procedures for transporting animals: Surgery will be conducted at the Large Animal Research Unit and transported to Macdonald Stewart building in a dog carrying cage via truck.

9. Standard Operating Procedures (SOPs)

Complete this section if you plan to use any of the UACC SOPs listed below. IT IS UACC POLICY THAT THESE SOPS BE USED WHEN APPLICABLE. Any proposed variation of the SOPs must be described and justified. The Standard Operating Procedures can be found at the UACC website at www.mcgill.ca/rgo/animal . The completed and signed SOP form must be attached to the protocol.

Check all SOPs that will be used:

Blood Collection (UACC#1)	☐	**Production of Monoclonal Antibodies (UACC#7)**	☐
Anaesthesia (rodents) (UACC#2)	☐	**Production of Polyclonal Antibodies(UACC#8)**	☐
Analgesia (rodents/larger species) (UACC#3)	☐	**Collection of Amphibian Oocytes (UACC#9)**	☐
Breeding (transgenics/knockouts) (UACC#4)	☐	**Rodent Surgery (UACC#10)**	☐
Transgenic Generation (UACC#5)	☐	**Neonatal Rodent Anaesthesia and Euthanasia (UACC#11)**	☐
Knockout/in Generation (UACC#6)	☐	**Stereotaxic Survival Surgery in Rodents (UACC#12)**	☐

10. Description of Procedures

a) **FOR EACH EXPERIMENTAL GROUP, DESCRIBE ALL PROCEDURES AND TECHNIQUES IN THE ORDER IN WHICH THEY WILL BE PERFORMED - surgical procedures, immunizations, behavioural tests, immobilization and restraint, food/water deprivation, requirements for post-operative care, sample collection, substance administration, special monitoring, etc. IF A PROCEDURE IS COVERED BY AN SOP, WRITE "AS PER SOP", NO FURTHER DETAIL IS REQUIRED. Appendix 2 of the Guidelines provides a sample list of points that should be addressed in this section.**

Research Design:
Pilot Study: 2 piglets will undergo surgery having catheters implanted and will be used to determine appropriate dose of dextran sulphate for the chronic model of IBD and determine tracer doses for new glutathione sysnthesis method.

Study 1: 16 piglets will be placed into 2 groups (n = 8; 1 group with DS induced IBD, 1 without DS induced IBD) to establish the effect of dextran sulphate IBD on zinc absorption and losses.

Study 2: 16 piglets will be placed into 2 groups (n = 8; both groups will have DS induced IBD) to examine the effect of probiotic supplementation in well nourished and calorie restricted piglets.

Total length of studies will 14 days: surgery on day 0 with 2 days for diet adaption and surgery recovery, 12 days of DS

treatment or sham (5 - 7 days required to obtain colitis-like symptoms) and amino acid isotope infusions on day 14.

Model of Gastrointestinal Inflammation: Sulphated polysaccharides, such as dextran sulphate (DS), produce an inflammatory response, which disrupts the mucosal lining of the colon. We developed the model in the previous study based on rodent models. Administration of DS will be via the stomach catheter as this method eliminates DS waste and ensures proper dosage. Pilot studies were conducted to determine the appropriate dose of DS. Piglets receiving 1g DS/kg body weight/day had a minor degree of loose stools, whereas 3g/kg/day produced bloody diarrhea. Piglets receiving 2g/kg/day (5ml of 20% DS solution/kg/day) showed loose dark stools and some diarrhea in both ad lib and restricted piglets. Histological examination of the gastrointestinal tract showed inflammation in the colon, specifically shortening of the crypts, epithelial thinning, and infiltration of inflammatory cells confined to the mucosa. The DS dose of 2g/kg/day was used to induce inflammation over the 7 day studies, as it is the minimal dose which consistently produces reduced weight gain and non-bloody diarrhea over this timeframe. The present studies will use a DS maximal dose of 1g/kg/day to obtain a chronic IBD model which produces the same indicators, reduced weight gain and occult blood, but over 12 days.

Monitoring and humane endpoints: The health (temperature, colour of mucus membranes, feces and urine production) and behavior (activity, interest in the environment, general demeanor) of the piglets receiving DS will be monitored closely. If severely bloody diarrhea or significant changes in the above criteria are observed, the next dose of DS will be skipped and the piglet monitored every four hours. If blood is still present 12 hours later the piglet will be killed with sodium pentobarbital (750 mg i.v.). Histology of the gastrointestinal tract of all piglets will be examined post mortem. Aside from surgery drugs, it is not anticipated that medications will be required.

Protocol, Housing and Diet: Thirty-four piglets of either sex will be obtained from Maconald Farm, McGill University. At 5 - 7 days of age (study day 0), they will be removed from the sow and transferred to the Large Animal Research Unit for surgery (catheters). One piglet from each litter will be assigned randomly to one treatment group. The piglets will be transferred to the animal facility and housed in individual cages with visual and aural contact with each other. They will have a ball and a squeaky toy for entertainment. Room temperature will be maintained at 25°C. Piglets will be adapted to full feeding with custom formula delivered by enteral pump which is based on skim milk/low-lactose whey consisting of 25% protein, 45% carbohydrates and 30% fat. The intake will be sufficent to promote normal growth under healthy stress free conditions. The calorie restricted diet will have the same compostion but piglets will receive 50% of the NRC requirements. All piglets will be weighed daily. Daily feed intake will be recorded daily throughout study, and their cages will be cleaned daily.

Surgery: On study day 0, catheters will be implanted into a jugular vein, femoral vein, bladder and stomach under aseptic conditions. Piglets will be premedicated with atropine (0.08 mg/kg i.m.) 15 minutes before induction of anesthesia with 5% isoflurane. Piglets will then be maintained at 2% isoflurane in oxygen by mask. Piglets will be kept warm during surgery on a heated operating table. The neck, groin and abdominal area will be shaved and then scrubbed sequentially with Hibitane, alcohol and iodine, before sterile draping on a heated operating table. Silastic catheters (0.8 mm id x 2.0 mm od x 1m) will be inserted into the left external jugular and femoral veins, anchored in place with grommets fitting snugly over the catheter wall, and sewn to stable underlying tissue. The stomach and bladder catheters will be placed through a hole in the gastric (or bladder) wall and held in place with a purse-string suture. The catheters will be tunneled subcutaneously to exit laterally on the side of the chest. The incisions will be closed with a double row of stitches, and covered with Polysporin. Buprenorphine (0.01 mg/kg i.m.) will be administered as incisions are being closed. The catheters will be filled with saline solution, capped, and secured in the pocket of a mesh jacket worn by the piglet. Each surgery will be performed by two of the named research personnel.

Postoperative Care: Following surgery, piglets will be kept warm and continuously observed with special attention paid to temperature, respiration, and colour of mucus membranes. They will be monitored for resumption of urinatoin, defecation and activity. Buprenorphine (0.01 mg/kg i.m.) will be administered 0, 12 and 24 hours after surgery and then repeated as necessary. Catheters will be flushed daily with 2 mL 0.9 g/L saline to maintain patency. Treatments (DS infusions or sham and probiotics or sham) will begin 2 days post surgery, once full feeding regimen has been achieved. Piglets will be monitored twice daily as per previous section.

Indirect Calorimetry: Energy expenditure will be determined in each piglet on study day 2 and 7 by indirect (respiratory gaseous exchange) calorimetry. Each piglet will be placed in a plexiglass box (20 x 16 x 16 inches) will feeding tubes entering through a small hole. Air will be pumped through the box and into a series of analyzers to measure rate of oxygen compsumptioon and carbon dioxide production. Metabolic rate will be calculated to determine the hypothesized reduction in metabolite rate due to malnutrition , and the increase due to the excess metabolic demands of the inflammatory response.

Zinc Isotope Infusion (16 Zn piglets only): On study day 7 or once DS induced colitis is confirmed sterile soulitions of stable non-radioactive isotopes of zine 70Zn and 65Zn will be infused via the femoral and stomach catheters, respectively. Following the infusion venous blood will be sampled at 2, 4, 8, 10, 14, 18, 22, 30 and 60 minutes during the first hour

followed by hourly sampling to a total of 14 - 0.5 ml samples (1.8% total blood volume based on 80 mL/kg x 5 kg = 400 mL blood volume). For the remainder of the study venous blood (0.5 ml) will be sampled once daily.

Stable Isotope Infusion (all piglets) On study day 14, a stable isotope infusion will be performed in a separate procedure room to determine the effect of intestinal inflammation on tissue and plasma protein synthesis. A sterile solution of [15N,1,2-13C2]glycine and L-[ring-2H5]phenylalanine will be infused through the jugular catheter for 5 hours. A priming dose will be given at the beginning of the infusion. The study will be conducted in the fed state with piglets being continiously fed during the infusions. Venous blood (4 mL) will be sampled at baseline and hourly thereafter. Total blood sampled will be 28mL or less than 10% blood volume. At the end of the 5 hour infusion, piglets will be killed with an intravenous injection of sodium pentobarbital (Euthanyl 750 mg). Tissues (gut sections, stomach, liver, lung, kidney, and muscle) will be sampled post mortem.

b) Field Studies – Provide all relevant details. Procedures to be conducted (e.g. surgery, blood collection, tagging etc.) should be described above.

Method of capture/restraint, duration of captivity, potential injury/mortality, monitoring frequency:

Transportation and /or housing of animals in the field:

Special handling required:

Capture of non-target species, potential injury/mortality:

Will captured animals be released at or near the capture site YES ☐ NO ☐
If not, specify if they will be relocated to other locations and/or populations.

Describe any potential ecological disruption this study may cause:

It is the responsibility of the investigator to obtain all necessary permits for work with wildlife. Copies of these permits must be forwarded to the Research Ethics Officer (Animal Studies) when they are obtained.

c) Pre-Anaesthetic/Anaesthetic/Analgesic Agents: List all drugs that will be used to minimize pain, distress or discomfort. Table will expand as needed. (*complete 1st column pressing 'enter' after each species, then 2nd column...)

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency
Pig	Atropine	0.08	0.1 mL/kg	i.m	pre-op
	Buprenorphine	0.01	0.01 mL/kg	i.m.	0,12,24 hours post op
	Isoflurane	5% to induce, 2% to maintain		inhaled	during surgery

d) Administration of non-anaesthetic substances: List all non-anaesthetic agents under study in the experimental component of the protocol, including but not limited to drugs, infectious agents, viruses (table will expand as needed). (*complete 1st column pressing 'enter' after each species, then 2nd column...)

Species	Agent	Dosage (mg/kg)	Total volume (ml) per administration	Route	Frequency
Pig	Dextran sulphate	1 g/kg/d		i.g.	b.i.d.

e) Endpoints : 1) Experimental – for each experimental group indicate survival time .
2) Clinical - describe the conditions, complications, and criteria (e.g. >20% wt.loss, tumour size, vocalizing, lack of grooming) that would lead to euthanasia of an animal before the expected completion of the experiment (specify per species and project if multiple projects involved).

1) Experimental - Piglets will be killed after the 6-h stable isotope study on study day 14 by i.v. pentobarbital
2) Clinical monitoring and humane endpoints: The health (temperature, colour of mucus membranes, feces and urine production) and behavior (activity, interest in the environment, general demeanor) of the piglets receiving DS will be monitored closely. If diarrhea becomes frankly bloody or significant changes in the above criteria are observed, the next dose of DS will be skipped and the piglet monitored every four hours. If blood is still present 12 hours later the piglet will be killed with sodium pentobarbital 750 mg i.v.). Histology of the gastrointestinal tract of all piglets will be examined post mortem. Aside from surgery drugs, it is not anticipated that medications will be required. If growth and behavior patterns are maintained, it is expected that the gastrointestinal discomfort is not sufficient to require medication. Surgical incisions and catheter exit sites will be checked daily. If a discharge or inflammation develops, a topical antibiotic (Polysporin) will be given. If the infection has not cleared up within 36 hours or goes systemic, the piglet will be killed with iv pentobarbital.

Specify person(s) who will be responsible for animal monitoring and post-operative care

Name: Linda Wykes	**Phone#:** 7843 or 697-0603
Scott Harding	7768/8736 or 457-2137
Keely Fraser	7768
Sean Mark	7768

f) Method of Euthanasia – According to CCAC guidelines, justification must be provided for use of any physical method of euthanasia without prior use of anaesthesia (justify here):

Specify Species		
Pig	☒	**anaesthetic overdose, list agent/dose/route:** Pentobarbital 750 mg.kg. i.v.
	☐	**exsanguination with anaesthesia, list agent/dose/route:**
	☐	**decapitation without anaesthesia** ☐**decapitation with anaesthesia,list agent/dose/route:**
	☐	**cervical dislocation**
	☐	**CO_2 chamber**
	☐	**other (specify)**
	☐	**not applicable (explain)**

11. Category of Invasiveness: B ☐ C ☐ D ☒ E ☐

Categories of Invasiveness (from the CCAC *Categories of Invasiveness in Animal Experiments*). Please refer to this document for a more detailed description of categories.

Category A: Studies or experiments on most invertebrates or no entire living material.

Category B: Studies or experiments causing little or no discomfort or stress. ***These might include holding animals captive, injection, percutaneous blood sampling, accepted euthanasia for tissue harvest, acute non-survival experiments in which the animals are completely anaesthetized.***

Category C: Studies or experiments involving minor stress or pain of short duration. ***These might include cannulation or catheterizations of blood vessels or body cavities under anaesthesia, minor surgery under anaesthesia, such as biopsy; short periods of restraint, overnight food and/or water deprivation which exceed periods of abstinence in nature; behavioural experiments on conscious animals that involve short-term stressful restraint.***

Category D: Studies or experiments that involve moderate to severe distress or discomfort. ***These might include major surgery under anaesthesia with subsequent recovery, prolonged (several hours or more) periods of physical restraint; induction of behavioural stresses, immunization with complete Freund's adjuvant, application of noxious stimuli, procedures that produce pain, production of transgenics (in accordance with University policy).***

Category E: Procedures that involve inflicting severe pain, near, at or above the pain threshold of unanaesthetized, conscious animals. ***Not confined to but may include exposure to noxious stimuli or agents whose effects are unknown; exposure to drugs or chemicals at levels that (may) markedly impair physiological systems and which cause death, severe pain or extreme distress or physical trauma on unanaesthetized animals.*** **According to University policy, E level studies are not permitted.**

12. Potential Hazards to Personnel and Animals **It is the responsibility of the investigator to obtain the necessary Biohazard and/or Radiation Safety permits before this protocol is submitted for review. A copy of these certificates must be attached, if applicable.**

No hazardous materials will be used in this study: ☒

a) Indicate which of the following will be used in animals:

☐**Toxic chemicals** ☐**Radioisotopes** ☐**Carcinogens** ☐**Infectious agents** ☐**Transplantable tumours**

b) Complete the following table for each agent to be used (use additional page as required).

Agent			
Dosage			
Route of administration			
Frequency of administration			
Duration of administration			
Number of animals involved			
Survival time after administration			

c) After administration the animals will be housed in: ☐ the animal care facility ☐ laboratory under supervision of laboratory personnel

Please note that cages must be appropriately labeled at all times.

d) Describe potential health risk (s) to humans or animals:

e) Describe measures that will be used to reduce risk to the environment and all project and animal facility personnel:

13. Reviewer's Modifications (to be completed by ACC only): The Animal Care Committee has made the following modification(s) to this protocol during the review process. Please make these changes to your copy. You must comply with the recommended changes as a condition of approval.

Appendix 2

Co-Author Waiver Letter

October 17, 2006

To Whom It May Concern:

This confirms that the co-authors (Keely Fraser and Linda Wykes) agree that the candidate (Scott Harding) includes the following manuscripts in his thesis entitled *Protein Synthesis and Gastrointestinal Pathophysiology in a Piglet Model of Colitis: Importance of Nutrition and Probiotics*:

Manuscripts:

1. PROBIOTICS STIMULATE LIVER AND PLASMA PROTEIN SYNTHESIS IN PIGLETS WITH DEXTRAN SULPHATE COLITIS AND MACRONUTRIENT RESTRICTION.

2. GROWTH AND TRACE ELEMENT NUTRITIONAL STATUS IN GROWING PIGLETS: IMPLICATIONS OF COLITIS AND PROBIOTIC SUPPLEMENTATION.

Mr. Harding was the sole PhD student working on this study. He was responsible assisting in the study design, diet formulation, conducting the animal surgeries and trial, isotope infusions and sample analysis (including calculations) associated with this study. He carried out sample analysis including GC-MS analysis for protein synthesis, histological grading, immunohistological staining, TUNEL staining, FRAP assay, F_2-isoprostane assay and all trace element analysis using AAS. Mr. Harding was also responsible for all statistical analysis of data relating to this study. Ms. Fraser was the principle author of the second manuscript but Mr. Harding contributed equally to the analysis and was involved in the development of this manuscript as well.

Scott Harding

I, the co-author, agree that the candidate Scott Harding, include the above noted manuscripts in his thesis.

Linda Wykes

Keely Fraser