Impact of Macronutrient Restriction and Probiotic Supplementation on Protein Synthesis and Growth in a Piglet Model of Dextran Sulphate-Induced Colitis

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July, 2006

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

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ISBN: 978-0-494-28487-2
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ISBN: 978-0-494-28487-2

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ABSTRACT

A dextran sulphate (DS) model of ulcerative colitis was used to examine the effects of macronutrient restriction with (MR+PRO) and without (MR) probiotic supplementation (VSL#3®) on protein metabolism and growth. MR and MR+PRO decreased weight and chest circumference gain, but had no effect on linear growth of piglets. MR decreased the protein fractional synthesis rate (FSR) of liver, masseter, longissimus dorsi, colon, as well as plasma albumin, measured by stable isotope tracer L-[ring-$^{2}H_{5}$]phenylalanine. MR+PRO increased the FSR of hepatic proteins by greater than 70% and increased both the FSR and absolute synthesis rate (ASR) of the total plasma protein pool, albumin and fibrinogen. Over 11 days, MR+PRO piglets showed a smaller decrease in plasma total protein concentrations than WN piglets, and maintained higher albumin levels than either WN or MR piglets. These findings highlight the importance of adequate nutrition during gastrointestinal inflammation and show that when food intake is decreased, probiotics stimulate liver protein synthesis and increase the acute phase protein response, similar to the well-nourished state.

RESUMÉ

A l'aide d'un modèle animal de colite ulcéreuse induite par le dextran sulfate (DS) cette étude vise à examiner les effets de la restriction en macronutrients, avec (MR+PRO) et sans (MR) supplément probiotique (VSL#3®), sur le metabolisme protéique et la croissance. MR et MR+PRO ont réduit le poids et le gain de circonférence de la poitrine des porcelets sans affecter leur croissance linéaire. Cependant, MR a entrainé une baisse du taux de synthèse fractionel des proteines (FSR) dans le foie, le masseter, le longissimus dorsi et le colon, ainsi que dans l'albumine du plasma, tel que mesuré par le traceur isotopique L-[ring $^{2}H_{5}$]phenylalanine. Il est à noter que dans le groupe MR+PRO, le FSR des protéines hépatiques a augmenté de plus de 40% de même que le FSR et le ASR des proteines totales plasmatique, l'albumine et le fibrinogène. Après 11 jours, les porcelets MR+PRO ont démontré une légère décroissance des protéines totale du plasma comparativement aux porcelets n' ayant aucune restriction nutritionelle (WN) et ont maintenu un niveau d'albumine plus haut que celui des porcelets WN et MR. Ces observations montrent l'importance de la nutrition durant une inflammation intestinale et suggèrent qu'en situation de restriction alimentaire les probiotiques stimulent la synthèse de protéines hépatiques et des protéines de phase aigüe de façon similaire au groupe WN.

ACKNOWKLEDGEMENTS

I would like to acknowledge the continuous support and guidance of my supervisor Dr. Linda Wykes who has shown unwavering kindness and confidence in my abilities. I am deeply thankful for your time, patience and all that you have taught me throughout this experience.

I would like to thank Scott Harding, for his friendship and assistance in completing this project, as well as Christine Gurekian for her help during isotope infusion studies. I would also like to acknowledge the contributions of Brian Lee and Edward Walker in their assistance in processing samples and data entry.

Finally, I would like to thank my Mom and Kal. I could not have done this without your love and support.

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LIST OF ABBREVIATIONS

ANOVA: analysis of variance

ASR: absolute synthesis rate

APR: acute phase response

CD: Crohn's disease

CFU: colony forming units

CRP: C-reactive protein

DNBS: dinitrobenzene sodium

DS: dextran sulphate

FSR: fractional synthesis rate

GC-MS: gas chromatography-mass spectrometry

GI: gastrointestinal

GSH: glutathione

HCl: hydrochloric acid

ig: intragastric

IBD: Inflammatory bowel disease

IgE: immunoglobulin E

IL-10: interleukin 10

INF γ: interferon gamma

LD: longissimus dorsi

LGG: Lactobacillus rhamnous GG

MPO: myeloperoxidase

MR : macronutrient restriction

mTOR: mammalian target of rapamycin

NOS: nitric oxide synthase

MR : protein energy malnutrition

Na_2EDTA: disodium ethylenediaminetetraacetic acid

PGE2: prostaglandin E2

PS: protein synthesis

SEM: standard error of mean

sc: subcutaneously

TCA: trichloroacetic acid

TJ: tight junction

TLR: toll like receptor

TNF: tumor necrosis factor-α

TNBS: trinitrobenenesulfonic acid

UC: ulcerative colitis

WN: well-nourished

CONTRIBUTION OF AUTHORS

A manuscript entitled "Probiotics stimulate hepatic protein synthesis and preserve protein concentrations despite macronutrient restriction in a piglet model of ulcerative colitis" has been prepared and is included as part of this thesis. Contributions of the author to this manuscript follow.

As first author, I was responsible for writing this manuscript and preparation of all figures and tables. I was responsible for surgery, daily piglet care, preparation of the liquid diet, administration of daily treatment and conducting stable isotope infusions. I prepared and analyzed all tissue and plasma protein samples on GC/MS, and measured plasma albumin and total protein concentrations in our laboratory. Two undergraduate students Edward Walker and Brian Lee assisted in isolation of albumin and fibrinogen for GC/MS analysis and data entry. Plasma fibrinogen concentrations were measured at McGill University Health Centre Laboratory.

Editorial assistance was provided by Dr. Wykes and Scott Harding. Scott Harding also participated in piglet surgeries, daily animal care, isotope infusions, isolation of proteins, and statistical analysis of data presented in this manuscript. Dr.Wykes was the principal investigator and provided guidance and expertise throughout this project.

1. INTRODUCTION

Malnutrition, muscle wasting, growth failure and hypoalbuminemia are well known complications of inflammatory bowel disease (IBD) (Bristian 1999). Specific changes in protein metabolism responsible for muscle wasting and growth failure in IBD are not well understood (Kirschner 1990; Seidman and LeLeiko 1991; Geerling et al. 1998). Our group has previously described the separate and combined effects of macronutrient restriction (MR) and acute GI inflammation on protein metabolism in piglets after 7 days of dextran sulphate (DS)-induced colitis (Mackenzie et al. 2003). Mackenzie and colleagues (2003) showed that whole body protein turnover was increased by inflammation and decreased by MR. There was a DS-induced doubling of albumin synthesis in piglets regardless of nutritional status, but in MR piglets, this increase was correlated with decreased muscle protein synthesis (Mackenzie et al. 2003). These findings underscored the critical role of adequate nutrition during active GI inflammation, and provided valuable insight into the potential mechanism of hypoalbuminemia and muscle wasting which occur in IBD (Mackenzie et al. 2003). Changes in protein metabolism which occur with more chronic nutritional deprivation and continued GI inflammation are not yet known.

Malnutrition and compromised immunity during infection and/or injury create a vicious cycle which negatively affects the acute phase protein response (Doherty et al. 1993), muscle protein (Reeds et al. 1994) and growth (Kirschner 1990). It is therefore important to investigate potential therapies which may disrupt this cycle either through decreased disease severity and/or improved immune function. Probiotic supplementation has proven beneficial in decreasing disease severity in individuals with pouchitis and ulcerative colitis (UC) (Venturi et al. 1999; Gionchetti et al. 2000; Bibiloni et al. 2005), as well as in experimental models of colitis (Madsen et al. 2001a; Shibolet et al. 2002; Rachmilewitz et al. 2004). However, the impact of probiotics on nutritional outcomes, including protein metabolism and growth is not yet known.

Therefore, our aim was to determine the impact of MR with and without probiotic supplementation on tissue and plasma protein synthesis, as well as growth in piglets after 11 days of DS-induced colitis. We hypothesized that probiotic supplementation in the MR state would decrease the metabolic demands for acute phase protein synthesis and therefore decrease plasma protein synthesis and improve both tissue protein synthesis and growth.

2. LITERATURE REVIEW

2. 1 INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is characterized by chronic recurrent episodes of gastrointestinal (GI) inflammation and encompasses a group of GI inflammatory diseases of unknown etiology, including but not limited to Crohn's disease (CD) and ulcerative colitis (UC). Inflammation, infection and injury have a profound effect on protein and energy metabolism, and stimulate an acute phase response (APR) (Klein et al. 1988) which plays a central role in healing (Doherty et al. 1993). The metabolic consequences of carrying out a sustained APR are evident in individuals with IBD (Grimble 1999). The APR involves a series of highly coordinated cellular and molecular-mediated reactions which stimulate the inflammatory process (Collins and Croitoru 1994) with the release of a host of inflammatory mediators and cytokines (Yamada and Grisham 1994). This response increases energy expenditure (Azcue et al. 1997), whole body protein turnover (Powell-Tuck et al. 1984), loss of body nitrogen and depletes muscle protein (Reeds et al. 1994).

2.1.1 Pathological Appearance of Crohn's Disease and Ulcerative Colitis

The pathological appearance of CD and UC can be distinguished by two characteristics, first by the location or section(s) of GI tract which is inflamed and secondly by the extent of tissue involvement. In UC, inflammation is continuous and limited to the mucosa of the colon, whereas CD can occur anywhere within the GI tract involving discontinuous transmural inflammation (Collins and Croitoru 1994). UC is characterized by edema, superficial ulcerations and erosion of the mucosa (Stenson and Korzenik 2003). There is an intense infiltration of neutrophils into the mucosa and submucosa, crypt abscess formation, mucus depletion and vascular congestion (Stenson and Korzenik 2003). Atrophy and branching of the crypts have been observed in both Crohn's disease and UC (Stenson and Korzenik 2003). Formation of granuloma, thickening of the bowel wall and subsequent narrowing of the lumen are characteristic of Crohn's disease (Stenson and Korzenik 2003). The gross structural damage observed in IBD is largely attributed to the action of

infiltrating leukocytes during active inflammation (Yamada and Grisham 1994). More specifically, leukocytes release reactive oxygen species (ROS) and indirectly stimulate enzymes which produce reactive nitrogen species and contribute to oxidative stress (Yamada and Grisham 1994). Consequently, vascular permeability increases and epithelial cells are destroyed, the mucosal lining is eroded and ulcerations appear (Yamada and Grisham, 1994).

2.1.2 The Acute Phase Protein Response

The APR to inflammatory stress results in an increased demand for protein. Amino acids are released from skeletal muscle protein at an accelerated rate and taken up by the splanchnic bed (Clowes et al. 1980). Hepatic synthesis of rapidly turning over acute phase proteins takes priority and muscle protein synthesis is impaired (Jahoor et al. 1999). Concentrations of positive acute phase proteins, such as fibrinogen and C-reactive protein increase, largely due to cytokine-induced changes which stimulate the liver to increase synthesis of these proteins (Fleck 1989). In contrast, concentrations of negative acute phase proteins such as albumin decrease (Fleck 1989). This decrease may not be attributed to decreased synthesis but rather increased disappearance and/or loss, through mechanisms including but not limited to increased vascular permeability (Casley-Smith 1980). The amino acid demands to maintain acute phase protein synthesis during continued stress and injury are high and in the absence of adequate exogenous protein, catabolism of muscle protein ensues as a survival mechanism to meet the demands of acute phase proteins (Reeds et al. 1994). Decreased protein and energy intake during active episodes of inflammation in IBD, therefore conflict with the increased metabolic demands of the APR for protein, perpetuating a vicious cycle of malnutrition and disease progression (Rigaud et al. 1994) (Bristian 1999) (O'Keefe and Rosser 1994).

2.1.3 Nutritional Status

Malnutrition is a well known complication of chronic gastrointestinal inflammation (Bristian 1999). The majority of individuals with CD experience at least one or more episodes of malnutrition, evident by muscle wasting and weight loss which increase with severity of disease activity (Harries and Heatley 1983; Rigaud et al. 1994). In children, the diagnosis of CD is frequently preceded by nutritional indicators of compromised nutritional status, such as decreased body weight and linear growth, with severe growth failure occurring in approximately one third of these children (Kanof et al, 1988).

Nutritional status is influenced by a number of factors. Decreased energy and protein intake is in part a result of cytokine-induced anorexia (O'Keefe and Rosser, 1994). Elevated levels of serum cytokines involved in systemic and localized proinflammatory responses, such as tumor necrosis factor-alpha (TNF-α) and interleukin 1 (IL-1) known to induce fever among many other effects, are believe to be partly responsible for the suppression of appetite observed in IBD (Fiocchi 1994). Other factors including self-restricting food behaviors, loss of appetite and pleasure associated with eating due to abdominal discomfort and depression may also contribute to malnutrition (Rigaud et al. 1994). Moreover, as disease activity increases, dietary intake decreases (Hodges et al. 1984).

2.1.4 Malnutrition and the Acute Phase Response

The combined stress of malnutrition and inflammation adversely affects immune function (Gallagher and Daly 1993), muscle protein synthesis, and contributes to negative nitrogen balance (Jahoor et al. 1999), muscle wasting (Seidman and LeLeiko 1991) and growth failure (Kanof et al. 1988). In uncomplicated malnutrition, whole body protein turnover and energy expenditure are decreased and nitrogen recycling becomes more efficient in an effort to preserve total body protein (Picou and Phillips 1972; Golden et al. 1977; Jahoor et al. 1996; Azcue et al. 1997). In protein-malnourished animals, synthesis of most major tissues and total protein in plasma

decrease, while total protein concentrations remain unchanged (Wykes et al. 1996). More specifically, protein-malnourished animals decrease the absolute synthesis rate (ASR) of some plasma proteins, such as albumin, fibrinogen and transferrin, but increase synthesis of others (e.g.,, transthyretin) (Jahoor et al. 1996). These kinetic changes are not always reflected in measures of concentration, suggesting that widely accepted measures such as albumin concentrations may not be the best predictors of nutritional status (McFarlane et al. 1969). Moreover, protein concentrations do not always reflect metabolic changes which occur in response to malnutrition (Jahoor et al. 1996).

Inflammatory stress, infection and trauma increase whole body protein turnover (Manary et al. 2004). More specifically, hepatic synthesis of rapidly turning over acute phase proteins is prioritized and muscle protein synthesis is impaired (Jahoor et al. 1999). In well-nourished pigs inflammatory stress stimulates plasma protein synthesis, and concentrations increase (Jahoor et al. 1999). Pigs fed a protein restricted diet for several weeks show a similar increase of plasma PS in response to inflammation, but concentrations of some plasma proteins, particularly albumin continue to decrease (Jahoor et al. 1999). This finding suggests that protein restriction decreases albumin concentrations due to decreased synthesis, whereas inflammation increases catabolic rates and/or losses (Jahoor et al. 1999). In contrast, both protein deficiency and inflammation decrease muscle PS (Jahoor et al. 1999). In short-term protein energy malnutrition and acute gastrointestinal inflammation there is a prioritization of acute phase protein synthesis at the expense of muscle protein and growth, which does not occur in the well-nourished state (Mackenzie et al. 2003).

In IBD, whole body protein turnover is increased (Powell-Tuck et al. 1984), yet accelerated rates of PS of tissues such as liver and colon make only a small contribution to this whole body increase (Heys et al. 1992). This suggests that PS must also be increased in other tissues and that further investigations are needed to more fully understand the impact of inflammation on protein metabolism (Heys et al. 1992). Furthermore, individuals with IBD also show increased energy expenditure and in

contrast to other malnourished/ starvation states, such as anorexia nervosa, they do not show decreased resting energy expenditure per unit of lean body mass (Azcue et al. 1997). This failure to decrease energy expenditure and concomitant increase in whole body protein turnover, despite malnutrition and weight loss may perpetuate disease-associated wasting and growth failure (Azcue et al. 1997). This disease-associated weight loss is thought to be a consequence of decreased food intake rather than other nutritional disturbances such as elevated energy expenditure and/or malabsorption (Rigaud et al. 1994). In children with active CD, an elemental diet and gradual reintroduction of food was as effective as high dose steroid therapy (adrenocorticotrophic hormone, and oral prednisolone with sulphasalazine) in inducing remission (Sanderson et al. 1987). This was evident by improved disease activity, body weight, CRP and albumin levels, and after 6 months, height velocity was greater in children fed an elemental diet (Sanderson et al. 1987). Despite this evidence to suggest that refeeding may be as effective as steroids in inducing remission of active disease, the focus of medical investigations often negates the critical role of nutrition in managing this disease (Thomas et al. 1992) (Sanderson et al. 1987).

2.1.5 Hypoalbuminemia in IBD

Hypoproteinemia, and more specifically hypoalbuminemia is common in IBD (Harries and Heatley 1983). Albumin levels play a critical role in maintaining oncotic pressure, antioxidant status, membrane permeability, molecular transport and preventing platelet aggregation (Mendez et al. 2005). Moreover, compromised albumin status has been associated with increased risk of mortality and morbidity in the critically ill and elderly (Covinsky et al. 2002), and preoperative levels are one of the most powerful predictor of postoperative outcomes in surgical patients (Gibbs et al. 1999). Much like other negative acute phase proteins such as transthyretin, pre-albumin, and retinol-binding protein, albumin concentrations decrease during the acute phase response to infection or injury (Jahoor et al. 1999). Despite its long half-life, plasma albumin levels decrease with malnutrition and even previously healthy individuals can

experience a greater than a 30% decrease in albumin levels within a few days of a serious physical trauma or infection (Mendez et al. 2005), suggesting increased catabolism (Ballantyne and Fleck 1973). However, this decrease in concentration of negative acute phase proteins may initially be due to increased vascular permeability and redistribution of proteins from plasma to tissue spaces, rather than immediate changes in synthesis or catabolism (Fleck et al. 1985). This loss of protein from plasma can be measured by the transcapillary escape rate and in individuals with septic shock this transvascular loss is increased by as much as 300%, and by greater than 100% within 7 hours in patients who have undergone cardiac surgery (Fleck et al. 1985). In healthy individuals the transcapillary escape rate is approximately 10 times the rate of synthesis and catabolism, therefore increases of this magnitude during infection and injury clearly have a profound effect on plasma concentrations (Fleck et al. 1985). In response to chronic protein deficiency pigs show a decrease in albumin concentrations, largely due to decrease synthesis (Jahoor et al. 1999). In contrast, piglets with DS-colitis double albumin synthesis in response to inflammation regardless of nutritional status, suggesting that albumin synthesis may not be impaired, but in fact prioritized during inflammation, at least in the short-term (Mackenzie et al. 2003). In hypoalbuminemic individuals with IBD, early isotope studies with ^{131}I – albumin showed that albumin synthesis is not sufficient to compensate for the increase in the rate of removal from circulation through gastrointestinal losses (Steinfeld et al. 1960). Both free and albumin bound ^{131}I were found in the feces of these individuals suggesting that some albumin lost into the gut may have been hydrolyzed to supply amino acids for synthesis of other functional proteins (Steinfeld et al. 1960). This is not unlikely, given the high sulfur amino acid content of albumin (Barker and Putman 1984) and the demand for amino acids such as cysteine necessary for synthesis of proteins such as glutathione, which plays a pivotal role antioxidant defense. It remains plausible that the prioritization of acute phase protein synthesis, despite malnutrition, may perpetuate disease-associated muscle wasting. It has been hypothesized that excessive muscle proteolysis may be necessary to supply aromatic amino acids essential to the synthesis of acute phase proteins (Reeds et al. 1994), although these changes are not well understood.

2.1.6 Animal Models of IBD

Due to the complex nature of this disease and its largely unknown etiology, animal models of IBD have been developed to investigate pathological changes which may trigger disease onset, progression and efficacy of potential treatments. Although, "no animal model exactly reproduces human IBD, nor could it" (Elson and Weaver 2003), variables can be more closely controlled in these models. Some animal models involve a genetic mutation, gene deletion or insertion, while others use chemical agents or environmental stressors to induce inflammatory stress (Elson and Weaver 2003).

Several well recognized models of GI inflammation have been developed in rodents. The IL-10 deficient model uses a genetic mutation to inactivate the interleukin-10 gene resulting in an inability to control normal immune response (Fedorak 1995). T cells do not develop or are dysfunctional resulting in copious amounts of Th1 cells being produced and activated by IL-12 and IFN-γ (Madsen 2001c). In this model rodents develop persistent colitis-like symptoms and with time show a marked decrease in growth accompanied by anemia (Elson and Weaver 2003). Other models use exogenous substances to induce colitis, such as trinitrobenzenesulfonic acid (TNBS)/ethanol, acetic acid and dextran sulphate (DS). In these models the chemical agent is typically introduced into the GI tract by way of a bolus injection (e.g.,, intragastric, intrarectal) or is added to drinking water. In the TNBS/ethanol model, a combination of both chemically induced injury and T cell mediated reactions lead to swelling, neutrophils penetrating the mucosa, large ulcerations and an increase in large bowel weight (Fedorak 1995) (Elson and Weaver 2003). There is full thickness inflammation, deterioration/degradation of distal colonic tissue due to oxidative damage within a very short time period and thickening of the gut lining (Fedorak 1995). Similar to TNBS/ethanol, acetic acid induces colitis through direct contact with intestinal tissue, resulting in inflammation and epithelial cell death (Stenson 1994). Alternatively, DS is a highly electronegative sulphated polysaccharide which induces a localized inflammatory response when ingested (Conner and Grisham 1996). The

mechanism by which DS elicits an acute phase response and inflammation within the colon similar to UC is not clear. There is mass infiltration of macrophages and eosinophils, accompanied by an increase in the number of neutrophils and crypt abscesses (Stevceva et al. 2001). The inflammatory response may involve phagocytosis of DS by macrophages which accumulate within mucosa lesions and in the spleen of animals following ingestion of DS (Ohkusa et al. 1995). In this model acute intestinal mucosal injury occurs within 3-5 days with continuous exposure (Conner and Grisham 1996). In the murine model of DS, clinical symptoms include diarrhea and/or stool containing intestinal blood loss, ulcerations and intestinal inflammation (Cooper et al. 1993). Histological examination reveals primary destruction of the lower one third of the mucosal crypt in early stages, eventually leading to total crypt destruction and breakdown of the musoca in less than 7 days (Cooper et al. 1993). The reproducibility of the DS model in terms of clinical symptoms and severity of intestinal injury make it a reliable and sensitive model for examining potential therapeutic interventions (Elson and Weaver 2003). The rodent model of DS-induced colitis has also been successfully adapted in the piglet, to study the interactive effects of nutrition and inflammatory stress (Mackenzie et al. 2003). The piglet's accelerated rate of growth, well-defined amino acid requirements and rates of protein synthesis make it an ideal model to study the impact of nutrition and inflammation on protein kinetic and growth (Wykes and Ball 1993; Ebner et al. 1994; Jahoor et al. 1996; Wykes et al. 1996).

2.2 BACTERIA AND INFLAMMATORY BOWEL DISEASE

2.2.1 Potential Role of Bacteria in IBD

While the etiology of IBD remains largely unknown, several well-supported hypotheses exist. There is evidence to suggest that the onset of IBD may be the result of a genetic predisposition, dysregulation of the immune response, and/or an environmental trigger (Shanahan and Targan 1994). Mounting evidence suggests that luminal bacteria and their products may be involved in the initiation and perpetuation of inflammation by stimulating an immune response (Desreumaux and Colombel

2003). This may also be linked to altered microbial balance of the intestinal lumen and a loss of host tolerance to normal bacteria resulting in an immune mediated response directed against the gut's own microflora (Madsen 2001c). This hypothesis is supported by the fact that IBD occurs at sites along the GI tract where levels of luminal bacteria are highest, the disease's marked similarities to enterocolonic infection, and measurable changes in concentration of luminal microbes (Sartor 1995) (Madsen 2001c). Also noteworthy is the marked improvement in disease activity when bacterial concentrations are reduced with interventions such as gut rest and antibiotics (Madsen et al. 2001a). This hypothesis is further supported by the observation that genetically modified rodents (e.g., IL-10 deficient mice) raised under sterile conditions, and animals with DS-induced colitis treated with antibiotics such as metronidazole do not usually develop colitis (Yamada and Grisham 1994; Rath et al. 1996; Dianda et al. 1997; Contractor et al. 1998; Sellon et al. 1998; Madsen et al. 1999). Taken together, this evidence suggests that bacteria may act as an environmental trigger for the onset and/or perpetuation of inflammation (Madsen et al. 1999).

With more than 400 varieties of microbial species inhabiting the human gut, these commensal microorganisms play an intricate and precarious role in host intestinal function (Francisco et al. 2000). Yet, our knowledge of this complex and dynamic microflora is limited (Desreumaux and Colombel 2003). Commensal bacteria and lumen colonic cells function together as a cohesive protective barrier from potential pathogens entering the gut (Madsen 2001b). However, during events of physiological stress or injury this barrier integrity is compromised, allowing opportunistic pathogens to invade and potentially become established (Madsen 2001b). An overpopulation of aggressive bacteria, a disruption to the lumen's microbial balance of protective verses hostile bacteria, and/or compromised gut barrier function may be responsible for lack of host tolerance and may play a role in pathogenesis of IBD (Desreumaux and Colombel 2003).

2.2.2 Probiotic Bacteria

The role of nutrition and the possibility that components, particularly bacteria, within the normal diet may play a role in improving overall immunity is not a new idea. The term "probiotic" was first introduced by Lilly and Stilwell in 1965 (Lilly and Stillwell 1965) in reference to organisms that contribute to the microbial balance of the gut (Madsen 2001c). In 1989, Fuller defined probiotics as "living organisms, which on ingestion in certain numbers improve the health of the host beyond their inherent basic nutrition" (Fuller 1989). As viable nonpathogenic microorganisms, probiotics normally exist in the healthy gut and have been found to exert beneficial effects on host microbial equilibrium when ingested (Marteau and Shanahan 2003). Probiotics may promote integrity of the gut barrier and immune regulation via gut associated lymphoid tissues (Francisco et al. 2000; Isolauri et al. 2001). Most widely recognized for their use in the treatment of infectious and antibiotic-induced acute diarrhea, probiotic strains such as *Lactobacillus* GG and/or *Saccharomyces boulardii* (a nonpathogenic yeast) have proven beneficial (Madsen et al. 1999). Recent evidence also supports the use of specific strains of probiotic bacteria in the treatment of inflammatory bowel diseases, food allergies, as well as acute gastroenteritis (Isolauri et al. 2001).

2.2.3 Probiotic Mixture VSL#3®

Given recent attention to the potential role of bacteria in the etiology of IBD, a probiotic mixture of various strains, known as VSL#3® (VSL Pharmaceuticals, United States) has become the center of much scrutiny and investigations. VSL#3® is a specially-formulated mixture of probiotic bacteria which is consumed as a supplement, and is resistant to stomach acids, digestive enzymes and bile salts, reaching the intestinal lumen as viable microorganisms. This preparation containing 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*. VSL#3® contains much higher concentrations of bacteria than other commercially

available preparations (4.5 x 10^{11} viable cells/g) and incorporates a variety of synergetic strains to maximize its therapeutic potential (Venturi et al. 1999). It is still not known whether such a large variety of species is required for VSL#3® to be effective (Madsen 2001c). Probiotic preparations containing only a single strain have provided less convincing evidence and have been relatively unsuccessful in colonizing the gut or modifying gastrointestinal microecology, if colonization is in fact necessary (Berg 1998).

2.2.4 Clinical Trials with VSL#3® and IBD

While there have been relatively few studies conducted in humans to investigate the potential efficacy of probiotic supplementation on disease activity and/or immunomodulation in IBD, the results of the few studies which have been conducted are both thought provoking and promising (**Table 2.1**). During clinical trials it is often difficult, if not impossible to control for variables such as use of pharmacological agents, naturopathic remedies, self treatment, and nutritional intake. Nevertheless, probiotics supplementation in both pouchitis (a serious complication of ulcerative colitis, following ileal pouch- anal anastomosis) and UC suggest that probiotics may maintain and/or extend periods of remission, decrease disease activity in those with mild to moderately active disease and improve quality of life (Venturi et al. 1999; Gionchetti et al. 2000; Gionchetti et al. 2003; Mimura et al. 2004; Bibiloni et al. 2005).

In a 12 month preliminary uncontrolled study, a twice daily supplement of VSL#3® (5 x 10^{11} cells/g; 6 g/day), as an alternative to traditional 5-acetylsalicylic acid (ASA) maintenance treatment, effectively maintained remission in 75% (12 out of 15) of individuals with UC (Venturi et al. 1999). Disease activity was clinically assessed bimonthly and endoscopically after 6 and 12 months or at time of relapse. Fecal cultures revealed that certain probiotic strains had effectively colonized the intestine (Venturi et al. 1999). Similarly, a randomized placebo-controlled double-blinded trial found that VSL#3® (5 x 10^{11} cells/g; 6 g/day) supplementation maintained antibiotic-

Table 2.1 Summary of Clinical Trials with Probiotic Supplementation in Inflammatory Bowel Disease

Author	Probiotic Dose	Study Design	Treatment Group	Duration	Results
Venturi et al. 1999	VSL#3 5×10^{11} CFU/g/day 6g/day	Uncontrolled Preliminary Study N= 20	UC patients in remission (allergic or intolerant to 5-ASA)	12 months	Maintained remission in 75% of patients with UC No adverse side effects
Gionchetti et al. 2000	VSL#3 $5x10^{11}$ CFU/g 6g/day	Double blind placebo-controlled N= 40	Chronic relapsing pouchitis after antibiotic induced remission	9 months	Maintained remission in 85% of patients with chronic pouchitis Placebo group 100% relapsed 4 months after probiotic therapy discontinued 100% relapsed
Mimura et al 2004	VSL#3 3×10^{11} CFU/day 6g/day	Double blind placebo-controlled N= 36	Chronic pouchitis and/or increased disease activity requiring continued antibiotics after antibiotic induced remission	12 months	Maintained remission in 85% of patients with chronic pouchitis Placebo group 94% relapsed VSL#3 associated with increased quality of life, decrease disease activity, and histological injury
Bibiloni et al 2005	VSL#3 3.6×10^{12} CFU/day	Randomized control led N=34	Ambulatory patients with mild to moderate active UC	6 weeks	Remission or response rate of 77% No response 9% Disease worsening 9% No adverse side effects associated with VSL#3

induced remission in 85% (17 of 20) of patients with refractory pouchitis, over a 9 month period (Gionchetti et al. 2000). Disease activity was clinically assessed once a month (Pouchitis Disease Activity Index), and an endoscopic exam and histological analysis were performed every 2 months or at time of relapse. The recurrence rate was 100% in patients receiving a placebo during the same period and four months following the cessation of probiotic therapy, 100% of the treatment group had also relapsed (Gionchetti et al. 2000). This may suggest that probiotics must be consumed continuously to maintain remission, as some probiotic strains (e.g., *Lactobacillus bulgaricus*) may not adhere to the mucosa and must be reintroduced on an ongoing basis (Madsen 2001b). These results were replicated by Mimura et al (2004) who likewise found that, after antibiotic-induced remission, a once daily dose of VSL#3® decreased histological injury scores and disease activity index, and increased quality of life in patients with recurring pouchitis and/or those requiring continued antibiotic treatment to control disease activity (Mimura et al. 2004). Antibiotic-induced remission was maintained for at least one year in 85% of patients receiving probiotics, compared to 6% in the placebo group (Mimura et al. 2004). VSL#3® has also proven beneficial to individuals with mild to moderately active UC (Bibiloni et al. 2005). After just 6 weeks of probiotic supplementation, 77% of individuals with UC showed signs of remission and/or response to treatment, confirmed by endoscopic assessment. Although no adverse effects were associated with supplementation, 9% experienced no effect and in another 9% the disease worsened (Bibiloni et al. 2005).

2.2.5 VSL#3® Supplementation in Animal Models of IBD

Animal models have been used extensively to investigate the mechanism of disease onset, progression and efficacy of clinical treatments in IBD (**Table 2.2**). Various animal models have been used to examine the mechanism by which probiotics may protect, prevent and/or decrease inflammation. Several main hypotheses have been proposed to explain the mechanism of probiotic action. There is evidence to suggest that probiotics may decrease severity of inflammation through colonization and competition with potentially pathogenic strains of bacteria in the bowel (Marteau et al. 2001), alternatively probiotics may improve intestinal barrier function (Madsen et al.

2001a) and/or have an immune stimulatory effect which extends beyond tissues of the GI tract to alter the production of inflammatory mediators, such as cytokines (Rachmilewitz et al. 2004).

2.2.6 Probiotics and Colonization

Elevated levels of certain aerobic (e.g., *E.coli*) and anaerobic strains (e.g., *Bacteroides fragilis)* as well as decreased levels of other commensal bacteria have been observed in patients with IBD (Giaffer et al. 1991). Although, the implications of these changes and the mechanism by which various bacteria strains may interact with the intestinal immune system are not well understood (Madsen 2001c) (Sartor 1995). On this note, various studies have demonstrated that the beneficial effects of probiotic supplementation are often accompanied by changes in concentrations of intestinal bacteria (Madsen et al. 1999; Venturi et al. 1999; Gionchetti et al. 2000; Gaudier et al. 2005). As a result, it has been hypothesized that probiotics may compete with potentially pathogenic strains for binding sites along the mucosa and/or prevent translocation (Marteau et al. 2001). In an *in vitro* model two probiotic strains, *Streptococcus thermophilus* and *Lactobacillus acidophilus* had a protective effect on intestinal epithelial cell lines (HT29/cl.19A and Caco-2) from the pathogenic invasion of *E. coli* (029:NM) (Resta-Lenert and Barrett 2003). Cell monolayers exposed to probiotics had reduced attachment and penetration of *E.coli* and decreased overall epithelial dysfunction caused by the invading pathogen (Resta-Lenert and Barrett 2003). Furthermore, dendritic cells of the lamina propria exposed to probiotics decreased the production of pro-inflammatory cytokines through contact with epithelial cells, and decreased the immune response of inflamed epithelium to competing microorganisms (Furrie et al. 2005). It is therefore plausible that specific probiotic strains may play a protective role for intestinal epithelial cells by inhibiting pathogenic bacteria from attaching to the mucosal lining and subsequent invasion (Resta-Lenert and Barrett 2003). This may also suggest that the beneficial effects of probiotics extend well beyond colonization and competitive inhibition to interact with both immunological and structural components of the gut.

Table 2.2 Summary of Probiotic Trials in Experimental Models of Colitis

Authors	Probiotics	Treatment initiated	Treatment Group	Control	Duration	Results
Moon et al (2004)	Lactobacillus rhamnosus GG 1×10^7 CFU/mL	Days 8-21 After induction of acute colitis	Chronic DS colitis BALB/c mice	Normal BALB/c mice Untreated PBS BALB/c mice with DS colitis	13 days	LGG ↓ Disease activity index ↓ IL-10 levels No affect on mucin gene expression
Madsen et al (2001)	VSL#3 2.8×10^8 cfu/day	Probiotics given after colitis was established	IL-10 deficient mice, 8 weeks old	IL-10 deficient mice, not receiving VSL#3	28 days	Barrier function & gut transport mechanisms normalized ↓ Histological injury score ↓TNF-α ↓IFN-γ mucosal secretion
Shibolet et al (2002)	VSL#3 0.142 mg/kg containing 3×10^9 bacteria or 1mL LGG 10^{10} cfu	Probiotics given for entire study Colitis induced on day 7	DNBS or Iodoacetamide induced colitis rats	Rats given probiotics for entire 14 days without colitis Or Rats with colitis on day 7 with normal diet	14 days	Iodoacetamide colitis ↓ lesion area, ↓colonic wet weight ↓PGE2 synthesis ↓ MPO ↓NOS No effect in DNBS colitis rats

Table 2.2 (continued) Summary of Probiotic Trials in Experimental Models of Colitis

Authors	Probiotics	Treatment initiated	Treatment Group	Control	Duration	Results
Dock et al 2004	Bifidobacterium lactis and Streptococcus thermophilus 10^6 cfu/g 4g/day	Probiotics given during refeeding after 3 days of starvation	Starved Wistar rats	Refed rats without probiotics; starved; standard rat chow	3 days	Probiotics ↑ albumin concentrations, ↑ mucosa DNA content ↑ feed efficiency compared to refed control
Rachmilewitz et al 2004	Viable, nonviable irradiated and heat killed VSL#3 2.8 x10^8 cfu/g	Probiotics given 10 days prior to induction of colitis and for 7 days after	DSS –induced colitis in Balb/c mice	DSS-Balb/c mice no treatment	7 days	Viable and irradiated probiotics ↓ severity of inflammation, ↓ MPO activity ↓ histological injury ig and sc injections of DNA proved beneficial in DSS, TNBS, and IL-10 mice
Gaudier et al 2005	VSL#3 4 x 10^9 cfu/day	Probiotics given after colitis was established	DSS- induced colitis in BALB/c mice	DSS- mice, with and without VSL#3	14 days	Modified cecal microflora VSL#3 did not heal colitis No effect on colonic mucus barrier, mucin gene expression or thickness of mucus layer

2.2.7 Probiotics and Intestinal Barrier Function

Under normal conditions the gastrointestinal epithelium acts a functional barrier between the tightly controlled environment within the body and contents of the lumen, which may contain potentially harmful foreign pathogens in addition to nutrients and commensal bacteria (Baumgart and Dignass 2002). Permeability is regulated primarily by intercellular tight junctions (TJ) (Kucharzik et al. 2001) which act as a barrier between enterocytes separating the intestinal lumen and vascular components (Schmitz et al. 1999b). Individuals with CD show evidence of abnormal intestinal permeability (Katz et al. 1989; Puspok et al. 1998). In UC there is decreased thickness of the colonic mucus layer (Pullan et al. 1994), apoptosis of epithelial tissue (Strater et al. 1997) and TJ structure between enterocytes is altered in inflamed sections of colonic segments (Schmitz et al. 1999b).

The mechanism responsible for this increased paracellular permeability in IBD is not entirely clear. Decreased synthesis of specific TJ proteins, such as occludin, at sites of inflammation, intestinal damage and destruction of crypts characteristic of acute inflammation (Kucharzik et al. 2001), and altered TJ structure (Schmitz et al. 1999b) may all contribute. Alternatively, changes in cytokine patterns both in serum and within the intestine may also be responsible for altered intestinal barrier function (Braegger and MacDonald 1994; Schmitz et al. 1999a). Cytokines, such as TNF- α may increase vascular permeability and decrease epithelial barrier function, changes which are intrinsically linked to the pathogenesis of both diarrhea and inflammation (Schmitz et al. 1999a). *In vitro* studies with HT-29/B6, a subclone of human colorectal cells, found TNF- α had a powerful dose-dependent reducing effect on resistance of epithelial barrier function, primarily through increased paracellular permeability (Schmitz et al. 1999a). Furthermore, impaired barrier function may in part account for hypoproteinemia observed in IBD, which cannot be attributed to impaired or decreased synthesis of proteins (Steinfeld et al. 1960). Increased vascular permeability may perpetuate intestinal losses of small proteins, such as albumin (MW ~68,000) which are leaked into the gut in individuals with IBD (Steinfeld et al. 1960). It is

therefore important to investigate means by which to reverse and/or decrease intestinal permeability due to the high metabolic costs associated with a carrying out a sustained acute phase response and its potential detriment to muscle protein and growth.

There is now evidence to suggest that probiotics may improve intestinal barrier function (Malin et al. 1996; Madsen et al. 2001a). In IL-10 gene deficient mice, VSL#3® effectively normalized mannitol flux, an *in vivo* measure of paracellular permeability, after just 4 weeks of supplementation (Madsen et al. 2001a). Histological appearance of the colon improved, while mucosal secretion of both TNF-α and IFN-γ decreased (Madsen et al. 2001a). Similarly, the probiotic strain *Lactobacillus GG* was also found to promote the immunological barrier of the gut, stimulating an IgA-mediated immune response in patients with CD (Malin et al. 1996). The effect of probiotics on intestinal barrier function and how these changes may impact protein status are not yet known. However, there is evidence to suggest that in healthy animals, probiotics may improve protein status and recovery from malnutrition (Dock et al. 2004). In normal rats, Dock and colleagues (2004) showed that after short-term fasting, rats refed an adequate diet plus probiotics had higher albumin levels and mean colonic mucosa DNA content (an indicator of cell proliferation) than refed controls not receiving probiotics. These findings suggest that probiotics may improve recovery after short-term fasting through increased feed efficiency and decreased mucosal atrophy. Dock and colleagues (2004) hypothesized that probiotics may alter the catabolic response to malnutrition and this effect may be mediated through decreased cytokine production (Dock et al. 2004). It is therefore plausible that probiotics may have a systemic effect, extending well beyond the intestinal wall.

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2.2.8. Probiotics and Immune Signaling

Probiotics may exert their beneficial effects systemically through activation of immune signaling pathways, rather than colonization (Rachmilewitz et al. 2004). Intragastric (ig) administration of VSL#3® in Balb/c mice decreased disease activity

of DS-induced colitis, myeloperoxidase (MPO) activity and histological injury scores (Rachmilewitz et al. 2004). Furthermore, these results were replicated in animals with DS and TNBS-induced colitis after subcutaneous (sc) injection of probiotic DNA, and in IL-10 deficient mice with established colitis (Rachmilewitz et al. 2004). Most surprising was that both viable and nonviable irradiated bacterial DNA proved beneficial in decreasing severity of colitis, suggesting that neither colonization nor substances produced by the bacteria were the primary mechanism responsible for their anti-inflammatory effects (Rachmilewitz et al. 2004). Rachmilewitz and colleagues (2004) showed that probiotic DNA is most likely absorbed through the upper GI tract and is biologically active exerting its effects systematically. This was confirmed when plasmid DNA was found in liver and spleen only a few hours after intragastric and subcutaneous, but not intrarectal injection (Rachmilewitz et al. 2004). This may suggest that the anti-inflammatory and/or protective effects of probiotics may be attributed to DNA which activates innate immunity through signaling of transmembrane proteins known as toll-like receptors (TLR), more specifically TLR9. This was confirmed when nonviable irradiated bacteria improved DS-induced colitis in mice deficient in other toll like receptors (e.g., TLR2 and TLR4) but not in TLR 9-deficient mice, the primary signaling pathway believed to mediate the protective and systemic effects of probiotics (Rachmilewitz et al 2004).

In the DS model, probiotics were found to have a protective effect when administered prior to inducing colitis (Rachmilewitz et al. 2004). Similarly, probiotics improved established colitis in IL-10 deficient mice and prevented iodoactamide-induced colitis in rats (Shibolet et al. 2002). However, probiotics did not prevent dinitrobenzene sodium (DNBS)-induced colitis (Shibolet et al. 2002) or promote healing after DS-induced colitis had already been established in mice (Gaudier et al. 2005). These results may suggest that the efficacy of treatment and/or the protective role of probiotics may be closely related to choice of experimental model and supplementation protocol (Gaudier et al. 2005). However, given the significant link between nutrition and immunity and the effect of probiotics in treating and/or preventing GI inflammation, their impact on protein metabolism and the acute phase

protein response to inflammation has not yet been examined. Furthermore, the nutritional implications of probiotic supplementation, beyond weight gain and feed efficiency in healthy animals have not yet been investigated (Dock et al. 2004). More specifically, how probiotic may affect nutritional complications such as muscle wasting, growth failure and hypoalbuminemia, which occur during active GI inflammation when food intake is decreased.

3. RATIONALE

Specific changes in protein metabolism responsible for muscle wasting and growth failure in IBD are not well understood (Kirschner 1990; Seidman and LeLeiko 1991; Geerling et al. 1998). The combined stress of decreased food intake and chronic recurring episodes of inflammation leads to weight loss, loss of body protein (Bristian 1999), compromised immune function (Doherty et al. 1993) and impaired gut barrier function (Schmitz et al. 1999b). Due to the complex nature of this disease and the significant link between nutrition and immunity (Doherty et al. 1993), the independent effects of malnutrition and acute inflammation on protein metabolism are often difficult to study clinically.

Our group has previously adapted the murine model of DS-induced colitis (Cooper et al. 1993) to the piglet, and examined the separate and combined effects of MR and acute GI inflammation on protein metabolism (Mackenzie et al. 2003). The piglet is an appropriate model to conduct protein kinetic studies of this nature due to its rapid rate of growth and response to nutritional deprivation, as well as marked physiological similarities between the GI tract of humans and pigs (Wykes and Ball 1993; Kararli 1995). Furthermore, the piglet has been extensively studied as a model of nutrition with well-defined protein requirements and amino acid metabolism (Ball et al. 1996).

Our group has previously described the impact of MR on protein metabolism in a 7 day model of acute DS-induced colitis (Mackenzie et al. 2003). Mackenzie and colleagues (2003) found that in the short-term, nutritional status had no impact on the fractional synthesis rate of proteins in liver and muscle during acute DS-induced colitis. Not surprisingly, whole body protein turnover was increased by inflammation and decreased by MR , however, the contribution of plasma protein synthesis to whole body protein turnover doubled in MR piglets with colitis but not in those which were well-nourished (Mackenzie et al. 2003). In MR piglets this increase in the contribution of plasma proteins to whole body protein turnover was largely attributed to an increase

in albumin synthesis (Mackenzie et al. 2003). DS-induced colitis more than doubled the rate of normal albumin synthesis regardless of nutritional status, yet concentrations remained unchanged, suggesting increased utilization and/or disappearance (Mackenzie et al. 2003). Unlike their WN counterparts, MR piglets prioritized the synthesis of plasma proteins, particularly albumin during acute inflammation, at the expense of muscle protein and weight gain (Mackenzie et al. 2003). These findings suggest that hypoalbuminemia observed in IBD may not be a result of decreased synthesis and highlight the importance of adequate nutritional during active GI inflammation.

To further understand changes in amino acid metabolism during the acute phase response, we aimed to adapt the piglet model to more closely mimic the clinical scenario of more chronic GI inflammatory stress and decreased food intake. We decreased the DS dose and extended the duration of exposure from 7 to 11 days. We developed a semi-purified liquid diet, infused into the stomach catheter to more precisely control dietary intake and confounding factors such as micronutrient deficiencies and fluid intake. Finally, we have collected more in depth growth data which has allowed us to differentiate weight loss, from wasting and stunting.

Malnutrition, particularly protein deficiency has a profound effect on tissue PS (Wykes et al. 1996). Yet, synthesis of acute phase proteins is not compromised in pigs with chronic protein deficiency exposed to a single systemic inflammatory insult (Jahoor et al. 1999) or in MR piglets with acute DS-induced colitis (Mackenzie et al. 2003). However, changes in protein metabolism which occur with more chronic nutritional deprivation and continued GI inflammation are not yet known. Malnutrition and compromised immunity during infection and/or injury (Amati et al. 2003; Cooper et al. 2004) create a vicious cycle which negatively affects the acute phase protein response, muscle protein and growth (Doherty et al. 1993). It is therefore important to investigate potential therapies which may disrupt this cycle either through decreased disease severity and/or improved immune function and protein status.

Probiotics have proven beneficial in both humans with pouchitis and active UC (Venturi et al. 1999; Gionchetti et al. 2000; Bibiloni et al. 2005), as well as in experimental models of colitis (Madsen et al. 2001a; Shibolet et al. 2002; Rachmilewitz et al. 2004), although, their impact on protein metabolism and growth is not yet known. Clinical trials have shown that probiotics maintain remission and improve quality of life in patients with pouchititis, and decreased disease activity in patients with UC (Venturi et al. 1999; Gionchetti et al. 2000). Similarly, animal trials have confirmed a potential role for probiotics in preventing and treating experimental colitis (Madsen et al. 1999; Madsen et al. 2001a; Shibolet et al. 2002; Rachmilewitz et al. 2004). However, the impact of probiotics on nutritional outcomes including protein metabolism and growth is not yet known.

We have proposed to investigate the effects macronutrient restriction (MR) with and without probiotic supplementation in the piglet model of DS-induced colitis to model the clinical scenario of both decreased food intake and continued inflammatory stress. This is a more in-depth continuation of our previous work (Mackenzie et al. 2003) to more completely understand the mechanisms responsible for wasting and growth failure in IBD, and the impact of probiotics on protein metabolism and growth.

4. HYPOTHESIS AND OBJECTIVES

4. 1 Hypothesis

Macronutrient restriction (MR) will decrease the availability of amino acids to meet the demands of acute phase response to DS-induced colitis. MR will decrease the fractional synthesis rate of proteins in tissues and plasma, lower protein concentrations, and compromise weight gain and growth. Probiotic supplementation in the MR state will decrease the metabolic demands for acute phase protein synthesis resulting in a decrease in the fractional synthesis rate of proteins appearing in plasma, and a re-prioritization of tissue protein synthesis and improve growth.

4.2 Overall Objectives

The objective of our study is to examine the effects of nutrition and probiotic supplementation in the macronutrient restricted state on protein metabolism and growth after 11 days of DS-induced colitis. More specifically, to determine if probiotics can effectively decrease nutritional complications of inflammation, such as muscle wasting, growth failure and hypoalbuminemia.

4.3 Specific Aims

To determine if probiotic supplementation with VSL#3 ® can mitigate the effects of macronutrient restriction during GI inflammation on:

a) Weight gain, chest circumference gain and linear growth
b) Concentrations of total protein in plasma, as well as positive and negative acute phase proteins, albumin and fibrinogen
c) Fractional and absolute synthesis rate of total plasma in protein, albumin and fibrinogen
d) Fractional synthesis rates of protein in tissues within (small intestinal mucosa, distal colon), as well as outside of, the GI tract (liver, longissimus dorsi, masseter).

Probiotics stimulate hepatic and plasma protein synthesis despite macronutrient restriction in a piglet model of ulcerative colitis

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To be submitted to Journal of Nutrition

5. MANUSCRIPT

5.1 ABSTRACT

Background: Muscle wasting, growth failure and hypoalbuminemia are well known complications of inflammatory bowel disease (IBD). Probiotics have been shown to maintain remission and decrease disease severity in individuals with ulcerative colitis. The impact of probiotic supplementation on nutritional outcomes including protein metabolism and growth are not yet known. **Objective**: To examine the effects of macronutrient restriction with (MR+PRO) and without (MR) probiotic supplementation with VSL#3® on protein metabolism and growth in piglets after 11 days of dextran sulphate (DS) -induced colitis **Design**: Piglets (n = 24) with DS-induced colitis were randomized to well-nourished (WN), MR or a MR+PRO diet. A 6-hour stable isotope L-[ring-$^{2}H_5$]phenylalanine infusion was conducted to determine the fractional synthesis rates (FSR) of liver, mucosa, colon, longissimus dorsi (LD), and masseter, as well as plasma proteins. **Results**: MR and MR+PRO decreased weight and chest circumference gain, but not linear growth. MR had no impact on mucosa, but decreased the FSR of proteins in colon ($P < 0.019$), masseter ($P < 0.001$), LD ($P < 0.042$), liver ($P < 0.028$) and plasma albumin ($P < 0.001$). MR+PRO had a stimulatory effect on the FSR of proteins in liver ($P < 0.036$) and increased total protein ($P < 0.010$), albumin ($P < 0.016$) and fibrinogen ($P < 0.038$) synthesis. MR+PRO resulted in a smaller decrease in total protein ($P < 0.039$) and albumin ($P < 0.037$) concentrations over time. **Conclusion:** MR+PRO increased the acute phase protein response to DS-induced colitis. The mechanism by which probiotics stimulate the liver is not clear, but may be a result of bacterial translocation and/or immune signaling. These findings merit further investigation to determine if probiotics may also increase acute phase protein synthesis in malnourished individuals with IBD.

5.2 INTRODUCTION

Malnutrition is a frequent complication of chronic gastrointestinal inflammation, as seen in both Crohn's disease and ulcerative colitis (UC) (Bristian 1999). Clinically, the diagnosis of inflammatory bowel disease (IBD) is often preceded by indicators of malnutrition such as growth failure (Kanof et al. 1988), loss of muscle protein (Christie and Hill 1990), and hypoalbuminemia (Bristian 1999). These changes may be attributed to increased metabolic demands for protein synthesis, manifested by accelerated rates of whole body protein turnover (Powell-Tuck et al. 1984), due to gastrointestinal inflammation and stimulation of the acute phase response (Bristian 1999). The metabolic demands of carrying out a sustained acute phase response are often compounded by reduced food intake, largely attributed to food avoidance (Rigaud et al. 1994) and/or cytokine-induced anorexia (O'Keefe and Rosser 1994). Due to the complex nature of IBD and the significant link between nutrition and immunity, the independent effects of malnutrition and acute inflammation on protein metabolism are often difficult to differentiate clinically.

Our group has established a piglet model of dextran sulphate (DS)-induced colitis to examine the effects of nutrition and acute GI inflammation on protein metabolism (Mackenzie et al. 2003). Previously, Mackenzie and colleagues (2003) showed that piglets mount an acute phase response to DS by increasing albumin synthesis, however, in the protein-energy malnourished (MR) state albumin synthesis is increased at the expense of muscle protein and growth (Mackenzie et al. 2003). Clearly, the metabolic demands of both the acute phase protein response and growth during GI inflammation cannot be met in the malnourished state. However, malnutrition is often difficult to ameliorate in individuals with IBD, therefore it is important to investigate strategies which may decrease disease severity and improve protein status, despite poor nutritional intake.

The role of nutrition and the possibility that components, particularly bacteria, within the normal diet may play a role in improving overall immunity is not a new idea (Lilly and Stillwell 1965). Probiotic bacteria have been found to exert favorable effects on host microbial equilibrium, proving particularly beneficial to individuals with GI inflammatory diseases (Marteau and Shanahan 2003). The probiotic mixture, VSL#3® (VSL Pharmaceuticals, United States) is a commercially available supplement which contains 8 strains of probiotic bacteria and has become the center of much scrutiny and investigation. Clinical trials have shown supplementation with VSL#3® to effectively decrease disease severity and the incidence of recurrent pouchitis in patients with UC (Gionchetti et al. 2003). More recently, VSL#3® has been shown to maintain antibiotic-induced remission for greater than 1 year in 85% of patients with chronic pouchitis, compared to 6% among those receiving a placebo (Mimura et al. 2004). VSL#3® has also proven beneficial to individuals with mild- to moderately-active UC (Bibiloni et al. 2005). After just 6 weeks of probiotic supplementation, 77% of individuals with UC showed signs of remission and/or response to treatment, confirmed by endoscopic assessment (Bibiloni et al. 2005).

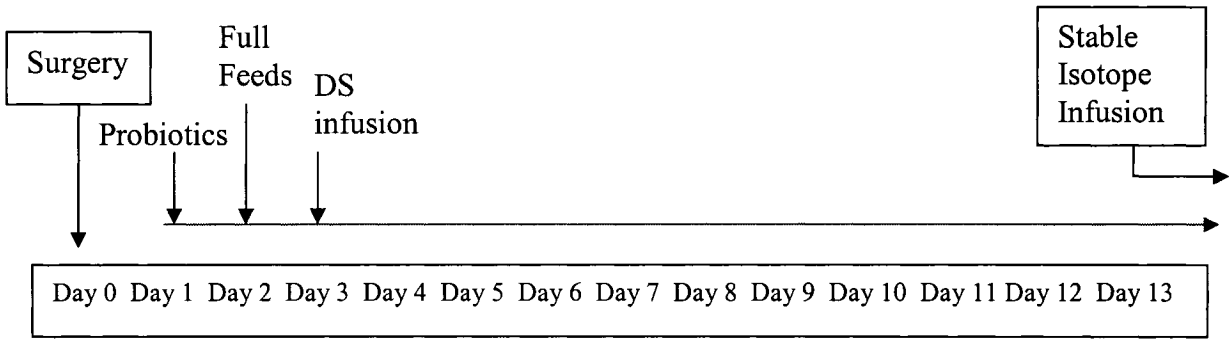
Probiotic supplementation has also proven beneficial in animal models of GI inflammation. In IL-10 gene deficient mice, VSL#3® decreased mucosal secretion of pro-inflammatory mediators TNF-α and IFN-γ, improved epithelial barrier function and histological appearance of the colon (Madsen et al. 2001a). In iodoacetamide-induced colitis, rats supplemented with either VSL#3® or *Lactobacillus* strain GG showed decreased intestinal lesion area, colonic wet weight, prostaglandin E2 synthesis, nitric oxide generation (both linked to the pathogenesis of colonic inflammation), and decreased myeloperoxidase activity compared to rats not receiving probiotics (Shibolet et al. 2002). Interestingly, both viable and non-viable probiotic DNA proved beneficial in decreasing severity of DS-induced colitis in Balb/c mice, myeloperoxidase (MPO) activity and histological injury scores, suggesting that neither colonization nor substances produced by probiotics were the primary mechanism responsible for their anti-inflammatory effects (Rachmilewitz et al. 2004).

While the mechanism remains unclear, these studies support a potential therapeutic role of probiotic bacteria in IBD. However, the impact of probiotic supplementation on nutrition parameters, including protein metabolism and growth has not yet been investigated. Our goal was to examine the effect of macronutrient restriction with and without VSL#3® supplementation on protein metabolism and growth in piglets after 11 days of DS-induced colitis. We adapted our piglet model to allow for a more precise control of macro and micronutrient intake and extended the duration of DS exposure to model the clinical scenario of decreased food intake and active GI inflammation. Our first objective was to examine the effect of MR on protein metabolism and growth, and secondly to determine if probiotics supplementation in the MR state could ultimately decrease nutritional complication and influence rates of tissue and plasma protein synthesis.

5.3 MATERIALS AND METHODS

Experimental protocol. Female piglets (n = 24; Landrace X Yorkshire), 5 days of age, between 2.2-3.0 kg were obtained from Macdonald Farm Swine Complex, McGill University. Piglets were randomly assigned to either a well-nourished (WN) or macronutrient restricted (MR) diet. MR piglets underwent a second randomization to receive either a probiotic (MR+PRO) bacteria preparation (VSL#3®) or vehicle only (MR) for a period of 13 days (**Figure 5.1**). Upon removal from the sow, piglets immediately underwent aseptic surgery (Wykes et al. 1993) to implant catheters into the stomach, bladder, jugular and femoral veins under isoflurane anesthesia (MTC Pharmaceuticals, Cambridge, ON) with pre operative intramuscular administration of atropine (atropine sulphate, MTC Pharmaceuticals, Cambridge, ON), Baytril™ (eurofloxacin/eurofloxacine, Bayer Inc., Etobicoke, ON) and Buprenex™ (buprenorphine hydrochloride, Reckitt & Colman Pharmaceuticals, Richmond, VA). Piglets were individually housed in metabolic cages for the duration of the study. Through the stomach catheter, all piglets were fed custom formulated liquid diets and received dextran sulphate (DS) twice daily, to induce colitis. After 13 days of treatment, plasma and tissue protein synthesis were determined during a 6-hour stable isotope infusion. The tracer L-[ring-$^{2}H_{5}$]phenylalanine was infused thought the jugular catheter. Blood was collected at baseline and hourly throughout the infusion, and tissues were sampled immediately following intravenous administration of Euthansol™ (750 mg sodium pentobarbital, Schering Canada, Pointe Claire). The study was approved by the McGill University Animal Care Committee in accordance with the guidelines of the Canadian Council of Animal Care.

Diet. All piglets were weaned to a custom formulated liquid diet (**Table 5.1**). The WN diet was designed to meet all nutrient requirements for normal piglet growth (National Research Council 1998). The MR diet was designed to model the malnutrition and food avoidance behavior frequently observed during relapses of IBD. The MR groups



Anthropometrics

Weight	×	×	×	×	×	×	×	×	×	×	×	×	×	×
Length & Chest	×													×

Blood Sampling

[Albumin]				×						×				×
[Total Protein]				×						×				×
[Fibrinogen]														×
Fecal Occult Blood				×				×	×	×				

Stable Isotope Study

Blood Sampling

Total Plasma Protein	×
Albumin & Fibrinogen	×
Hematocrit	×

Tissue Sampling

L. Dorsi	×
Masseter	×
Distal Colon	×
Liver	×
Mucosa	×

Figure 5.1 Experimental protocol and study timeline

Table 5.1 Composition of Well-Nourished and Macronutrient Restricted Diets

Macronutrient Composition [1]	**Well-Nourished**	**Macronutrient Restricted**
Energy (ME) (kJ/L)	3088	1544
Carbohydrate (g/L)	103	51
Protein (g/L)	46	23
Lipid (g/L)	16	8
Ingredients (g/L)		
Egg Albumin[a]	42	21
Avonlac Whey [b]	35	17.5
Maltodextrin (DE10) [a]	88.2	44.1
Soybean Oil [a]	11.4	5.7
Coconut Oil [a]	2.9	1.45
Flax Seed Oil [c]	0.4	0.2
Vitamin Mix*	0.01	0.01
Mineral Mix**	5	5
Total (g/L)	185	95

[1] NRC (1998), [a] Harlan Teklad, Madison, WI; [b] Glanbia Nutritionals Inc., Monroe, WI; [c] MP Biomedical, Irvine, CA; *Vitamin Mix (amount/kg dry diet): Vitamin A Palmitate (500,000 IU/g) - 5.9mg; Vitamin D3 (cholecalciferol) - 0.007mg; Vitamin E Acetate (335 ug/g) - 0.0435mg; Menadione Sodium Bisulfite (62.5% Menadione) - 0.93mg; Biotin - 0.35mg; Choline Chloride - 0.21g; Cyancocobalamin, 0.1% - 19mg Folic Acid - 0.16mg; Nicotinic Acid - 20.5mg; Pantothenic Acid - 0.6mg; Thiamin - 0.62mg; Pyridoxine-HCl - 1.75mg. **Mineral Mix (amount/kg dry diet): Calcium Phosphate, Dibasic (29.5% Ca, 22.8% P) - 24.2g; Sodium Chloride - 0.5g; Cupric Carbonate (57.5% Cu) - 11.7mg; Ferrous Citrate (22.8% Fe) - 0.185g; Manganous Sulfate (36.4% Mn) - 11.3mg; Sodium Selenite (45.7% Se) - 0.9mg; Zinc Sulphate (22.4% Zn) - 0.068g.

received a 50% energy and protein restricted diet based on normal growth requirements (National Research Council 1998). Both WN and MR diets provided >120% of all vitamin and mineral requirements (National Research Council 1998) and were prepared by suspending 185 and 95 g of dry ingredients per liter of water, respectively. Piglets received an equal fluid volume (300 mL · kg^{-1} per day) to eliminate any potential differences in weight gain or plasma volume, due to hydration. The diets were composed of spray dried egg white and low-lactose whey protein concentrate with non-protein energy from maltodextrin (DE10) and three lipid sources, soybean, coconut and flaxseed oil (providing a n6:n3 ratio of 8.2:1). To overcome binding/inactivity of biotin, due to avidin in the egg albumin, biotin was added to supply 10 times NRC requirement (0.003 mg · kg^{-1} per day). Prepared onsite in our laboratory (modified from (Weiler and Fitzpatrick-Wong 2002) the diet was delivered continuously over a 16 hour period through the gastric catheter (COMPAT™ enteral delivery system; Novartis Nutrition). WN and macronutrient restricted piglets (MR and MR+PRO) received target metabolizable energy intakes of 221 kcal · kg^{-1} per day (14 g protein/kg, 31g carbohydrate/kg, 4.7 g lipids/kg) and 110 kcal · kg^{-1} per day (7 g protein/kg, 15 g carbohydrate/kg, 2.4 g lipids/kg), respectively. Intake was adjusted daily according to weight gain. Energy intake and body weight was recorded daily.

Dextran sulfate. Previously, our group has successfully adapted the DS induced rodent model of colitis (Cooper et al 1993 and Murthy et al 1993) to the piglet (Mackenzie et al. 2003). In our current study we have decreased the DS dose from 2 to 1 mg · kg^{-1} per day delivered by bolus rather than continuous infusion. These changes allowed us to extend the duration of DS exposure from 7 to 11 days, creating a slightly more chronic response to colitis. DS (200 g/L, 40,000 MW, ICN Biomedical Inc, Aurora, OH) was administered twice daily as a bolus dose through the stomach catheter, adjusted daily according to weight gain. DS administration began on day three and continued for the duration of the study, a total of 11 days. To confirm the presence of colitis, fecal occult blood was tested on day 8 with Hemoccult® strips (Beckman Coulter,

Montreal, QC). Observational changes in fecal smell, consistency and color were also noted to accompany and confirm the presence of colitis.

Probiotic supplementation. Macronutrient restricted piglets in the probiotics group (MR+PRO) 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 the piglet's usual feed. 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 weight$^{0.73}$ to scale between metabolic body sizes (Madsen et al. 1999; Venturi et al. 1999; Gionchetti et al. 2000; Shibolet et al. 2002). The bacterial suspension was divided equally and delivered twice daily as a 15 mL bolus, infused directly into the gastric catheter. WN and MR piglets received the vehicle only.

Growth and weight gain. Snout to rump length and chest circumference were measured at the beginning of the study, preoperatively while under anaesthesia and at the conclusion of the study immediately after death. Weight was recorded daily and both feed intake and DS dose were adjusted accordingly.

Stable isotope infusion. On day 13 a stable isotope infusion was conducted in the fed state to determine protein synthesis of tissues and plasma, in response to DS-induced colitis. The tracer L-[ring-$^2H_5$]phenylalanine (Cambridge Isotope Laboratories, Cambridge MA, 99% enriched) was dissolved in 0.9% sterile saline to create a tracer dilution of 35 μmol/L. The tracer solution was filtered through a 0.22 μm filter and infused at 35 μmol · kg^{-1} per hour into the jugular vein following a priming dose of 52.5 μmol/kg. Femoral blood samples (3 mL) were taken at baseline and hourly thereafter in prechilled vacutainers containing Na_2EDTA, sodium azide, merthiolate and soybean trypsin inhibitor to prevent breakdown of plasma proteins (Jahoor et al. 1999). Blood samples were immediately centrifuged at 2500 x g for 15 minutes at 4°C.

Plasma was removed and frozen in liquid nitrogen and then held at -80°C. Hematocrit levels were determined at hour 1 and 5, using whole blood collected in heprinized capillary tubes. Immediately post mortem, samples from the masseter, longissimus dorsi, small intestinal mucosa, distal colon and liver were collected and frozen immediately in liquid nitrogen, then stored at -80°C.

Sample Analysis

Plasma concentrations of total protein and albumin were determined using a Biuret method (modified Lowry protein assay kit, Pierce, Rockland, IL) and by ELISA (Bethyl Laboratories Inc, Montgomery, TX), respectively, using an Ultra Microplate Reader (El_x808_{IU}, Bio-TEK Instruments, Inc. Winooski, VT). Plasma fibrinogen concentration was determined using the clotting method of Clauss (Clauss 1957) measured on a STA® analyzer.

To determine rates of synthesis, total proteins in plasma were precipitated from 100 μL plasma with an equal volume of 0.6 mol/L trichloroacetic acid (TCA). The pellet was washed twice, then suspended in 4 mol/L HCL, capped under nitrogen and heated to 110 °C for 18 hours.

Fibrinogen and albumin were isolated from 100 μL of plasma. Fibrinogen was precipitated with the addition of ethanol then purified under non-reducing conditions on an 8% native sodium dodecyl sulfate polyacrylamide gel (PAGE) on a MINI PROTEAN II System (Bio Rad Laboratories, Hercules, CA). Proteins were precipitated from 100 μL of fibrinogen-free plasma with TCA. Albumin was re-solubilized in ethanol and purified using electrophoresis with a 10% PAGE. Both albumin and fibrinogen bands were excised from the gels, hydrolyzed in 4 mol/L HCL, capped under nitrogen and heated to 110°C for 18 hours.

Frozen tissues (100-200 mg) were homogenized in ice cold TCA (0.6 mol/L) and centrifuged at 3000 x g for 20 minutes. Free amino acids were isolated from the supernatant. The pellet, containing bound tissue proteins, was washed twice with

TCA (0.6 mol/L), then hydrolyzed in 4 mol/L HCl, capped under nitrogen and heated for 18 hours at 110°C.

Amino acids from tissues and plasma proteins were isolated by passing hydrolyzed samples through a cation exchange column (Dowex-50-X8, Bio Rad Laboratories), then drying under vacuum (Speed Vac® Plus AR, SC110AR, Savant). Phenylalanine was esterified and derivatized to its n-propyl ester heptoflurobutyramide derivative, using n-propanol and acetyl chloride, followed by hepatofluorobutyric anhydride (Mackenzie et al. 2003).

Phenylalanine enrichment was analyzed by Gas Chromatography / Mass Spectrometry (Hewlett-Packard 5988A Gas Chromatograph / Mass Spectrometer, Palo Alto, CA). Under methane negative chemical ionization conditions, $[M-FH]^-$ ions were monitored at mass to charge ratio of 383 and 388, unlabelled and labeled ions respectively. Using raw ion abundances and analysis of the tracer and natural abundance of phenylalanine, tracer:tracee ratios were determined (Mackenzie et al. 2003).

Calculations

The fractional synthesis rate (FSR) of mixed proteins in each tissue was calculated as:

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

Where ΔE_{bound} is the net tracer:tracee ratio of protein-bound phenylalanine above baseline in each tissue at the end of the infusion time; and E_{free} is the tracer:tracee ratio of tissue free phenylalanine at isotopic steady state (Wykes et al. 1996; Ljungqvist et al. 1997; Reeds and Davis 1999; Mackenzie et al. 2003).

The FSR for plasma proteins (total proteins in plasma, albumin, and fibrinogen) was calculated as:

$$\text{FSR (\%/day)} = \frac{(Et_2 - Et_1) \times 24 \text{ (h/day)} \times 100\%}{E_{\text{free}} \times (t_2 - t_1)}$$

Where Et_2-Et_1 is the increase in tracer:tracee ratio of phenylalanine incorporated into plasma proteins determined from the slope of the linear regression line over the final 3 hours of the infusion, assuming no isotopic enrichment at time zero. E_{free} is the net tracer:tracee ratio above baseline at steady state of free liver phenylalanine, which represents the free amino acid pool enrichment, from which plasma proteins are synthesized (Jahoor et al. 1999; Mackenzie et al. 2003).

The absolute synthesis rate (ASR) of plasma proteins was calculated as:

$$\text{ASR (mg/(kg·day))} = \frac{\text{FSR (\%/day) x concentration (g/L) x plasma volume (ml/kg)}}{100\% \text{ x } 1000 \text{ mL/L}}$$

Where hematocrit was taken twice during the infusion and averaged to calculate plasma volume. An average of 80 mL/kg body weight was used to approximate blood volume (Ramirez et al. 1963).

Statistics

SPSS software (version 11, SPSS Inc. Chicago, Illinois) was used to analyze all statistical data. Statistical significance was defined as $p < 0.05$. All data was expressed as mean +/- SEM. Differences in means were determined using one-way ANOVA and group means were compared using a post hoc least significant difference (LSD) test. Changes in variables over time (e.g., concentration) were determined with repeated measures ANOVA.

5.4 RESULTS

Nutrient intake. WN piglets received a metabolizable energy intake of 200 ± 9 kcal · kg^{-1} per day, meeting their estimated requirement of 200 kcal · kg^{-1} per day (National Research Council 1998). MR and MR+PRO piglets received approximately 50% of their metabolizable energy requirements, 90 ± 3 kcal · kg^{-1} per day. All groups met or exceeded requirements for all vitamins and minerals (National Research Council 1998) (**Table 5.1**). MR and MR+PRO piglets had a slightly higher fluid intake ($P < 0.035$) than the WN group, 298 ± 5 and 282 ± 5 mL · kg^{-1} per day, respectively. Urinary output was measured over 24 hours on day 12 and there was no difference in volume excreted between groups (160 ± 10 mL· kg^{-1} per day). After 5 days of DS administration, all piglets tested positive for fecal occult blood which was accompanied by loose stools and a distinct change in both fecal color and odor.

Weight gain and growth. Initial body weight, chest circumference, and snout to rump length did not differ among groups. WN piglets gained 37% of their initial body weight, whereas, MR and MR+PRO decreased weight gain by 50% ($P < 0.001$) (**Figure 5.2**). WN piglets gained 12% of their initial chest circumference, while MR and MR+PRO attenuated that growth by 30% ($P < 0.001$). MR and MR+PRO had no impact on linear growth, as all piglets gained 20% of their initial snout to rump length (**Table 5.2**).

Fractional synthesis rates of proteins in tissues. Protein synthesis in small intestinal mucosa was rapid, with ~80% of mucosal proteins newly synthesized each day (**Figure 5.3**). The FSR of hepatic mixed proteins and distal colon in WN piglets were approximately 40% and 35% per day, respectively. Proteins in skeletal muscles, masseter and longissimus dorsi (LD) had the slowest FSR, with approximately 10% of proteins newly synthesized each day.

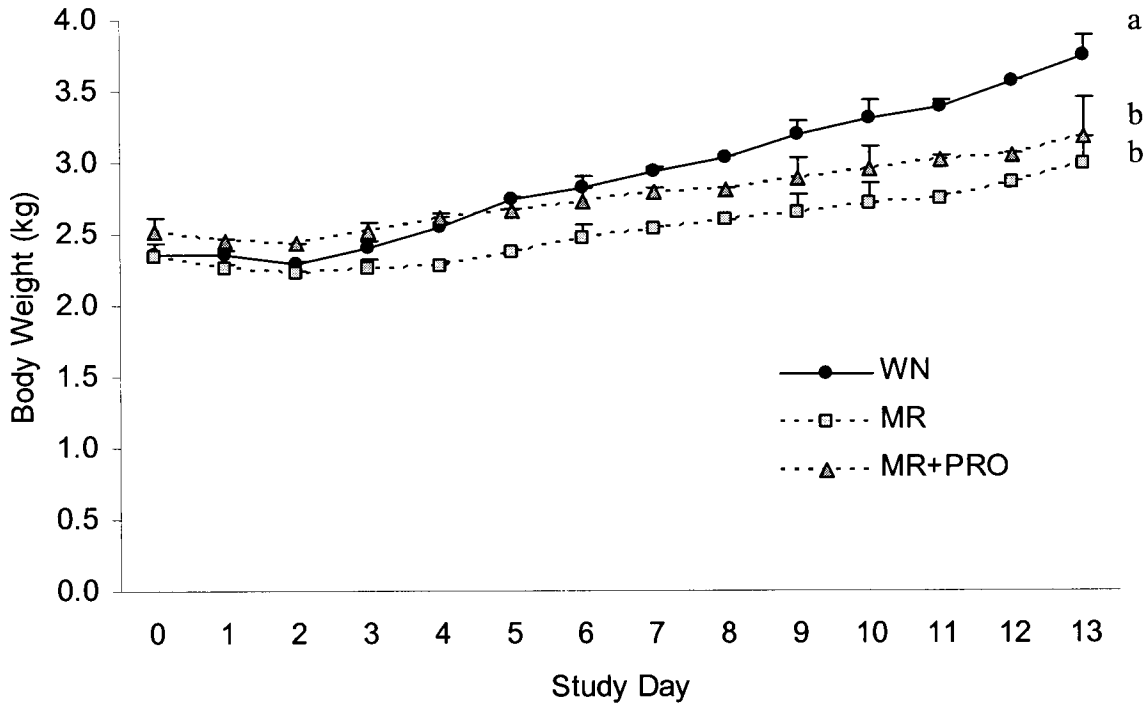


Figure 5.2 Weight gain of piglets fed an adequate (well-nourished diet, WN) or a macronutrient restricted diet with (MR+PRO) and without (MR) probiotics for 13 days. Initial weight for all piglets averaged 2.40 ± 0.06 kg. Values are means ± SEM, n = 8 per group. Means without common superscript differ, $P < 0.05$.

	Chest Circumference (cm)			Length (cm)		
Group	Day 1	Day 13	Total Gain	Day 1	Day 13	Total Gain
WN	27.3 ± 0.4 [a]	30.5 ± 0.4	3.2 ± 0.4 [a]	41.7 ± 0.6 [a]	47.8 ± 2.8 [a]	8.9 ± 0.9 [a]
MR	26.9 ± 0.4 [a]	27.9 ± 0.4	1.0 ± 0.3 [b]	40.6 ± 0.9 [a]	48.6 ± 1.1 [a]	8.0 ± 0.4 [a]
MR+PRO	27.9 ± 0.6 [a]	28.7 ± 0.5	1.0 ± 0.3 [b]	41.8 ± 0.8 [a]	49.8 ± 1.3 [a]	8.0 ± 0.9 [a]

Table 5.2 Initial (day 1), final (day 13) and total chest circumference and length gain in piglets with dextran sulphate (DS)- induced colitis consuming an adequate (well-nourished, WN), macronutrient restricted (MR) diet or a macronutrient restricted diet with probiotic supplementation (MR+PRO) for 13 days. Values are means ± SEM, n =8 per group. Means across without common superscript differ, $P < 0.05$

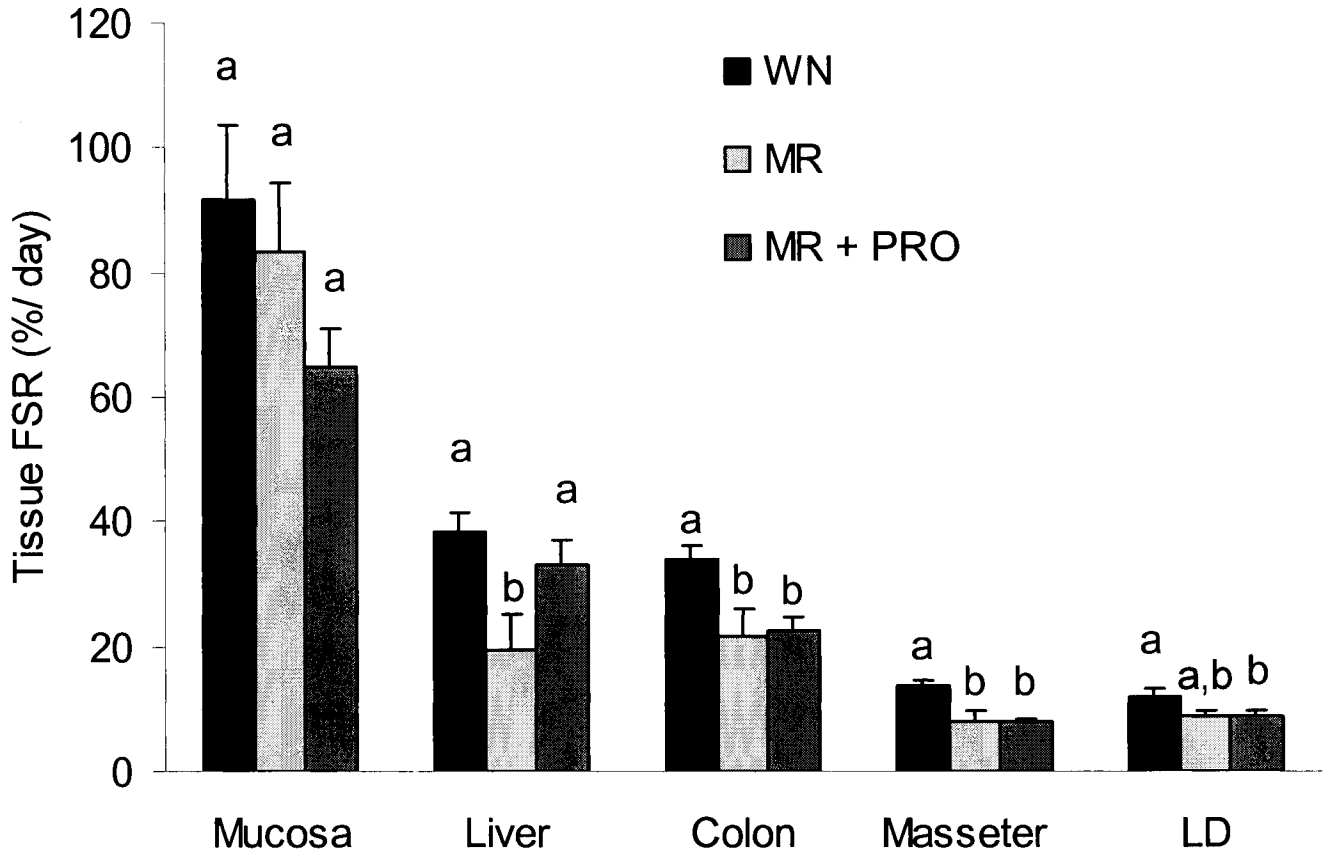


FIGURE 5.3 Fractional synthesis rate of proteins in mucosa, liver, colon, masseter and longissimus dorsi (LD) in piglets with dextran sulphate (DS)-induced colitis consuming either an adequate (well-nourished, WN) or macronutrient restricted (MR) diet with and without probiotics for 13 days. Values are means ± SEM, n = 8. Means without common letter differ, $P < 0.05$.

Impact of MR on tissue protein synthesis. Compared to the WN state, MR had no impact on the FSR of proteins in the small intestinal mucosa, but decreased the FSR of proteins in full thickness of the colon wall by approximately 35% ($P < 0.013$). MR also decreased the FSR of masseter by 40% ($P < 0.001$) and tended to decrease the FSR of proteins in LD ($P < 0.054$). In liver, MR decreased the FSR of proteins by 50% ($P < 0.028$).

Impact of MR on plasma proteins. The rate of increase in isotopic enrichment of the total plasma protein pool was linear throughout the duration of the infusion (**Figure 5.4**). MR had no impact on the fractional and absolute synthesis rate (ASR) of total protein in plasma (**Figure 5.5**). In contrast, MR tended to decrease the FSR of fibrinogen ($P < 0.059$) but not the ASR (**Figure 5.6**). Initial total protein in plasma concentration did not differ among groups and MR had no impact on total protein concentration over time (by repeated measures ANOVA), or day 13 fibrinogen concentration. In contrast, MR decreased the FSR ($P < 0.001$) (**Figure 5.7**) and ASR of albumin by 50% ($P < 0.002$), but this had no effect on albumin concentration over time, by repeated measures ANOVA

Impact of MR+PRO on tissue protein synthesis. MR+PRO had no impact on the FSR of protein in the small intestinal mucosa (**Figure 5.3**). The effect of MR+PRO on the FSR of proteins in colon, masseter and LD was not different from MR alone. In contrast, MR+PRO increased the FSR of proteins in liver by approximately 70%. This increase in hepatic protein synthesis affected both constitutive liver proteins and proteins appearing in plasma.

Impact of MR+PRO on plasma proteins. Compared to MR alone, MR+PRO increased the FSR (**Figure 5.5**) of total protein pool by greater than 80% ($P < 0.010$) and more than doubled the ASR of total protein ($P < 0.004$), an increase which was 60% higher ($P < 0.037$) than even WN piglets. As a result, MR+PRO piglets showed a smaller decrease in total protein in plasma concentration than WN piglets over time, by repeated measures ANOVA ($P < 0.039$). MR+PRO also increased the FSR of

fibrinogen ($P < 0.038$) (**Figure 5.6**) and albumin by approximately 70% ($P < 0.016$) (**Figure 5.7**) and increased the ASR of fibrinogen ($P < 0.009$) and albumin ($P < 0.005$) by 100%. This increase in synthesis had no impact on day 13 fibrinogen concentration. In contrast, MR+PRO piglets showed a smaller decrease in albumin concentration than either MR or WN piglets over time, by repeated measures ANOVA ($P < 0.037$).

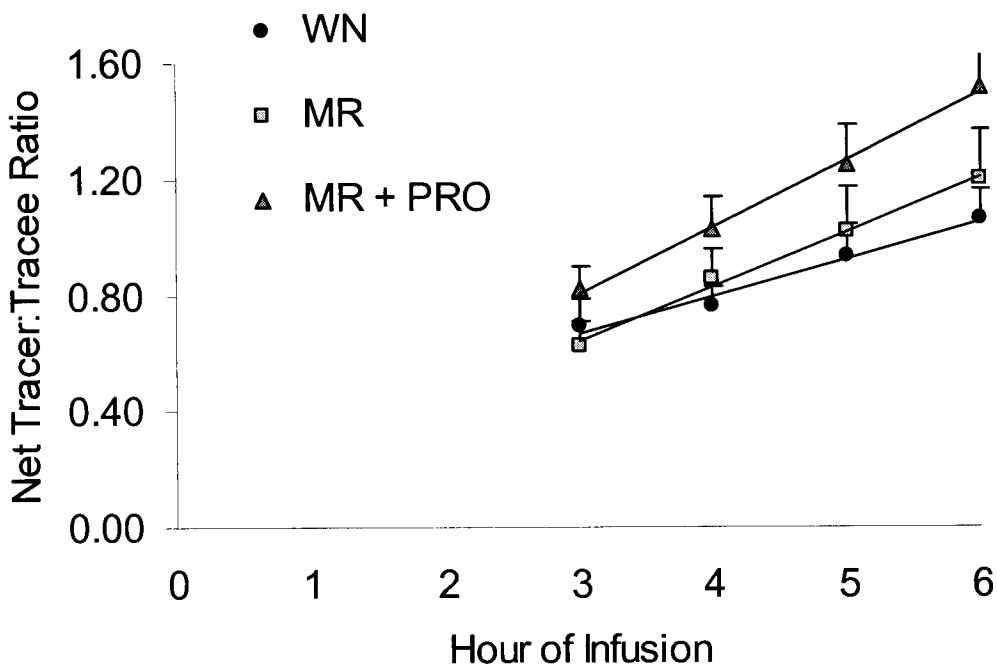
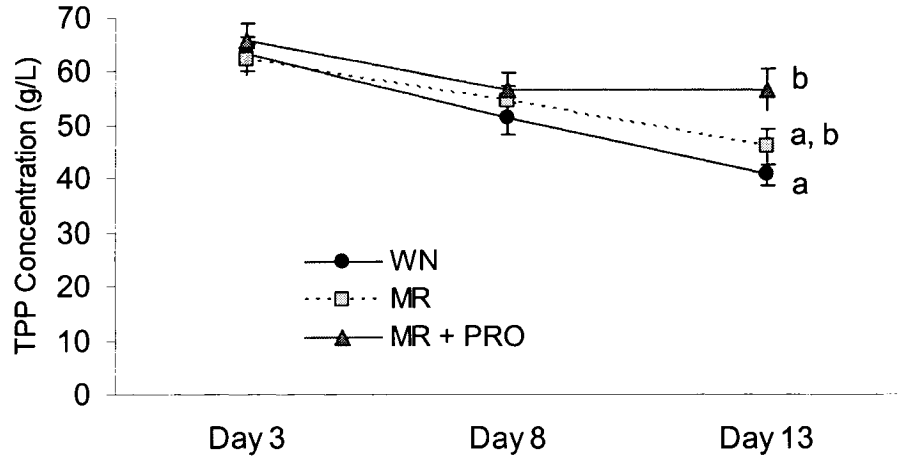
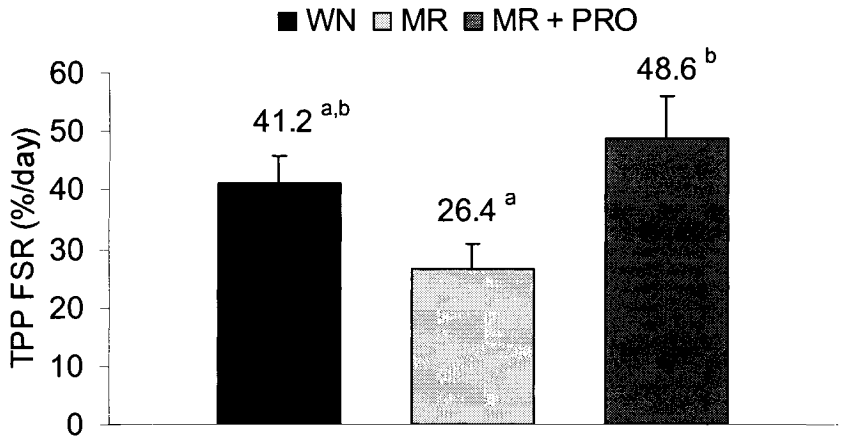


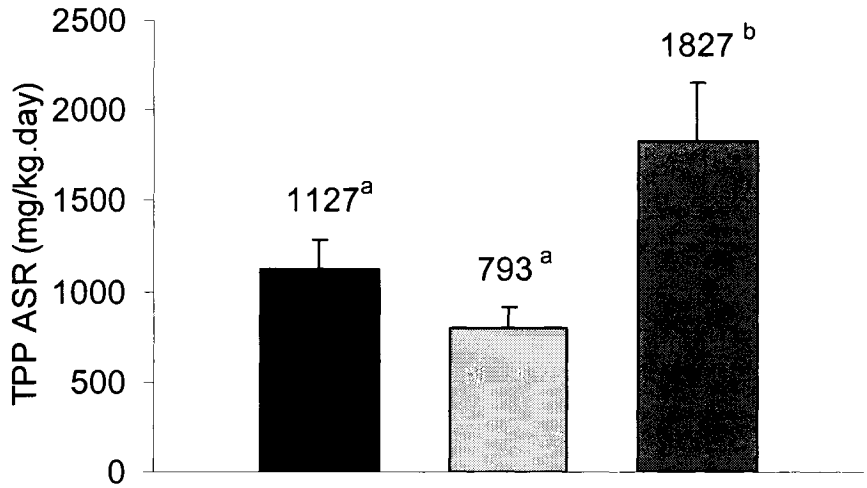
FIGURE 5.4 Isotopic enrichment of total plasma protein pool over time course of infusion in piglets with dextran sulphate (DS)-induced colitis, consuming either an adequate (well-nourished, WN) or macronutrient restricted diet with (MR+PRO) and without (MR) probiotics for 13 days. Values are means ± SEM



a)

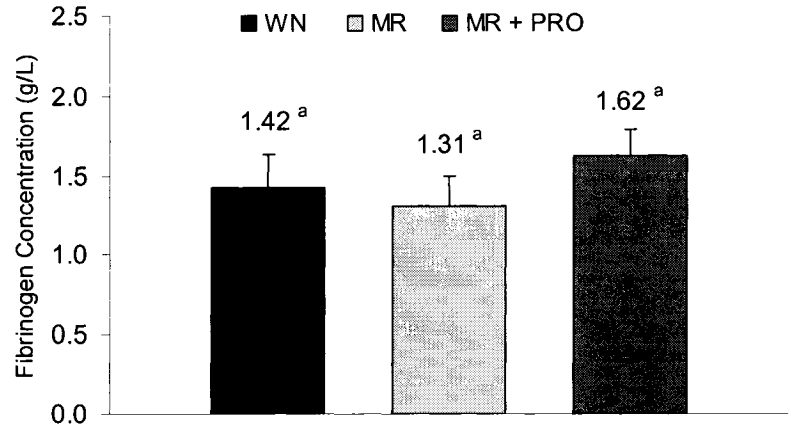


b)

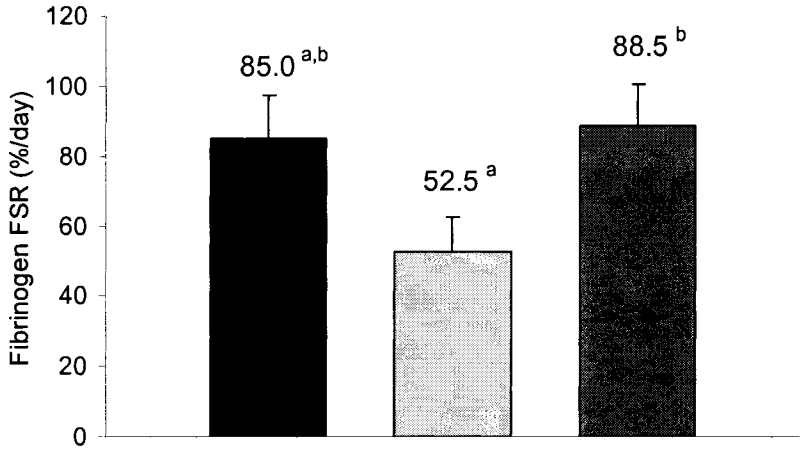


c)

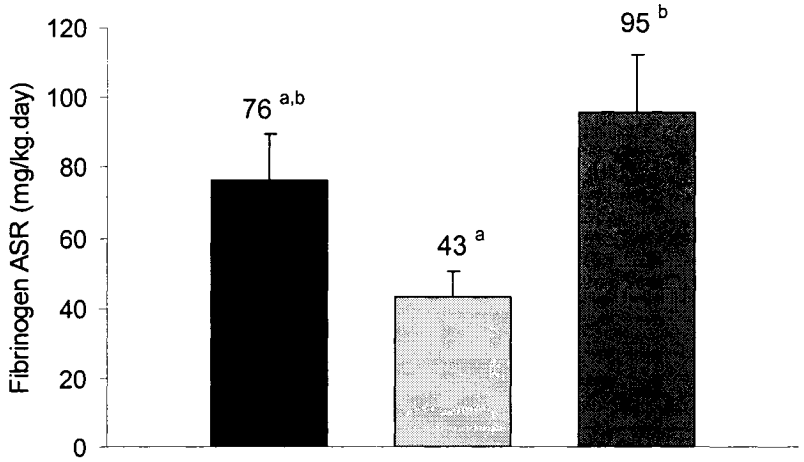
FIGURE 5.5 a) Time course of total plasma protein concentration, in piglets with dextran sulphate (DS)-induced colitis consuming either an adequate (well-nourished, WN) or macronutrient restricted diet with (MR+PRO) and without (MR) probiotics for 13 days. Values are means ± SEM, n = 8. Means without common letter differ by repeated measures ANOVA, $P < 0.05$. b) Fractional synthesis rate (FSR) of total plasma protein pool on day 13. c) Absolute synthesis rate (ASR) of total plasma proteins in piglets on day 13.



a)

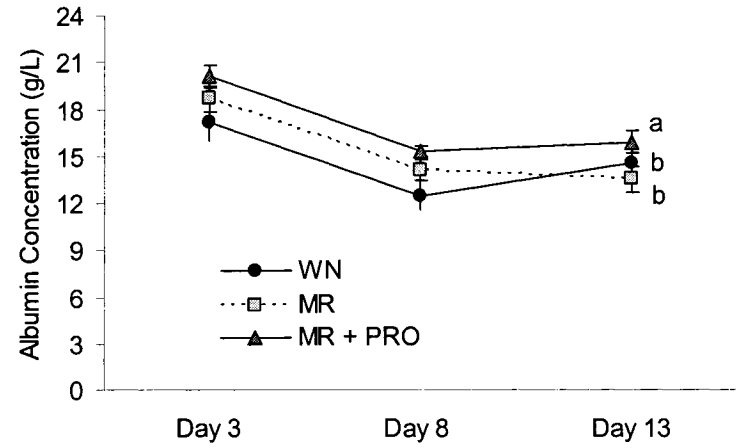


b)

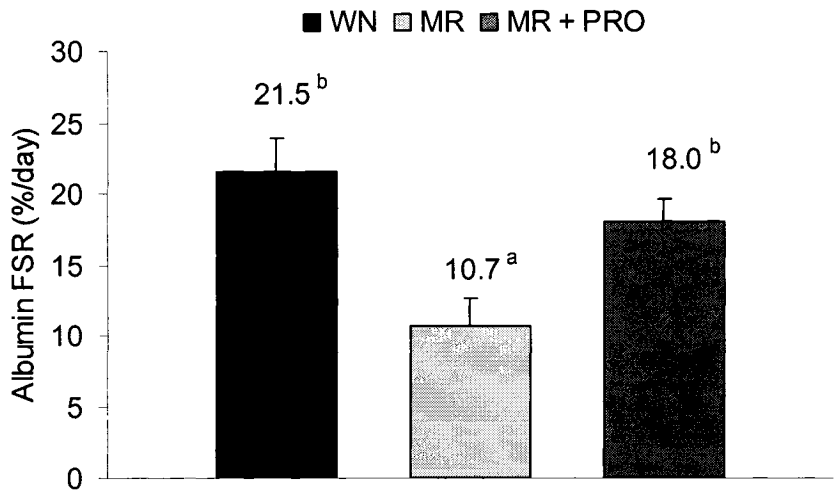


c)

FIGURE 5.6 a) Day 13 fibrinogen concentration, in piglets with dextran sulphate (DS)-induced colitis consuming either an adequate (well-nourished, WN) or macronutrient restricted diet with (MR+PRO) and without (MR) probiotics for 13 days. Values are means ± SEM, n = 8. Means without common letter differ, $P < 0.05$. b) Fractional synthesis rate (FSR) of fibrinogen on day 13. c) Absolute synthesis rate (ASR) of fibrinogen in piglets on day 13.



a)



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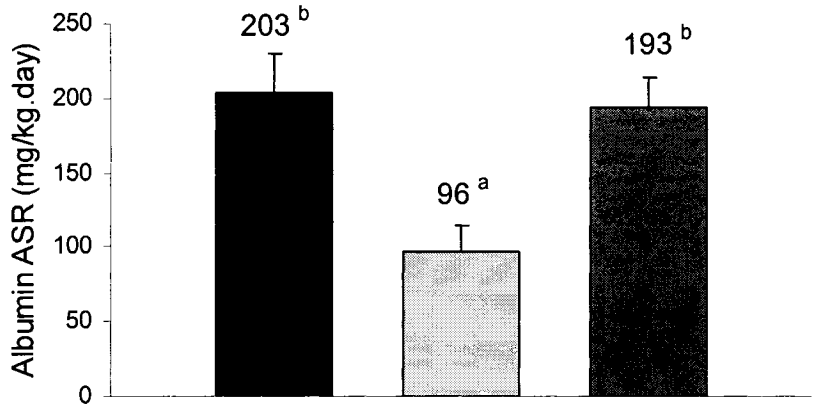

c)

FIGURE 5.7 a) Time course of albumin concentration, in piglets with dextran sulphate (DS)-induced colitis consuming either an adequate (well-nourished, WN) or macronutrient restricted diet with (MR+PRO) and without (MR) probiotics for 13 days. Values are means ± SEM, n = 8. Means without common letter differ by repeated measures ANOVA, $P < 0.05$. b) Fractional synthesis rate (FSR) of albumin on day 13. c) Absolute synthesis rate (ASR) of albumin in piglets on day 13.

5.5 DISCUSSION

The first objective of our study was to examine the impact of MR on protein metabolism and growth in piglets after 11 days of DS-induced colitis, in comparison to WN piglets. Our second objective was to determine if probiotic supplementation in the MR state might affect protein metabolism and growth differently than MR alone. More specifically, we aimed to determine if probiotics could potentially decrease nutritional complications of DS-induced colitis and improve growth. This discussion will begin by describing the consequences of MR and MR+PRO on weight and growth, followed by the impact of MR on protein synthesis, and lastly the effect of probiotic supplementation.

Weight Gain and Growth

A 50% decrease in macronutrient intake, decreased weight gain by 50% compared to WN controls with colitis. Mackenzie and colleagues (2003) showed that a similar restriction of intake after 7 days decreased weight gain in piglets by 60%, and this gain was further compromised by superimposed DS-induced colitis. Rats with chronic DS-induced colitis show a 17% loss of initial body weight in comparison to pair fed controls (Mercier and Breuille 2002). Clearly the increased metabolic demands of colitis compromise weight gain, as adequately-nourished piglets with DS-induced colitis showed an increase in weight gain similar to that of healthy controls (Mackenzie et al. 2003). Probiotics did not increase weight gain in MR piglets with DS-induced colitis. To our knowledge only one other study has measured weight gain in probiotic supplemented animals (Dock et al. 2004). In this study, rats refed a diet containing probiotics after short-term fasting regained 39% of their initial weight while controls consuming an isocaloric diet regained only 21% (Dock et al. 2004). This difference was not significant, but suggests that probiotics may increase feed efficiency (Dock et al. 2004). Compared to WN piglets, MR decreased chest circumference gain, but had no impact on linear growth. Wasting is well-documented in both Crohn's disease and UC, and often precedes diagnosis (Fisher 1999).

Malnutrition is largely to blame for this disease-associated wasting, and is reversible when nutritional demands are met (Howard et al. 1995; Schneeweiss et al. 1998). In contrast, children with IBD often show decreased height velocity (Kanof et al. 1988; Fisher 1999) and below average adult stature (Griffiths et al. 1993). Decreased linear growth or stunting often reflects more chronic deficiencies, particularly micronutrients required for bone mineralization. Linear growth was not compromised by MR after 13 days, most likely due to the duration of MR and adequate provision of micronutrients in the diet. Probiotic supplementation had no impact on either chest circumference gain or linear growth in MR piglets with colitis.

Effect of Macronutrient Restriction on Protein Synthesis

After 11 days of DS-induced colitis, MR decreased the FSR of proteins in all tissues sampled, with the exception of small intestinal mucosa. In general, the mucosa is highly sensitive to changes in nutritional status. Gut atrophy occurs with starvation (Chappell et al. 2003), parenteral feeding (Dudley et al. 1998) and chronic protein deficiency (Wykes et al. 1996). Pigs fed a protein-deficient diet for 8 weeks adapt by decreasing the FSR of most major tissues, with the greatest decrease occurring in tissues with highest rates of turnover, such as jejunal mucosa (Wykes et al. 1996). In contrast, rats with DS-induced colitis show an increase in the FSR of proteins in full wall sections of ileum, but not duodenum or jejunum (Mercier and Breuille 2002) suggesting that inflammatory stress may have a stimulatory effect on the FSR of proteins in some regions of the small intestine. In our piglets with colitis, MR had no impact on the FSR of proteins in mucosa. This is not surprising, given that the gut has first pass to luminal nutrients (Van Der Schoor et al. 2002) and the protein content of our diet. It is therefore reasonable to expect that MR would have a greater impact in tissues without this same first pass advantage.

Not surprisingly, MR decreased the FSR of proteins in colon, liver, and masseter in our piglets with colitis. Given that the colon is the primary site of disease activity, previous studies have shown that protein turnover is increased within this tissue (Mercier and Breuille 2002). This is most likely due to increases in both protein

synthesis and degradation (Mercier and Breuille 2002). Rats with chronic DS-induced colitis (Mercier and Breuille 2002) and HLA-B27 transgenic rats with spontaneous colitis (El Yousfi et al. 2003) show increased colonic protein synthesis, compared to normal controls. In patients with moderately active chronic colitis undergoing elective surgery, Heys and colleagues (1992) used a flooding dose technique to assess protein synthesis of the rectal mucosa. The FSR of proteins isolated from colonic mucosa was increased in individuals with colitis, as well as individuals with benign and malignant tumors, compared to healthy controls (Heys et al. 1992). However, these individuals (with IBD) had normal body mass index (21 ± 1 kg/m^2) and albumin levels (39 ± 1 g/L), suggesting that they may have been adequately-nourished (Heys et al. 1992). Therefore, the findings of Heys and colleagues (1992) may represent rates of colonic protein synthesis in well-nourished individuals, but not those with compromised nutritional status. In our piglets with colitis, MR decreased the FSR of proteins in full thickness of the colon wall. Clearly the demands for protein synthesis within the colon cannot be met in the MR state, as amino acids resources may be used sparingly to sustain other more urgent processes.

MR decreased the FSR of proteins in masseter, but not in LD. Rats with chronic DS-induced colitis showed significant gastrocnemius muscle atrophy which corresponded with a 23% decrease in proteins synthesis (Mercier and Breuille 2002). In contrast, Mackenzie and colleagues (2003) showed that a similar restriction of intake after 7 days had no impact on muscle protein synthesis, whereas superimposed DS-induced colitis decreased muscle protein synthesis compared to adequately-nourished control piglets (Mackenzie et al. 2003). Our findings show that with slightly longer exposure to DS (11 versus 7 days), MR piglets adapt by decreasing the FSR of masseter muscle proteins to a rate which is in fact slower than WN piglets with colitis. Both inflammation and chronic protein deficiency have an inhibitory effect on muscle protein synthesis, although protein-deficient animals exhibiting a more pronounced decrease in muscle protein synthesis in response to inflammation than their well-nourished counterparts (Jahoor et al. 1999). This is not surprising given that malnutrition generally increases nitrogen recycling and concomitant sepsis or trauma

results in a redistribution of amino acid away from peripheral tissues to liver for synthesis of proteins to support defense processes of the acute phase response (Jahoor et al 1999). This adaptation is critical to survival and is evident in various catabolic states including protein energy malnourished children with infection (Schelp et al 1979) as well as surgical patients (Essen et al. 1993). Loss of muscle protein ensues due to an imbalance between synthesis and proteolysis and during infection or injury muscle protein likely becomes a reservoir of amino acids (Reeds et al. 1994). There is evidence to suggest that fast twitch muscle fibers such as masseter which are more metabolically active than slow twitch fibers (e.g., LD) may be more sensitive to changes in nutritional status (Garlick et al. 1975; El Haj et al. 1986; Wykes et al. 1996). This may in part explain why pigs fed a protein-deficient diet for 8 weeks (Wykes et al. 1996) and our MR piglets with colitis both show a decrease in the FSR of proteins in masseter, but not LD.

In individuals with IBD, the FSR of proteins in liver was approximately 43% higher than patients with benign or malignant colorectal tumors (Heys et al. 1992). Similarly, experimental animals with systemic or localized inflammation have increased hepatic protein synthesis. This has been demonstrated in rats with chronic DS-induced colitis (Mercier and Breuille 2002) and after turpentine-induced systemic inflammation (Ballmer et al. 1990). Increased hepatic protein synthesis during inflammation may involve an increase in both structural and liver-secreted proteins, which play a central role in the acute phase response (Heys et al. 1992). Individuals with IBD show increased concentrations of various hepatically synthesized acute phase proteins, including α-1-acid glycoprotein, α-1- antichymotrypsin and orosomucoid (Weeke and Jarnum 1971; Buckell et al. 1979). After 11 days of MR our piglets with colitis decreased the FSR of liver proteins by 50%. Whereas, after 7 days of colitis and a similar restriction in intake, piglets showed no decrease in liver protein synthesis and maintained a rate which was similar to adequately-nourished piglets with or without colitis (Mackenzie et al. 2003). Taken together these findings suggest that after 11 days of MR piglets adapt to nutritional deprivation, by decreasing the FSR of various tissues including liver. Therefore, our findings agree more closely with studies in

protein-deficient pigs, which showed a decrease in the FSR of hepatic secretory proteins after 4 weeks (Jahoor et al. 1996) and a 25% decrease in hepatic proteins after 8 weeks of consuming a low protein diet (Wykes et al. 1996). It is therefore consistent that MR also decreased or tended to decrease the synthesis of acute phase proteins in plasma, even in the presence of colitis.

While MR had no impact on total protein in plasma synthesis, albumin synthesis was decreased and the FSR of fibrinogen tended to be lower. It has previously been demonstrated that even pigs fed a chronic protein-deficient diet can increase acute phase protein synthesis of albumin and fibrinogen in response to acute systemic inflammation (Jahoor et al. 1999). However, plasma concentrations of these proteins remained lower in protein-deficient pigs than in well-nourished controls (Jahoor et al. 1999). In contrast, Mackenzie and colleagues (2003) showed that after 7 days of DS exposure, MR piglets maintained total protein, albumin and fibrinogen synthesis similar to that of WN piglets with colitis. However, fibrinogen concentration was lower in MR piglets, which may imply increased utilization (Mackenzie et al. 2003). Our current findings agree with those of Mackenzie and colleagues (2003), and suggest that extending the duration of MR and DS exposure from 7 to 11 days had no impact on either the FSR or ASR of total plasma proteins or their concentrations. In contrast, MR tended to decrease the FSR of fibrinogen, but had no impact on the ASR or its concentrations. This difference in fibrinogen concentrations between Mackenzie's (2003) findings and our may be attributed to the lower dose of DS (1 g/(kg· day) versus 2 g/(kg· day) used in our protocol. Our piglets with colitis may have experienced a lower grade of inflammation within the colon but over a slightly longer period of time, which may have decreased fibrinogen utilization in clotting and repair, therefore piglets preserved concentration despite a tendency towards decreased synthesis.

MR had its most profound effect on albumin synthesis, and decreased both the FSR and ASR by 50%, although this had no impact on concentrations over time. Inflammation and malnutrition have a profound reducing effect on albumin

concentrations, yet synthesis is critical to survival and must be maintained during infection and/or injury at any cost (Fleck 1989). Other studies in protein-deficient animals have suggested that the FSR of albumin may be decreased immediately in response to decreased protein intake, while catabolic rates adjust more slowly and only after concentrations have substantially decreased (Kirsh et al 1968). In uncomplicated protein malnutrition, rats show a 20% decrease in both serum albumin and total protein concentrations after 14 days of consuming a 2% casein diet, compared to adequately-nourished controls (Ling et al. 2004). Interestingly, these protein-malnourished rats appear to exhibit normal systemic response to inflammation, and increase both cytokine and acute phase protein levels in the first 24 hours after inducing the inflammatory response (Ling et al. 2004). Mackenzie and colleagues (2003) similarly found that after 7 days of DS exposure both, WN and MR piglets doubled the FSR and ASR of albumin. However, our findings in MR piglets agree with those in malnourished children with infection (Doherty et al. 1993) which suggest that malnutrition decreases the acute phase protein response, and with chronicity of infection or injury, these demands cannot be met in the malnourished state (Doherty et al. 1993; Jahoor et al. 1999; Reid et al. 2002). This may therefore explain why MR had no impact on albumin synthesis in piglets after 7 days of colitis (Mackenzie et al. 2003), but decreased synthesis after 11 days. This decrease in albumin synthesis had no impact on concentrations over time, suggesting a concomitant decrease in albumin disappearance, either through decreased catabolism and/or extravascularization. In rats fed a protein-free diet, Jeejeebhoy and colleagues (1973) demonstrated that fibrinogen concentrations were maintained despite a decreased FSR, suggesting that adaptation to inadequate dietary protein may involve a decrease in catabolic rates as well as synthesis.

Clearly, MR adversely affects growth and protein synthesis during active GI inflammation, yet nutritional status is often difficult to ameliorate during relapse of IBD. Malnutrition and infection create a vicious cycle which decreases immune function (Amati et al. 2003; Cooper et al. 2004), and negatively affects muscle protein and growth (Doherty et al. 1993). Therefore, it is important to investigate potential

therapies which may disrupt this cycle either through decreased disease severity and/or improve protein status and immunity. We now shift our focus to the effect of probiotic supplementation in the MR state compared to MR alone.

Effect of Probiotics during Macronutrient Restriction on Protein Synthesis

As compared to MR alone probiotics increased the FSR of proteins in liver, but had no effect on the FSR of proteins in mucosa, colon, LD or masseter. Despite evidence to suggest that the small intestine (SI) may be involved in absorption of probiotic DNA (Rachmilewitz et al. 2004), few studies have focused on the effect of probiotics within the upper GI tract and have reported cellular and immunological changes only. In normal rats, probiotics increased epithelial cell proliferation by 25% and 40% in the jejunum and ileum respectively, and to a greater extent in distal segments of the colon (Ichikawa et al. 1999). Similarly, piglets supplemented with *Bifidobacterium breve, B. animalis,* and *Lactobacillus acidophilus* showed increased crypt cell proliferation as well as immunological changes including increased fibrocytes, fibroblasts, lymphocytes and lymphoid cells in the mucosal lamina propria after probiotic supplementation (Babinska et al. 2005). In *In vitro* studies with HT29 colonic cells, probiotics increased expression of some mucin genes (Otte and Podolsky 2004) although it is not known whether mucin protein synthesis was also increased. While it remains possible that probiotics may alter the expression of various mucosal proteins, the SI mucosa has not been a major site of investigations and in our MR piglets with colitis, probiotics had no impact on the rate at which mucosal proteins were synthesized.

Despite evidence to suggest that the colon may be the primary site of bacterial action, probiotics had no impact on the FSR rate of colonic proteins in MR piglets. Patients with UC show decreased inflammation and regeneration of epithelial tissue within the rectum after one month of *Bifidobacterium longum* (Furrie et al. 2005) and a remission or response rate of 77% of mild or moderately active disease (confirmed by endoscopic assessment) after just 6 weeks of VSL#3® supplementation (Bibiloni et al. 2005). Experimental animals receiving probiotics similarly show morphological and

immunological changes within the colon. In IL-10 deficient mice with established colitis, VSL#3® supplementation decreased histological injury scores and improved gut barrier function; mice also showed decreased secretion of pro-inflammatory cytokine tumor necrosis factor (TNF)-α in both ileum and colon (Madsen et al. 2001a). Similarly Balb/c mice supplemented with VSL#3® prior to the induction of DS-induced colitis and for 7 days thereafter showed decreased histological injury scores and oxidative stress measured by myeloperoxidase activity (Rachmilewitz et al. 2004). Whereas, VSL#3® had no impact on mucus barrier and did not heal chronic DS-induced colitis in Balb/c mice after colitis had already been established (Gaudier et al. 2005). Taken together, these findings may suggest that supplementation protocol rather than choice of experimental model may influence the efficacy of probiotics in decreasing and/or preventing damage within the colon. Furthermore, there is evidence to suggest that probiotics may influence cell proliferation within the colon (Dock et al. 2004) as normal rats refed a diet containing probiotics after short-term starvation had higher colonic mucosal weight and mean DNA content than refed controls (Dock et al. 2004). It is not clear why probiotics had a stimulatory effect on cell proliferation in the colon of individuals with UC (Furrie et al. 2005) and refed rats (Dock et al. 2004) but did not increase protein synthesis in the colon of MR piglets with colitis. The combined stress of malnutrition and repeated localized injury may decrease the potential for tissue repair and this may explain why we saw no difference in colonic protein synthesis. The effect of probiotics within the colon is not fully understood and we do not yet know the impact of probiotics on composition of proteins synthesized within the colon, and what proportion may be involved in damage versus repair.

While measuring changes in gut microflora concentration was not one of our specific aims, traditional measures such as fecal cultures and rectal swabs may not be sensitive enough to represent the true microbial population of the gut (Greenquist et al. 2005). Furthermore, ingestion of live bacteria and repopulation may not be necessary, or the primary mechanism by which probiotics exert their beneficial effects (Rachmilewitz et al. 2004). Rachmilewitz and colleagues (2004) showed that both viable and nonviable probiotic DNA isolated from VSL#3® elicit a systemic immune response through

signaling of toll like receptors, a family of transmembrane proteins involved in recognition of microbial components and stimulating innate immunity. Moreover, both intragastric and subcutaneous administration of bacterial DNA effectively decreased the severity of DS-induced colitis in mice, suggesting that immune signaling rather than recolonization may be the primary mechanism by which probiotics exert their anti-inflammatory effects (Rachmilewitz et al. 2004).

This mechanism may explain why probiotics had a stimulatory effect on the liver and nearly doubled hepatic protein synthesis in our MR piglets with colitis. Our findings agree with those of Rachmilewitz and colleagues (2004) and suggest that probiotics may affect hepatic protein synthesis through immune signaling or alternatively through translocation. Various studies have shown that probiotics may alter cytokine levels (Madsen et al. 2001a; Furrie et al. 2005; Sauter et al. 2005) which, in combination with certain hormones, regulate gene expression of acute phase proteins (Lyoumi et al. 1998). In an *ex vivo* model of duodenal inflammation, tissue samples cultured with a probiotic mixture containing three strains of *Lactobacilli spp.* showed decreased secretion of key pro-inflammatory cytokines and increased levels of regulatory cytokines (IL-10 and TGF-β) secreted by T-lymphocytes, and favorably altering the overall balance of pro-inflammatory versus regulatory cytokines (Sauter et al. 2005). *In vivo*, VSL#3® decreased mucosal secretion of TNF-α and IFN-γ, in IL-10 deficient mice (Madsen et al. 2001a). While one month of supplementation with *Bifidobacterium longum* and a prebiotic substrate promoted re-growth of epithelial tissue and decreased levels of TNF- α and IL-1 in rectal biopsies of patients with UC (Furrie et al. 2005).

Furthermore, hepatic protein synthesis may also be increased due to translocation, as most bacterial DNA has immunstimulatory effects (Rachmilewitz et al. 2004). Translocation is not unlikely given that even healthy animals supplemented with live bacteria (e.g., *Bifidobacterium breve)* show evidence of translocation (Mullie et al. 2004). In wild-type mice and those with DS-induced colitis, plasmid DNA fragments were found in both spleen and liver after intragastric, but not intrarectal administration

of irradiated probiotics (Rachmilewitz et al. 2004). Furthermore, both subcutaneous and intragastric administration of probiotic DNA decreased the severity of DS-induced colitis, suggesting that probiotic DNA is biologically active and that ingested bacteria may be absorbed by the upper GI tract (Rachmilewitz et al. 2004). While the mechanism by which probiotics affect hepatic protein synthesis is not entirely clear probiotics most likely exert their effects systemically through immune signaling.

The effect of probiotics on liver protein synthesis was no limited to constitutive proteins but also increased the synthesis of plasma proteins produced by the liver as well. Probiotics increased the FSR of total protein in plasma and more than doubled the ASR, compared to MR alone. However, this increase in total protein synthesis came at a high nutritional cost. Given the protein content of the MR diet (7 g/kg·day), piglets receiving probiotics used approximately 25% of their daily protein intake to maintain the ASR of total protein in plasma, whereas WN and MR piglets not receiving probiotics used approximately 11 and 8%, respectively. Therefore, piglets receiving probiotics increased total protein synthesis but at a higher nutritional cost than either WN or MR piglets. This increase in synthesis had only a small effect on concentrations, as piglets receiving probiotics showed a smaller decrease in total protein concentrations than WN, but not MR piglets over time.

Piglets receiving probiotics also increased the FSR of fibrinogen and more than doubled the ASR, but had no impact on plasma fibrinogen concentration. This increase in synthesis, yet no change in concentration, may suggest a corresponding increase in fibrinogen disappearance and/or utilization perhaps in tissue repair and clotting within the colon. Increased utilization is the more likely cause given the unlikelihood of extravascularization of this large protein. In other animals with experimental colitis, probiotic supplementation has been associated with outcomes such as decreased disease activity and indicators of oxidative stress (e.g., myeloperoxidase and nitric oxide) (Shibolet et al. 2002; Rachmilewitz et al. 2004), as well as decreased rectal bleeding (Osman et al 2004) and intestinal lesion area (Shibolet et al 2002). However, in MR piglets this corresponding increase in

fibrinogen disappearance may suggest that probiotics did not decrease disease severity.

Gene expression of fibrinogen and other acute phase proteins including α-1-antichymotrypsin and α-1-antitrypsin (Ling et al. 2004) is primarily regulated by IL-6. While this cytokine (IL-6) has both pro and anti-inflammatory functions, levels often correspond with increased C-reactive protein (CRP) concentrations, disease activity (Raddatz et al. 2005), and acuity of allergic reactions (Lin et al. 2001). Furthermore, IL-6 may increase secretion of other regulatory cytokines including IL-10 and IL-1, as well as hormones such as cortisol (Steensberg et al. 2003). In individuals with IBD, IL-6 levels are dramatically increased in inflamed sections of intestinal mucosa (Raddatz et al. 2005). Certain probiotic strains may affect IL-6 levels and potentially the synthesis of specific acute phase proteins. In infants with IgE associated atopic eczema-dermatitis syndrome, treatment with *Lactobacillus rhamnosus* GG (LGG) increased IL-6 levels which stimulated gene activation and hepatic secretion of C-reactive protein (Viljanen et al. 2005). It is therefore plausible that complex interactions between probiotics and immune signaling of IL-6 may also increase fibrinogen synthesis in MR piglets with colitis.

Albumin synthesis was also increased by probiotics. MR piglets receiving probiotics increased the ASR of albumin by more than 100% and these piglets showed a smaller decrease in albumin concentrations than either WN or MR piglets over time. Our findings agree with those of Dock and colleagues (2004) who showed that rats refed a diet containing probiotics after short-term fasting had higher albumin levels than refed controls not receiving probiotics. Dock and colleagues (2004) hypothesized that probiotics may alter cytokine production of T cells and decrease the catabolic response to malnutrition. Cytokines, such as TNF-α have a profound reducing effect on albumin concentration (Henning et al. 1988), although this may not reflect changes in rates of synthesis. Probiotics stimulated an increase in albumin synthesis but this had only a minimal effect on plasma concentrations therefore catabolism and/or extravascular losses must also be increased.

Protein malnutrition and oxidative stress adversely affect intestinal barrier function (Darmon et al. 1993). Altered cytokines levels may play a role in barrier dysfunction, as cytokines are intrinsically linked to the pathogenesis of both diarrhea and inflammation (Schmitz et al. 1999a). *In vitro,* TNF-α increased paracellular permeability and had a powerful dose-dependant reducing effect on resistance of epithelial barrier function in HT-29/B6 colon cells (a subclone of human colorectal cells) (Schmitz et al. 1999a). In individuals with IBD impaired epithelial barrier function may be a result of altered tight junction structure (Schmitz et al. 1999b) and may contribute to hypoalbuminemia. Using ^{131}I labeled albumin, Steinfeld and colleagues (1960) demonstrated that in individuals with IBD albumin synthesis is not sufficient to compensate for the increase in the rate of removal from circulation through gastrointestinal losses (Steinfeld et al. 1960). Both free and albumin-bound ^{131}I were found in the feces of these individuals suggesting that some albumin lost into the gut may have been hydrolyzed to supply amino acids for synthesis of other functional proteins (Steinfeld et al. 1960). This is not unlikely, given the high sulfur amino acid content of albumin (Barker and Putman 1984) and the demand for amino acids namely cysteine necessary for synthesis of proteins such as glutathione, which plays a pivotal role in antioxidant defense mechanisms (Wu et al. 2004). Alternatively, probiotics have been shown to improve human and murine epithelial barrier function (Madsen et al. 2001a) although supplementation did not reinforce mucus barrier in mice with chronic DS-induced colitis (Gaudier et al. 2005). Our findings in MR piglets suggest that while albumin synthesis may be increased, probiotics do not substantially slow albumin catabolism and/or losses, as albumin status was not greatly improved by supplementation.

While both albumin and fibrinogen synthesis were increased by probiotics, these two proteins do not account for or explain the dramatic increase in the total protein pool. The acute phase response stimulates the liver to produce a "mélange" of proteins and concentration measures have shown increases as great as 1000-fold in proteins such C-reactive protein and serum amyloid-A (Boosalis et al. 1989; Doherty et al. 1993).

However, we do not yet know the effect of probiotics on other plasma protein pools such hapatoglobin, α1-antitrypsin, α-1-acid glycoprotein, hemopexin or their effect on hormones such as insulin, thyroxine and cortisol which influence protein synthesis (Mendez et al. 2005).

In summary, piglets adapt to MR by decreasing the FSR of proteins in most major tissues, as well as plasma albumin synthesis. The combined stress of decreased food intake and active GI inflammation lead to decreased weight gain and muscle wasting. Our findings, and those of others highlight the importance of adequate nutrition in mounting an acute phase response to injury and infection (Doherty et al. 1993; Jahoor et al. 1999; Mackenzie et al. 2003; Jahoor et al. 2005). To our knowledge, these findings are the first to demonstrate the effect of probiotic supplementation on nutritional outcomes in a model which reflects the clinical scenario of reduced food intake and GI inflammation in growing animals. We have shown that while probiotics do not reverse wasting, supplementation had a stimulatory effect of hepatic protein synthesis which increased the acute phase protein response to DS-induced colitis. While the mechanism remains unclear it seems that probiotics may exert their effects through a systemic immune response, as a result of translocation and/or toll-like receptor signaling (Rachmilewitz et al. 2004). These findings show that probiotics affect protein metabolism and increase acute phase response to GI inflammation in the MR state without further compromising protein synthesis of other tissues or growth.

6. CONCLUSION

6.1 Summary of Results

After 11 days of DS-induced colitis, macronutrient restriction decreased weight gain, growth, and tissue protein synthesis of liver, colon, masseter and longissimus dorsi, but not small intestinal mucosa. Macronutrient restriction also decreased albumin synthesis and tended to decrease fibrinogen synthesis. Plasma protein concentrations did not accurately reflect changes in rates of synthesis. Contrary to earlier findings in macronutrient restricted piglets after 7 days of DS-induced colitis (Mackenzie et al. 2003) we have now shown that with more chronic exposure to inflammatory stress, piglets do not maintain liver or muscle protein synthesis at the same rate as well-nourished piglets with colitis. Instead, macronutrient restricted piglets adapt by decreasing protein synthesis in most major tissues, as well as plasma proteins. Alternatively, probiotic supplementation increased hepatic protein synthesis to a rate not different from well-nourished piglets with colitis. Compared to MR alone, piglets receiving probiotics doubled total plasma protein, albumin and fibrinogen synthesis without further compromising protein synthesis of other tissues or growth. As a result, piglets receiving probiotics maintained higher total protein concentrations than MR piglets, and higher albumin concentrations than either WN or MR piglets over time. The mechanism by which probiotics affect hepatic protein synthesis is not entirely clear but is likely the result of systemic immune signaling. These findings underscore the critical role of adequate nutrition during GI inflammation and show that probiotics increase the acute phase protein response to inflammation when food intake is decreased.

6.2 Limitations of Current Findings and Future Research

The mechanism by which probiotics stimulate hepatic protein synthesis is not clear. During malnutrition and/or infection and injury, skeletal muscle protein becomes an important reservoir of amino acids to synthesize acute phase proteins (Reeds et al. 1994). This adaptation is critical to survival but may lead to excessive muscle protein breakdown due to the relatively low aromatic amino acid content of muscle and the

high demand for these amino acids to synthesize acute phase proteins (Reeds et al. 1994). Probiotics increased acute phase protein synthesis during macronutrient restriction and DS-induced colitis, without decreasing muscle protein synthesis. However, we do not yet know the effect of probiotics on muscle protein catabolism or proteolytic rates in general. It is therefore important to investigate impact of probiotics on ubiquitin mediated proteolysis and how this might affect changes in muscle protein and growth both in the short and long term.

The effect of probiotic supplementation on molecular regulators of cell growth including components of the mammalian target of rapamycin (mTOR) pathway is not yet known. A more in depth understanding of how probiotics may affect nutrient derived and growth factor signaling which affect the mTOR pathway is merited. Cytokines, such as TNF- α have a profound reducing effect on both plasma proteins concentrations (Henning et al. 1988) and epithelial barrier function (Schmitz et al. 1999a). Probiotics have been shown to improve epithelial barrier function in IL-10 deficient mice (Madsen et al. 2001a), and albumin status in refed rats after short-term starvation (Dock et al. 2004). Therefore, future research should focus on the effect of probiotics on cytokine secretions and/or gut barrier function, and what impact these changes might have on protein status during malnutrition and GI inflammation.

During malnutrition, antioxidant status is compromised (Jahoor et al. 1995). The combined stress of decreased food intake and inflammation within the colon increases oxidative stress, the driving force behind cellular and tissue damage in many disease processes (White et al. 1994). Intracellular glutathione (GSH) produced by hepatocytes is one of the body's most powerful antioxidants (Sido et al. 1998). GSH synthesis is dependent on sufficient dietary protein intake, and concentrations are inherently linked to immune function (White et al. 1994). During protein energy malnutrition GSH synthesis is decreased (Jahoor et al. 1995) and in IBD concentrations of this powerful antioxidant are decreased in intestinal mucosa (Sido et al. 1998). Probiotics have been shown to decrease indicators of oxidative stress including myeloperoxidase and nitric oxide synthase activity in experimental models

of colitis (Shibolet et al. 2002; Rachmilewitz et al. 2004), yet their effect on antioxidant synthesis is not yet known. Future investigations should examine the impact of probiotics on erythrocyte and tissue specific GSH synthesis. More specifically, how probiotics might affect indicators of oxidative stress during malnutrition and colitis, including MPO activity, isoprostane production and histological injury scores.

6.3 Significance of Findings

Our findings highlight the critical role of adequate nutrition in maintaining growth, as well as plasma and tissue protein synthesis during active GI inflammation. To our knowledge this study was the first to examine the impact of probiotic supplementation on protein synthesis and growth in an experimental model which reflects the clinical scenario of reduced food intake during GI inflammation. We have shown that probiotic stimulate hepatic and plasma protein synthesis similar to the well-nourished state. Our findings suggest that probiotics increase the acute phase protein response to GI inflammation during MR, without further compromising muscle protein synthesis and growth. These findings merit further investigation to determine the mechanisms responsible for this increase in hepatic protein synthesis and to determine if probiotics may also increase the acute phase response in individuals with IBD and affect disease severity.

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8. APPENDIX

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: ACC

☐ 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 #:** **Emergency #:**
Name: Scott Harding **Work #:** **Emergency #:**

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
CC		
DB	J	Jan 30 '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: C **Date:** 2/3/04

Approval Signatures:

Chair, Facility Animal Care Committee: **Date:** 17 March 2004

University Veterinarian: **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.

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.**

April 24th, 2006

To Whom it May Concern:

Co-authors, Scott Harding and Linda Wykes of the manuscript entitled: *Probiotics stimulate hepatic and plasma protein synthesis despite macronutrient restriction in a piglet model of ulcerative colitis*, agree that the candidate (Keely Fraser) may include this manuscript as part of her thesis.

The candidate was responsible for performing piglet surgeries, daily animal care, treatment delivery, stable isotope infusions, sample preparation, GC/MS analysis of data, preparing all tables and figures, and writing the manuscript included as part of this thesis. This manuscript was prepared and modified under the co-authors guidance.

Keely Fraser

I, the co-author agree that the candidate, Keely Fraser may include the manuscript entitled: *Probiotics stimulate hepatic and plasma protein synthesis despite macronutrient restriction in a piglet model of ulcerative colitis* as part of her thesis.

Scott Harding

Linda Wykes