NRC Publications Archive Archives des publications du CNRC

Potential of magnetic resonance spectroscopy in assessing the effect of fatty acids on inflammatory bowel disease in animal model

Varma, Sonal; Eskin, Michael; Bird, Ranjana; Dolenko, Brion; Raju, Jayadev; Ijare, Omkar B.; Bezabeh, Tedros

This publication could be one of several versions: author's original, accepted manuscript or the publisher's version. / La version de cette publication peut être l'une des suivantes : la version prépublication de l'auteur, la version acceptée du manuscrit ou la version de l'éditeur.

For the publisher's version, please access the DOI link below. / Pour consulter la version de l'éditeur, utilisez le lien DOI ci-dessous.

Publisher's version / Version de l'éditeur:

<https://doi.org/10.1007/s11745-010-3455-7>

Lipids, 45, 9, pp. 843-854, 2010-09-01

NRC Publications Record / Notice d'Archives des publications de CNRC:

<https://nrc-publications.canada.ca/eng/view/object/?id=728d25a0-a9dc-40e1-b0fe-1da271cafe41>

<https://publications-cnrc.canada.ca/fra/voir/objet/?id=728d25a0-a9dc-40e1-b0fe-1da271cafe41>

Access and use of this website and the material on it are subject to the Terms and Conditions set forth at

<https://nrc-publications.canada.ca/eng/copyright>

READ THESE TERMS AND CONDITIONS CAREFULLY BEFORE USING THIS WEBSITE.

L'accès à ce site Web et l'utilisation de son contenu sont assujettis aux conditions présentées dans le site

<https://publications-cnrc.canada.ca/fra/droits>

LISEZ CES CONDITIONS ATTENTIVEMENT AVANT D'UTILISER CE SITE WEB.

Questions? Contact the NRC Publications Archive team at

PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca. If you wish to email the authors directly, please see the first page of the publication for their contact information.

Vous avez des questions? Nous pouvons vous aider. Pour communiquer directement avec un auteur, consultez la première page de la revue dans laquelle son article a été publié afin de trouver ses coordonnées. Si vous n'arrivez pas à les repérer, communiquez avec nous à PublicationsArchive-ArchivesPublications@nrc-cnrc.gc.ca.

**Potential of magnetic resonance spectroscopy in assessing
the effect of fatty acids on inflammatory bowel disease in
animal model**

Journal:	<i>Lipids</i>
Manuscript ID:	LIPIDS-10-0006.R1
Manuscript Type:	Article
Date Submitted by the Author:	15-Jul-2010
Complete List of Authors:	Varma, Sonal; National Research Council-Inst for Biodiagnostics Eskin, Michael; University of Manitoba, Human Nutritional Sciences Bird, Ranjana; University of Waterloo, Dept of Biology Dolenko, Brion; National Research Council-Inst for Biodiagnostics Raju, Jayadev; University of Waterloo, Dept of Biology Ijare, Omkar; National Research Council-Inst for Biodiagnostics Bezabeh, Tedros; National Research Council-Inst for Biodiagnostics; University of Manitoba, Human Nutritional Sciences
Keywords:	Fatty acid analysis < Analytical Techniques, NMR < Analytical Techniques, Polyunsaturated fatty acids (PUFA) < Specific Lipids, n- 6 fatty acids < Specific Lipids, n-3 Fatty acids < Nutrition, Cancer < Physiology, Inflammation < Physiology

Potential of magnetic resonance spectroscopy in assessing the effect of fatty acids on inflammatory bowel disease in animal model

Sonal Varma^{1,2,#}, Michael Eskin², Ranjana Bird³, Brion Dolenko¹, Jayadev Raju⁴,
Omkar B. Ijare, Tedros Bezabeh^{1,2,*}

¹National Research Council Institute for Biodiagnostics, Winnipeg, Manitoba, Canada,

²Department of Human Nutritional Sciences, University of Manitoba, Winnipeg, Manitoba, Canada,

³Department of Biological Sciences, University of Windsor, Windsor, Ontario, Canada

⁴Department of Biology, University of Waterloo, Waterloo, Ontario, Canada

CORRESPONDING AUTHOR:

*Dr. Tedros Bezabeh
National Research Council Institute for Biodiagnostics
435 Ellice Ave.
Winnipeg, MB
R3B 1Y6 Canada
Email: tedros.bezabeh@nrc-cnrc.gc.ca
Phone: (204) 983-0994
Fax: (204) 984-7036

#Current address: Department of Pathology and Molecular Medicine, Queen's University, Kingston, Ontario, Canada

KEY WORDS

Beef tallow, Corn oil, Flaxseed oil, Inflammatory bowel disease, Proton magnetic resonance spectroscopy, Statistical classification strategy

ABBREVIATIONS

FID, free induction decay; GA_ORs, genetic-algorithm-based optimal region selection; GC, gas chromatography; H & E, haematoxylin and eosin; HFB, High fat beef tallow; HFC, High fat corn oil; HFF, High fat flaxseed oil; HPLC, high performance liquid chromatography; IBD, inflammatory bowel disease; LDA, linear discriminant analysis; LFC, Low fat corn oil; LT, leukotriene; MRS, magnetic resonance spectroscopy; PAF, platelet activating factor; PBS/D₂O, phosphate-buffered saline in deuterium oxide; PUFA, polyunsaturated fatty acids; SCS, statistical classification strategy; TSP, 3-trimethylsilylpropionic acid-d₄ sodium salt

SYMBOLS

Omega – ω , Proton - ¹H

ABSTRACT

People with inflammatory bowel disease (IBD) are at risk for developing colorectal cancer and this risk increases at a rate of 1% per year after 8-10 years of having the disease. Saturated and ω -6 polyunsaturated fatty acids (PUFAs) have been implicated in its causation. Conversely, ω -3 PUFAs may have the potential to confer therapeutic benefit. Since proton magnetic resonance spectroscopy (^1H MRS) combined with pattern recognition methods could be a valuable adjunct to histology, the objective of this study was to analyze the potential of ^1H MRS in assessing the effect of dietary fatty acids on colonic inflammation. Forty male Sprague-Dawley rats were administered one of the following dietary regimens for 2 weeks: low fat corn oil (ω -6), high fat corn oil (ω -6), high fat flaxseed oil (ω -3) or high fat beef-tallow (saturated fatty acids). Half of the animals were fed 2% carrageenan to induce colonic inflammation similar to IBD. ^1H MRS and histology were performed on *ex vivo* colonic samples and the ^1H MR spectra were analyzed using a statistical classification strategy (SCS). The histological and/or MRS studies revealed that different dietary fatty acids modulate colonic inflammation differently, with high fat corn oil being the most inflammatory and high fat flaxseed oil the least inflammatory. ^1H MRS is capable of identifying the biochemical changes in the colonic tissue as a result of inflammation and when combined with SCS, this technique accurately differentiated the inflamed colonic mucosa based on the severity of the inflammation. Our data also suggests that both the type and the amount of fatty acids in the diet are critical in modulating IBD.

INTRODUCTION

Inflammatory bowel disease (IBD) is a very common condition in industrialized countries including in North America. It is estimated that about 1.4 million people in the United States and 2.2 million people in Europe suffer from IBD (1). The exact cause and pathogenesis of IBD is not known, however, interplay of genetic, environmental and immune factors is considered to be involved in its causation. Many epidemiological and experimental studies have pointed out that there is a strong connection between diet and IBD. Diet particularly that is rich in ω -6 fatty acids and saturated fats is one of the major environmental factors implicated in the etiology of IBD (2). Conversely, ω -3 polyunsaturated fatty acids (PUFAs) have been considered to be partially agonistic compared to ω -6 PUFAs and may confer therapeutic benefit in IBD (3, 4). IBD is not a point event, it's a continuum of a disease process with remitting and relapsing course. People with longstanding IBD have a significant risk of developing colorectal cancer and this risk increases at the rate of 1% per year after 8-10 years of the disease (5). As such, early diagnosis and intervention can help prevent the progression of IBD to colon cancer. Currently, the data from experimental or randomized trials on the benefits of pharmacologic or dietary intervention on this window between IBD and colon cancer is very limited.

Presently, colonoscopy with biopsy of grossly inflamed and random parts of the colon is the gold standard for diagnosing IBD. However, it is known that in diseased state, biochemical and physiological changes precede the histological manifestations of the disease and as such, the technique exploiting metabolic abnormalities may be suitable to aid in early diagnosis. One such technique that can provide extensive biochemical information from tissue samples is proton magnetic resonance spectroscopy (^1H MRS) (6, 7). MRS makes use of magnetic properties of

atomic nuclei and provides information about the molecular structures of biochemical/biomolecules. It is non-destructive, quantitative and highly reproducible. It has been utilized in lipid analysis of intact tissues and their aqueous/organic extracts as well as dietary fats due to its unique advantage in differentiating various lipid classes without the need for any chemical modification (8-11). It has been used as an alternative to conventional techniques such as gas chromatography (GC) and/or high performance liquid chromatography (HPLC) for the analysis of lipids from various biological specimens and dietary fats (8,11). It has been shown that the accuracy of MRS-based lipid analysis is comparable to that of GC and/or HPLC (9). Although recent developments in MRS technology, such as high resolution magic angle spinning (HR MAS), have greatly simplified the spectral data obtained from tissues/biopsied samples, the spectra are still very complex and require specialized methods for thorough data analysis (12). The pattern recognition methods have proven to be valuable in the classification of biomedical data. These methods are being used in discriminating data obtained from humans and laboratory animals in pathologic and healthy states and have shown diagnostic value (13,14). In this study, we are using a statistical classification strategy (SCS) – a pattern recognition methodology developed in-house – for the data analysis. SCS methodology has been used for the classification of MRS data in biomedical applications (15,16). The study is based on the hypothesis that various dietary fatty acids modulate colonic inflammation differently and that ^1H MRS combined with SCS methodology has the potential to detect changes in the colonic metabolite profile induced by these dietary fatty acids.

MATERIALS AND METHODS

Forty male 3-4 weeks old Sprague-Dawley rats (Charles River, Canada) were used for this study. The rats were kept on a basic laboratory chow for two weeks before starting the study. For the two week duration of the study, the animals were divided into two major groups with twenty animals in each group. The animals in both groups were further divided into four subgroups (five animals in each subgroup) depending upon the composition of fatty acids in their diet. Low fat corn oil (LFC) subgroup animals received AIN 76A diet containing 5% corn oil, high fat corn oil (HFC) subgroup received 23% corn oil, high fat flaxseed oil (HFF) subgroup animals received 14% corn oil with 9% flaxseed oil and high fat beef tallow (HFB) subgroup received 5% corn oil with 18% beef tallow. The detailed composition of the diet mixture for each subgroup is provided in Table 1. The amount of cellulose (as Cellufil), vitamin mix, mineral mix, and casein in the diet was adjusted to ensure that animals were fed isocaloric diets. In addition, all the animals in group 1 were fed with 2% carrageenan in their diet for the entire 2 week period of the study. Carrageenan is an extract of red seaweed and has been shown to cause colon-specific inflammation. The animals in group 2, however, were not fed with carrageenan. The grouping of animals is schematically depicted in Figure 1. Table 2 lists the detailed percent fatty acid composition of the different diet groups.

All the animal housing, specialized feeding and sample collection procedures were undertaken at the animal facility in the department of Biology, University of Waterloo. At the end of 2 weeks, the animals were sacrificed using CO₂ asphyxiation method. This project was approved by the animal care committee at the Institute for Biodiagnostics, National Research Council of Canada and the University of Waterloo. The colon of each animal was excised after sacrificing the

animal. Mucosal layer of the colon was scraped off using a glass slide by the method previously described by Briere et al. (17). The mucosal samples were coded and stored in cryovials containing phosphate-buffered saline in deuterium oxide (PBS/D₂O) medium at -70 °C. These coded cryovials were shipped on dry ice to the National Research Council of Canada, Winnipeg for MRS and histological assessment.

¹H MRS analysis

One-dimensional (1D) experiments

For ¹H MRS experiments, the samples were thawed and cut into 5-7 mm long pieces along the longitudinal axis of the colon. This yielded 18-20 samples from each animal. ¹H MRS was conducted on alternate samples from each animal. Thus, a total of 119 spectra from group 1, and 127 spectra from group 2 were generated for our study. Each piece from the colon was counted as an independent observation for both MR analysis and histology. This was done as it is known that IBD can be very focal and doesn't involve all the parts of the colon in a consistent manner. This holds true in the clinical setting too as not all of the biopsies taken during a single colonoscopy at a single point of time show IBD-related changes. As we were comparing MR spectra from each piece with the histology of that piece, we found it more appropriate to compare the pieces as separate samples rather than group them together per animal.

The sample preparation technique used for conducting ¹H MRS was similar to the technique described by Kuesel et al. (18). Briefly, the colon sample was placed in a glass capillary tube filled with PBS/D₂O with one end plugged. This capillary was inserted into the NMR tube containing 300 µl of PBS/ D₂O solution along with 5 µl of chemical shift reference, 3-trimethylsilylpropionic acid-*d*₄ sodium salt (TSP). ¹H MRS was conducted on these samples

using a Bruker Avance 360 MHz spectrometer at 25 °C with pre-saturation of the water signal. The acquisition parameters included: 90° pulse at 9.30 µs, number of scans = 256, spectral width = 4990.02 Hz, relaxation delay = 3 s and time domain data points = 8k. The samples were fixed in 10% neutral buffered formalin immediately after ¹H MRS experiments for histological assessment. Since the experimental time was only ~16 mins, and the spectra were recorded without sample spinning, any mechanical damage to the tissue should be precluded.

Two-dimensional (2D) MRS experiments

¹H-¹H correlated spectroscopy (COSY) experiments using gradient pulses were performed on representative colon mucosal samples to unambiguously identify the metabolites whose signals are overlapping in 1D spectra or are present at very low concentration. The acquisition parameters used were: spectral width = 4990.02 Hz in both dimensions; time domain data points = 2048; number of free induction decays (FIDs) with t_1 increments = 256; relaxation delay = 1 s and number of transients = 128. The resulting data were zero filled to 1024 points in the t_1 dimension and Fourier transformed along both dimensions after multiplying the data by sine-bell window function shifted by $\pi/2$.

Gas chromatography

Fatty acid composition of dietary lipids used in this study was analysed by GC following the method described in AOCS- Official methods and protocols (19). In brief, samples of all dietary groups underwent fat extraction/evaporation cycles using a mixture (1:1, v/v) of HPLC grade chloroform and methanol (Fisher Scientific Limited, Nepean, ON, Canada). Total amount of fat extracted from each dietary lipid was weighed before GC analysis. To 50 µl of extracted fat, 1

μl isooctane containing exact amount of internal standard (C17: 1) and 12 ml 2% H₂SO₄ in methanol were added. The resulting mixture was heated with vortex for 2 hrs at 65-70 °C to obtain a monophasic system. After cooling to room temperature, 6 ml isooctane and 6 ml distilled water were added, stirred and allowed to stand. One ml of upper clear layer of the esterified sample was subjected to automated GC analysis. Contribution of individual fatty acids to the dietary fat was calculated with reference to the internal standard.

Histology

Histology was performed on representative animals from both groups 1 and 2, using the same tissue used for the MRS experiment. Each sample was coded to avoid analysis bias, fixed in 10% neutral buffered formalin and finally embedded into paraffin blocks. The paraffin blocks were cut into 5 μm thin sections and collected on coated glass slides. The sections were stained with haematoxylin and eosin (H & E) stain and examined at 60× magnification under a light microscope. Cellularity, crypt architecture and fibrosis were the broad criteria used for grading inflammation in the samples. Predominantly, the density of lymphocytes within the surface epithelium, lamina propria and invasion within the crypt epithelium were considered when assessing increased cellularity. Destruction of crypt architecture by inflammatory cells and formation of crypt abscess were assessed under architecture. Fibrosis denoting chronicity of the inflammatory process was also assessed. When assessing each sample, these criteria were independently assigned mild, moderate or severe grade and then a compiled composite score was given to the sample. The degree of inflammation was graded based on the proportion of total sample inflamed. If 30% or less of the total section was inflamed, it was graded as mild. Sections

having 30-60% inflammation were graded as moderate and those with over 60% inflamed area were graded as having severe inflammation.

Statistical classification strategy

The Statistical Classification Strategy (SCS) (16) is comprised of several stages, and depending on the data, some or all of the stages may be invoked. We employed the following stages:

Preprocessing

All spectra were subjected to normalization (scaling to unit area), smoothing and peak alignment (with respect to the external reference, TSP). This step also involves transformations (e.g., forming derivatives or ranking the spectral intensities). Only the subregion 0.5 – 4.5 ppm (1184 data points) was used for SCS analysis. This region was selected to avoid artefacts due to the residual water signal at 4.7 ppm and also the external reference (TSP) peak at 0 ppm. Finally, the resultant 1184-point spectra were rank-ordered to eliminate baseline differences between the spectra (The actual intensity values were replaced by their ranks to eliminate or reduce the influence of excessively large resonance intensities).

Feature selection/extraction

The feature selection/extraction is a genetic-algorithm-based optimal region selection algorithm (GA_ORS) which involves the reduction of feature space dimension but retains the spectral identity (16). A small number of discriminatory subregions are selected from the spectra, in which adjacent spectral intensities are averaged. In an extension of the feature selection method,

the data are split randomly into a number (N, e.g., N=50) of training – test set pairs, one half of the split is for the training set, the other half the test set. For each split, a set of features are determined using a simple classifier, in this case linear discriminant analysis (LDA). This process produces N feature sets (generally different), and a histogram is constructed of feature selection frequency. The features most frequently selected are the eventual inputs for the final classifier. This stage is essential for reliable classifier development. Subregions identified in this study have been listed in Tables 3 and 4 for groups 1 and 2 (with and without carrageenan treatment).

Classifier generation

In this step, the subregions determined by histogramming form the feature set that is submitted to the ultimate classifier, also an LDA classifier. The classifier's coefficients are obtained by our bootstrap-inspired approach. This involves splitting the data randomly 5-10000 times into training and test sets, determining the individual coefficients for LDA and using these to test the corresponding test sets. A weighted average of the above coefficients is taken, weights determined by the classifier's performances on the test sets.

RESULTS

Figure 2 shows the 2D ^1H - ^1H COSY spectrum of a typical colonic mucosa showing assignments for various metabolites. ^1H MR spectrum of the same sample is shown above the 2D plot. These assignments were also compared with those from recent reports (12, 20). Owing to an extensive broadening of the lipid signals, the chemical shift region 2.70 – 2.90 ppm representing diallylic methylene protons $[-\text{CH}=\text{CH}-(\text{CH}_2-\text{CH}=\text{CH})_n-]$ was not well-resolved. This obviates the identification of individual PUFAs present in the colonic mucosa. Similar observations were

made in recent studies despite the use of advanced NMR techniques such as HR MAS (12, 20). In addition to the lipid components, we have also detected choline-containing phospholipids such as phosphatidylcholine and other small molecules such as creatine, leucine, valine, lysine, alanine, threonine, aspartate, glutamine, glutamate, ethanolamine, uracil, tyrosine, and phenylalanine.

SCS methodology was applied to the spectra obtained from colonic mucosa in three stages of the study. In the first stage, all the specialized dietary subgroups were compared with the LFC fed subgroup. The results of the first stage of SCS analysis from group 1 (treated with 2% carrageenan) and group 2 (not treated with carrageenan) are shown in Table 3. In group 1, the classifier assigned the spectra to their respective groups with high accuracy ranging from about 91 - 100%. The lowest accuracy of 91% was seen for the comparison between LFC and the HFB fed subgroups. In this comparison, 4 out of 32 spectra from LFC subgroup were misclassified and 2 out of 34 spectra from the HFB subgroup were misclassified. The spectral subregions identified by SCS being discriminatory have been listed in Table 3. These regions could be attributed to the resonances due to the protons of the fatty acyl chain, creatine, leucine/isoleucine, valine, and glyceryl protons of phospholipids. Similar observations have been made in group 2 (see Table 3). The classification accuracy was very high, ranging from 96 to 100%. A major difference observed between the two groups was that creatine signals were found to be discriminatory in the classification of LFC and HFB in group 2 whereas glyceryl signals of phospholipids were found to be discriminatory in group 1 (for detailed assignments see Table 3). The first stage of SCS analysis from group 1 and group 2 shows that dietary fat causes enough differences in the metabolite profile of rat colon that it can be discerned by SCS methodology.

The results hold true when there is no extrinsic inflammatory agent and even when there is carrageenan-induced colonic inflammation. These differences could be due to difference in the amount of fatty acids, type of fatty acid or extent of inflammation within these groups.

Therefore, the first stage of SCS analysis was followed by a second stage to assess if ^1H MRS could identify the differences between the effects of various dietary fats containing different fatty acids when keeping the total fat content in each dietary group constant.

In the second stage, the three specialized diet groups were compared against each other. All the animals in these three groups received 23% fat by weight in their diets; however, the type of fatty acid differed. The results of the second stage of SCS analysis of samples from groups 1 and 2 are shown in Table 4. ^1H MRS combined with SCS analysis depicted an accuracy of 98-100% in classifying the spectra to their respective groups. In addition to the regions identified during comparison of each group to the LFC group, resonances due to glutamic acid, total choline and glycerophosphoethanolamine were also ascribed to be discriminatory in this analysis. In the second stage of group 2 analysis, ^1H MRS with SCS attained an accuracy of 97% in differentiating HFC from HFF, 98.4% between HFC and HFB, and 100% between HFB and HFF subgroups.

These results indicate that ^1H MRS can detect subtle differences in the metabolite profile of colon when the animals receive the same amount but different types of fats. The stage 1 analyses showed that MRS combined with SCS can detect differences between the LFC subgroup and HFC, HFF and/or HFB subgroups. The combination of these two results shows that ^1H MRS can detect differences in the colon caused not only by different amounts but also different types of

fatty acids. The metabolites found to be discriminatory in the SCS analysis included creatine, glyceryl signals of glycerophospholipids, glycerophosphoethanolamine, glutamic acid, total cholines and signals from fatty acyl chain, particularly, the allylic/diallylic methylene protons ($\text{-H}_2\text{C-CH=CH-/-CH=HC-CH}_2\text{-CH=CH-}$) including ($\text{-CH}_2\text{-}$) chain, terminal -CH_3 , and $\text{-CH}_2\text{-CH}_2\text{-COO}^-$ groups. It is worthwhile to note that the diallylic methylene protons ($\text{-CH=CH-CH}_2\text{-CH=CH-}$) are the predominant entities responsible for the classification of different dietary PUFAs in both groups 1 and 2. Metabolites resonating in the region 1.1 and 1.22 ppm could be assigned to the amino acids leucine/isoleucine and valine.

Having established that both the amount and type of dietary fat caused enough differences in the fatty acid profile of the rat colon and that ^1H MRS can accurately detect these changes, we proceeded to the third stage of analysis. The goal this stage of analysis was to assess the accuracy of ^1H MRS in detecting changes in the metabolite profiles of the rat colons predominantly due to inflammation. For this, each subgroup in group 1 was compared to the corresponding subgroup in group 2 of our study. As shown in Table 5, the SCS analysis was highly accurate in classifying the spectra from each subgroup in group 1 versus the corresponding subgroup in group 2. SCS identified resonances due to total cholines, creatine, valine, leucine, phosphatidylcholine, and methyl & diallylic methylene protons of fatty acid chain as discriminatory.

Details of the criteria used to grade inflammation have been described in the Methods section. In group 1, sections from 21 out of 23 samples analyzed from the HFF subgroup were either not inflamed or had very mild inflammation. Similarly, out of 21 samples from the LFC subgroup only 1 sample had severe inflammation. On the other hand, all of the samples from HFB (n=20)

and HFC (n=23) subgroups showed moderate to severe inflammation. The representative sections of H&E stained samples from each group are shown in Figure 3. The ^1H MR spectra corresponding to these sections are shown in Figure 4. Due to extensive broadening of the ^1H MRS signals, it was difficult to monitor the modulation in the levels of various dietary fatty acids. However, from Figure 4, it is obvious that creatine and total-cholines are decreased with the severity of the inflammation. In group 2 animals, very mild or no inflammation was observed in all the sections examined (data not shown).

DISCUSSION

The main objective of this work was to study the effect of various dietary lipids on the modulation of the colonic inflammation in an IBD model. We utilized ^1H MRS combined with pattern recognition methods such as SCS to study the above effect. Inflammatory reactions cause biochemical changes and MRS has the potential to detect these changes before histological manifestations (21). In our earlier studies, we have confirmed that ^1H MRS in combination with pattern recognition methods could be a valuable adjunct to histology for the assessment of tissue whose status is intermediate between normal and malignant (22, 23). Hence, ^1H MRS could be used for indirectly assessing the inflammatory processes.

The data was analyzed in three stages to answer three specific objectives of our study, sequentially. The first step was to identify whether ^1H MRS was able to identify differences between low fat diet and different types of high fat diet in inflamed (group 1) and non-inflamed (group 2) colons. Those results showed that MRS was able to categorize the samples in their respective group with high sensitivity. Then the questions arose whether the differences seen

were purely because of difference in the amount of total fat in the diet or whether MRS was sensitive enough to categorize the samples accurately even when same concentration, but different type of fatty acids were introduced in the diets. This led to stage 2 of the analyses where we compared the MR spectra from different high fat diet groups against each other in both inflamed (group 1) and non-inflamed (group 2) colons. The results showed that ^1H MRS with SCS methodology was able to accurately classify the samples to their respective groups again. At this stage, we had confirmed the basic hypothesis of our overall project that different amounts and types of dietary fats affect the colon differently and that ^1H MRS is a sensitive technique to identify these differences. Therefore, we were at a stage where we could proceed to assess whether ^1H MRS could still categorize the samples with confidence when comparing the difference in inflammation between 'normal' and 'IBD-like' states of the colon. This was the reasoning for doing stage 3 of the analyses where we compared each dietary subgroup from the 'carrageenan treated' (group 1) with the corresponding subgroup from the 'non-carrageenan treated' (group 2) separately. Since the dietary composition was the same any differences seen in the MR spectra could be attributed to the difference in inflammation between the two subgroups. As we aim to eventually apply this methodology to assess the effect of different dietary fatty acids on modulation of inflammation in IBD, this was a critical step to confirm that ^1H MRS has the capability to categorize samples based on differences in the degree of inflammation.

The ^1H MRS signals found to be discriminatory in the SCS analysis included those from creatine, glyceryl signals of glycerophospholipids, glycerophosphoethanolamine, glutamine/glutamic acid, total cholines and parts of fatty acyl chain, particularly, the allylic/diallylic methylene protons. The implications of these metabolites are discussed herewith.

Creatine is a metabolite that is found in normal tissues and serves as an energy reservoir. It is converted into phosphocreatine by adding a high energy phosphate bond in the presence of the enzyme creatine kinase. This is a reversible reaction and whenever energy is needed, phosphocreatine is broken down to creatine while releasing the energy as ATP. Significant concentrations of creatine have previously been detected in human colonic mucosa (24). The creatine kinase isoenzyme BB has been isolated from normal (25) and infarcted colonic mucosa (26). In an earlier study, the presence of significant amounts of creatine in normal colon tissue was attributed to a more developed muscular morphology of colon (12). In this study, we observed relatively high levels of creatine (2.88 – 3.10 ppm) in the LFC subgroup, whereas creatine was detected in negligible amounts in animals fed with HFC and HFB. However, the creatine levels were intact in the animals fed with HFF (Figure 4). These observations indicate that muscular morphology was not disturbed in the animals fed with HFF, whereas it was upset in animals fed with HFC/HFB. As colonic inflammation causes cellular damage, demand for the energy would be high, which may result in the depletion of creatine stocks in these cells.

The region around 2.7 - 2.8 ppm denotes diallylic methylene protons from PUFAs (9). PUFAs are critical for maintaining the membrane structure and function of various cells in the body (27). Linoleic acid is an abundant PUFA in the cell membrane and a precursor of arachidonic acid. Arachidonic acid is the precursor in the synthesis of many inflammatory mediators such as the products of lipoxygenase pathway LT B₄ and LT C₄. These inflammatory markers are increased in many disorders in the human body including IBD (28). Nishida et al. observed an increase in arachidonic acid concentration in the colonic mucosa of patients with ulcerative colitis (29). Nieto et al. also observed an increase in linoleic acid concentration in the colonic mucosa of rats

2 weeks after inducing experimental colitis (30). The linoleic acid incorporated into colonic cells may be used to synthesize arachidonic acid and inflammatory mediators. The GI tract is well known to be affected by dietary lipids. Colonocytes have fatty acid synthase including in colorectal cancer (31). A high fat diet does affect eicosanoid production in colonic mucosa. However, we recognize that inflammation is mediated via a complex network of different cell types in the colon and almost every cell type is affected in the body by dietary lipids (32, 33). These changes in colonic mucosal lipid profile are similar in human ulcerative colitis as well as experimental colitis induced in animals. Using a gas chromatography technique, Hawthorne et al. observed that the levels of unsaturated fatty acids, EPA and DHA increased in rectal mucosa after ω -3 PUFA supplementation (34). A similar effect on colonic fatty acid profile was seen by Bartoli et al. in Sprague-Dawley rats after ω -3 supplementation for 12 weeks (35). As mentioned earlier, due to extensive broadening of the ^1H MRS signals, we could not determine which specific PUFA contributed to the peak due to protons in the unsaturated region based solely on these spectra. We deduced that the predominant PUFA in a particular dietary group must have been responsible for the selection of allylic/diallylic methylene protons peak as discriminatory (Tables 3 and 4).

Glutamine is the major fuel for intestinal cells. Significant levels of glutamine were observed both in human colon (36) and rat colonic mucosa (37). Glutamate acts as an excitatory neurotransmitter in the intestinal neuronal cells. Intense glutamate staining was observed in the neural plexuses of human colon. It is being postulated that glutamate regulates acetylcholine release and motility in colon (38). Glutamic acid is converted to γ -aminobutyric acid by glutamic acid decarboxylase enzyme. Recently, it was shown that glutamic acid decarboxylase and γ -

aminobutyric acid are present in the lamina propria of colonic mucosa of rats and may be involved in maturation and differentiation of colonic epithelial cells (39). In the present study, although we detected small amounts of glutamine/glutamate in colonic mucosa using 2D COSY experiments, they were overlapping with those of lipid signals. As a result, we were not able to quantify these metabolites.

The comparison between group 1 and group 2 spectra shows that ^1H MRS combined with SCS can differentiate between inflamed and non-inflamed samples from the animals fed the same amount and type of fatty acids. In this comparison, total cholines were found to be discriminatory in addition to the metabolites mentioned above. In this study, total cholines were decreased in HFC/HFB fed animals. Martin et al. observed significant levels of phosphatidylcholine, creatine, glycerol and triglycerides in the normal colon (12).

Phosphatidylcholine is the predominant phospholipid present in the lipid bilayer of cell membranes (40) and in colonic mucosal lining (41). It is synthesized via the CDP-choline pathway, and the final step of this *de novo* synthesis is catalyzed by cholinephosphotransferase (42,43). Cholinephosphotransferase has also been implicated in the synthesis of platelet activating factor (PAF) which is an inflammatory mediator and necessary signal for steps in the inflammatory cascade such as platelet activation and increase in vascular permeability. The active species of PAF have been shown to increase significantly in mucosal biopsies from ulcerative colitis patients (44). Levels of PAF in stool are even considered to be diagnostic and indicative of severity in patients with ulcerative colitis (45). The decreased levels of phosphatidylcholine in animals with severe inflammation seen in our study could be due to increased levels of PAF in colonic inflammation. We speculate that the

cholinephosphotransferase could be more active towards the synthesis of PAF under inflammatory conditions. As there is increased apoptosis and cell turnover in IBD, there is increased demand for phosphatidylcholine to repair and regenerate the cells in colonic mucosa. This may have also contributed to the decreased levels of phosphatidylcholine/total-cholines seen in our spectra in line with earlier reports (14, 46, 47).

The histological assessment of group 1 (Figure 3) samples showed that both LFC and HFF may be protective against inflammation. It points to the fact that both the amount and type of fatty acids supplemented may be important in modulating colonic inflammation. HFC and HFB subgroups showed significant inflammation in our study. HFC diet supplied a high concentration of ω -6 fatty acids, which are pro-inflammatory and may have caused severe inflammation in that subgroup. The same trend holds true for HFB diet which supplies high amounts of saturated fats.

On the contrary, LFC diet; although it provides mainly ω -6 PUFAs, did not have high enough concentration of these PUFAs to manifest severe pro-inflammatory effects. In a randomized control trial on patients with IBD, Bamba et al. observed that low fat diet induced remission in 80% patients as compared to only 25% in the high fat diet group (48). Interestingly, the fat they supplemented in the diet was soybean oil, which has significant amount of ω -3 PUFAs. Similar effects were observed by Reddy et al. in a study on colon cancer (49). They showed that rats fed with 20% corn oil had significantly higher incidence of colon cancer than rats fed with 5% corn oil for the same duration (49). More recently, Rao et al. reported that rats fed with high fat fish oil (ω -3) and LFC (ω -6) exerted similar protective effect on colon tumour development as compared to a high saturated fat diet (50). Our results are in agreement with all these reports. In addition, the duration for which these PUFAs are administered is also important in regulating the

outcome of the therapy. The effects of ω -3 and ω -6 fatty acids on IBD need to be studied in long-term studies. It is necessary to assess whether the effects of these fatty acids on IBD are reversible and the relapse and remission rates for patients who become non-compliant to the dietary modifications. Very mild or no inflammation seen in animals from group 2 could be due to variation in the normal mucosa, as usually some inflammatory cells are always present in the colon.

The results of this study show that ^1H MRS combined with SCS methodology can detect differences in the prevalence of inflammation in the colon of animals fed with both low fat and high fat diet. It can also differentiate between the subtle changes caused by the different fatty acids on the colon of animals fed with specialized diet. The fact that both LFC and HFF subgroups showed very mild or no inflammation implies that both the amount and the type of fatty acid in the diet are crucial in modulating inflammation. Our study will serve as an adjunct to histologic examination of colon biopsies. The role of ^1H MRS as an adjunct for diagnosing and differentiating between Crohn's disease and ulcerative colitis has gained footing (6). This study will facilitate future studies to identify the utility of ^1H MRS in assessing disease progression/response to dietary modification. One of our future research goals is to conduct a study using different timelines for each dietary group of animals to show the temporal separation between biochemical and histological changes in IBD. We intend to use a combination of serum markers, ^1H MRS and histology at various time-points of that study.

ACKNOWLEDGEMENTS

We would like to thank Saro Bascaramurty who was the key resource for tissue sectioning and staining for histological assessment.

CONFLICT OF INTEREST and FUNDING DISCLOSURE

There are no conflicts of interest to report. We thank the Natural Sciences and Engineering Research Council of Canada (NSERC) for the strategic project grant to R. P. Bird, M. Eskin and T. Bezabeh.

For Peer Review

References

1. Loftus EV. (2004) Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence and environmental influences. *Gastroenterology* 126:1504-1517.
2. Shoda R, Matsueda K, Yamato S, Umeda N. (1996) Epidemiologic analysis of Crohn disease in Japan: increased dietary intake of n-6 polyunsaturated fatty acids and animal protein relates to the increased incidence of Crohn disease in Japan. *Am J Clin Nutr* 63: 741-745.
3. Stenson WF, Cort D, Rodgers J, Burakoff R, DeSchryver-Kecskemeti K, Gramlich TL, Beeken W. (1992) Dietary supplementation with fish oil in ulcerative colitis, *Annals Internal Med* 116:609-614.
4. Lands WE. (1992) Biochemistry and physiology of n-3 fatty acids, *FASEB J* 6, 2530-2536.
5. Eaden JA, Abrams KR, Mayberry JF. (2001) The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 48:526-535.
6. Bezabeh T, Somorjai RL, Smith ICP, Nikulin AE, Dolenko B, Bernstein C.N. (2001) The use of ^1H magnetic resonance spectroscopy in inflammatory bowel diseases: distinguishing ulcerative colitis from Crohn's disease. *Am J Gastroenterology* 96:442-448.
7. Varma S, Bird R, Eskin M, Dolenko B, Raju J, Bezabeh T. (2007) Detection of inflammatory bowel disease by proton magnetic resonance spectroscopy (^1H MRS) using an animal model. *J Inflamm (Lond)* 4:24.

8. Kriat M, Vion-Dury J, Confort-Gouny S, Favre R, Viout P, Sciaky M, Sari H, Cozzzone PJ. (1993) Analysis of plasma lipids by NMR spectroscopy: application to modifications induced by malignant tumors. *J Lipid Res* 34:1009-1019.
9. Adosraku RK, Choi GT, Constantinou-Kokotos V, Anderson MM, Gibbons WA. (1994) NMR lipid profiles of cells, tissues, and body fluids: proton NMR analysis of human erythrocyte lipids. *J Lipid Res* 35:1925-1931.
10. Singer S, Sivaraja M, Souza K, Millis K, Corson JM. (1996) ^1H -NMR detectable fatty acyl chain unsaturation in excised leiomyosarcoma correlate with grade and mitotic activity. *J Clin Invest* 98:244-250.
11. Gunstone FD. (1994) High resolution ^{13}C NMR. A technique for the study of lipid structure and composition. *Prog Lipid Res* 33:19-28.
12. Martin FP, Wang Y, Sprenger N, Holmes E, Lindon JC, Kochhar S, Nicholson JK. (2007) Effects of probiotic *Lactobacillus paracasei* treatment on the host gut tissue metabolic profiles probed via magic-angle-spinning NMR spectroscopy. *J Proteome Res* 6:1471-1481.
13. Sands CJ, Coen M, Maher AD, Ebbels TM, Holmes E, Lindon JC, Nicholson JK. (2009) Statistical total correlation spectroscopy editing of ^1H NMR spectra of biofluids: application to drug metabolite profile identification and enhanced information recovery. *Anal Chem* 81:6458-6466.
14. Bjerrum JT, Nielsen OH, Hao F, Tang H, Nicholson JK, Wang Y, Olsen J. (2010) Metabonomics in ulcerative colitis: diagnostics, biomarker identification, and insight into the pathophysiology. *J Proteome Res* 9:954-962.

15. Nikulin AE, Dolenko B, Bezabeh T, Somorjai RL. (1998) Near-optimal region selection for feature space reduction: novel preprocessing methods for classifying MR spectra. *NMR Biomed* 11:209-217.
16. Somorjai RL, Alexander M, Baumgartner R, Booth S, Bowman C, Demko A, Dolenko B, Mandelzweig M, Nikulin AE, Pizzi N, Pranceviciene E, Summers R, Zhilkin P. (2004) A data-driven, flexible machine learning strategy for the classification of biomedical data. In: Azuaje F, and Dubitsky W. (eds). *Computational Biology, Volume 5: Artificial Intelligence Methods and Tools for Systems Biology*, Boston: Kluwer Academic Publishers, 67-85.
17. Briere KM, Kuesel AC, Bird RP, Smith ICP. (1995) ^1H MR visible lipids in colon tissue from normal and carcinogen-treated rats. *NMR Biomed* 8:33-40.
18. Kuesel AC, Kroft T, Saunders JK, Prefontaine M, Mikhael N, Smith ICP. (1992) A simple procedure for obtaining high-quality NMR spectra of semiquantitative value from small tissue specimens: cervical biopsies. *Magn Reson Med* 27:349-355.
19. AOCS Official Method Ce 1-62. (1998) Fatty Acid Composition by Packed Column Gas Chromatography, American oil chemist's society-Official methods and recommended protocols, AOCS Press, Champaign.
20. Righi V, Durante C, Cocchi M, Calabrese C, Di Febo G, Lecce F, Pisi A, Tugnoli V, Mucci A, Schenetti L. (2009) Discrimination of healthy and neoplastic human colon tissues by ex vivo HR-MAS NMR spectroscopy and chemometric analyses. *J Proteome Res* 8:1859-1869.

21. Lean CL, Newland RC, Ende DA, Bokey EL, Smith ICP, Mountford CE. (1993) Assessment of human colorectal biopsies by ^1H MRS: correlation with histopathology. *Magn Reson Med* 30:525-533.
22. Smith ICP, Blandford DE. (1998) Diagnosis of cancer in humans by ^1H NMR of tissue biopsies. *Biochem Cell Biol* 76:472-476.
23. Bezabeh T, Smith ICP, Krupnik E, Somorjai RL, Kitchen DG, Bernstein CN, Pettigrew, NM, Bird RP, Lewin KJ, Briere, K.M. (1996) Diagnostic potential for cancer via ^1H magnetic resonance spectroscopy of colon tissue. *Anticancer Res* 16:1553-1558.
24. Ende D, Rutter A, Russell P, Mountford CE. (1996) Chemical shift imaging of human colorectal tissue (ex vivo). *NMR Biomed* 9:179-183.
25. Jockers-Wretou E, Giebel W, Pflleiderer G. (1977) Immunohistochemical localization of creatinkinase isoenzymes in human tissue. *Histochemistry* 54:83-95.
26. Graeber GM, Cafferty PJ, Wolf RE, Harmon JW (1984) An analysis of creatine phosphokinase in the mucosa and the muscularis of the gastrointestinal tract. *J Surg Res* 37:376-382.
27. Clandinin MT, Cheema S, Field CJ, Garg ML, Venkatraman J, Clandinin TR. (1991) Dietary fat: exogenous determination of membrane structure and cell function. *FASEB J* 5:2761-2769.
28. Simopoulos AP. (2002) Omega-3 fatty acids in inflammation and autoimmune diseases. *J Am Coll Nutr* 21:495-505.
29. Nishida T, Miwa H, Shigematsu A, Yamamoto M, Iida M, Fujishima M. (1987) Increased arachidonic acid composition of phospholipids in colonic mucosa from patients with active ulcerative colitis. *Gut* 28:1002-1007.

30. Nieto N, Giron MD, Suarez MD, Gil A. (1998) Changes in plasma and colonic mucosa fatty acid profiles in rats with ulcerative colitis induced by trinitrobenzene sulfonic acid. *Dig Dis Sci* 43:2688-2695.
31. Rashid A, Pizer ES, Moga M, Milgraum LZ, Zahurak M, Pasternack GR, Kuhajda FP, Hamilton SR. (1997) Elevated expression of fatty acid synthase and fatty acid synthetic activity in colorectal neoplasia. *Am J Pathol* 150:201-208.
32. Robblee NM, Farnworth ER, Bird RP. (1998) Phospholipid profile and production of prostanoids by murine colonic epithelium: effect of dietary fat. *Lipids* 23:334-339.
33. Robblee NM, Bird RP. (1994) Effects of high corn oil diet on preneoplastic murine colons: prostanoid production and lipid composition. *Lipids* 29:67-71.
34. Hawthorne AB, Daneshmend TK, Hawkey CJ, Belluzzi A, Everitt SJ, Holmes GK, Malkinson C, Shaheen MZ, Willars JE. (1992) Treatment of ulcerative colitis with fish oil supplementation: a prospective 12 month randomised controlled trial. *Gut* 33:922-928.
35. Bartolí R, Fernández-Bañares F, Navarro E, Castellà E, Mañé J, Alvarez M, Pastor C, Cabré E, Gassull MA. (2000) Effect of olive oil on early and late events of colon carcinogenesis in rats: modulation of arachidonic acid metabolism and local prostaglandin E(2) synthesis. *Gut* 46:191-199.
36. James LA, Lunn PG, Elia M. (1998) Glutamine metabolism in the gastrointestinal tract of the rat assessed by the relative activities of glutaminase (EC 3.5.1.2) and glutamine synthetase (EC 6.3.1.2). *Br J Nutr* 79:365-372.
37. James LA, Lunn PG, Middleton S, Elia M. (1998) Distribution of glutaminase and glutamine synthetase activities in the human gastrointestinal tract. *Clin Sci* 94:313-319.

38. Giaroni C, Zanetti E, Chiaravalli AM, Albarello L, Dominioni L, Capella C, Lecchini S, Frigo G. (2003) Evidence for a glutamatergic modulation of the cholinergic function in the human enteric nervous system via NMDA receptors. *Eur J Pharmacol* 476:63-69.
39. Wang FY, Zhu RM, Maemura K, Hirata I, Katsu K, Watanabe M. (2006) Expression of gamma-aminobutyric acid and glutamic acid decarboxylases in rat descending colon and their relation to epithelial differentiation. *Chin J Dig Dis* 7:103-108.
40. Cullis PR, Hope MJ (1992) Physical properties and functional roles of lipids in membranes. In: Vance DE (ed) *Biochemistry of lipids, lipoproteins and membranes*, Amsterdam: Elsevier Science publishers, 1-41.
41. White DA. (1973) The phospholipid composition of mammalian tissue. In: Ansell GB, Hawthorne JN, Dawson RMC. (eds) *Form and function of phospholipids*, Amsterdam: Elsevier Scientific publishing Co, 441-482.
42. Snyder F. (1997) CDP-choline:alkylacetyl glycerol cholinephosphotransferase catalyzes the final step in the de novo synthesis of platelet-activating factor. *Biochim Biophys Acta* 4:1348:111-116.
43. Henneberry AL, Wistow G, McMaster CR. (2000) Cloning, genomic organization, and characterization of a human cholinephosphotransferase. *J Biol Chem* 275:29808-29815.
44. Thyssen E, Turk J, Bohrer A, Stenson WF. (1996) Quantification of distinct molecular species of platelet activating factor in ulcerative colitis. *Lipids* 31:255-259.
45. Hocke M, Richter L, Bosseckert H, Eitner K. (1999) Platelet activating factor in stool from patients with ulcerative colitis and Crohn's disease. *Hepatogastroenterology* 46:2333-2337.

46. Sharma U, Singh RR, Ahuja V, Makharia GK, Jagannathan NR. (2010) Similarity in the metabolic profile in macroscopically involved and un-involved colonic mucosa in patients with inflammatory bowel disease: an in vitro proton (^1H) MR spectroscopy study. *Magn Reson Imaging* (in press).
47. Balasubramanian K, Kumar S, Singh RR, Sharma U, Ahuja V, Makharia GK, Jagannathan NR. (2009) Metabolism of the colonic mucosa in patients with inflammatory bowel diseases: an in vitro proton magnetic resonance spectroscopy study. *Magn Reson Imaging* 27:79-86.
48. Bamba T, Shimoyama T, Sasaki M, Tsujikawa T, Fukuda Y, Koganei K, Hibi T, Iwao Y, Munakata A, Fukuda S, Matsumoto T, Oshitani N, Hiwatashi N, Oriuchi T, Kitahora T, Utsunomiya T, Saitoh Y, Suzuki Y, Nakajima M. (2003) Dietary fat attenuates the benefits of an elemental diet in active Crohn's disease: a randomized, controlled trial. *Eur J Gastroenterol Hepatol* 15:151-157.
49. Reddy BS, Narisawa T, Vukusich D, Weisburger JH, Wynder EL. (1976) Effect of quality and quantity of dietary fat and dimethylhydrazine in colon carcinogenesis in rats. *Proc Soc Exp Biol Med* 151:237-239.
50. Rao CV, Hirose Y, Indranie C, Reddy BS. (2001) Modulation of experimental colon tumorigenesis by types and amounts of dietary fatty acids. *Cancer Res* 61:1927-1933.

Figure Legends

Figure 1: Schematic diagram showing the grouping of the animals into various study groups.

Figure 2: 2D ^1H - ^1H COSY spectrum (360 MHz, 25 °C) of a typical colonic mucosa with labelling of various fatty acids signals along with other predominant biochemicals.

Corresponding 1D ^1H spectrum is shown on the top of the 2D spectrum with assignments of lipid signals. Abbreviations: L, Lipid; Leu, Leucine; Ile, Isoleucine; Val, Valine; Lys, Lysine; Glu, Glutamate; Gln, Glutamine; Ala, Alanine; Asp, Aspartate; Thr, Threonine; Tyr, Tyrosine; Phe, Phenylalanine; PC, phosphatidylcholine.

Figure 3: Representative H & E stained sections from each dietary group: Low fat corn oil (LFC) group (a) showing normal architecture with no inflammation while the section from high fat corn oil (HFC) group (b) shows moderate inflammation with lymphocytic infiltrate and fibrosis. The section from high fat flaxseed oil (HFF) (c) shows unremarkable architecture with no evidence of inflammation and the section from high fat beef tallow (HFB) group (d) shows moderate inflammation with distortion of crypt architecture and interstitial fibrosis.

Figure 4: ^1H MR spectra (360 MHz, 25 °C) of the corresponding colonic tissue specimens whose histology is shown in Fig. 3 from each dietary group: (a) Low fat corn oil group (LFC) (b) High fat corn oil (HFC) group (c) High fat flaxseed oil (HFF) group and (d) High fat beef tallow (HFB) group. Some of the peaks/metabolites that showed change in their relative levels have been marked in the spectra.

759x478mm (96 x 96 DPI)

137x177mm (300 x 300 DPI)

145x108mm (300 x 300 DPI)

(a) Low fat corn oil

(b) High fat corn oil

(c) High fat flaxseed oil

(d) High fat beef tallow

159x109mm (600 x 600 DPI)

Review

Table 1: Detailed composition of the diets fed to animals in each group (gms/kg of the diet)

Ingredients	LFC	HFC	HFF	HFB
Corn Starch	520	337.5	337.5	337.5
Casein	200	230	230	230
Dextrose	130	85.2	85.2	85.2
Cellufil	50	59	59	59
AIN-76 mineral mix	35	41.1	41.1	41.1
AIN-76 vitamin mix	10	11.3	11.3	11.3
Methionine	3	3	3	3
Choline bitartrate	2	2.4	2.4	2.4
Corn oil	50	230	140	50
Flaxseed oil	0	0	90	0
Beef tallow	0	0	0	180

Abbreviations: LFC, Low fat corn oil; HFC, High fat corn oil; HFF, High fat flaxseed oil, HFB, High fat beef tallow.

Table 2. Fatty acid composition of low/high fat corn oil, high fat flaxseed oil, and high fat beef tallow diets (expressed as % weight of the total fat content)

Fatty acids	LFC/HFC	HFF	HFB
C 10:0			0.066
C 12:0			0.067
C 14:0	0.041	0.044	1.882
C 14:1			0.268
C 15:0		0.02	0.292
C 16:0	10.817	8.482	21.830
C 16:1	0.111	0.091	1.833
C 17:0	0.084	0.082	0.945
C 18:0	1.872	2.693	16.995
C 18:1ω9	29.131	25.295	34.182
C 18:1ω11	0.584	0.577	1.420
C 18:2ω6	55.282	39.259	18.343
C 18:3ω6			0.029
C 18:3ω3	0.958	22.401	
C 20:0	0.491	0.434	0.336
C 20:1	0.278	0.240	
C 20:2	0.039	0.036	0.177
C 20:3ω4			0.067
C 20:3ω3		0.026	0.028
C 20:4			0.105
C 22:0	0.165	0.152	0.075
C 22:4			0.057
C 22:5			0.050
C 22:6			
C 24:0	0.199	0.163	0.505

Abbreviations are same as in Table 1

Table 3: SCS analysis of low fat corn oil group vs. each dietary subgroup in Groups 1 (N=127) and 2 (N=119)

Group 1*					Group 2			
Class	N	Accuracy	Regions (ppm)	Metabolites	N	Accuracy	Regions (ppm)	Metabolites
LFC	32	100%	2.68-2.86	=HC-CH ₂ -CH=	24	96.6%	2.69-2.79	=HC-CH ₂ -CH=
			1.95-2.0	-H ₂ C-CH=CH-			2.05-2.11	-H ₂ C-CH=CH-
HFC	31		1.59-1.61	-CH ₂ -CH ₂ -COO-	35		1.35-1.4	(-CH ₂ -) chain
			1.12-1.19	Leucine/isoleucine, valine			1.1-1.22	Leucine/isoleucine, valine
LFC	32	98.4%	3.02-3.05	Creatine	24	98.2%	3.0-3.08	Creatine
			2.78-2.95	=HC-CH ₂ -CH=			2.69-2.83	=HC-CH ₂ -CH=
HFF	30		2.66-2.88	=HC-CH ₂ -CH=	32		1.15-1.22	Leucine/isoleucine, valine
							0.87-0.94	-CH ₃
LFC	32	90.9%	3.75-3.80	Glyceryl moiety	24	100%	3.0-3.05	Creatine
			2.66-2.68	=HC-CH ₂ -CH=			2.76-2.9	=HC-CH ₂ -CH=
HFB	34		2.20-2.27	-CH ₂ -CH ₂ -COO-	28		2.04-2.08	-H ₂ C-CH=CH-

*In Group 1, all animals were treated with 2% carrageenan to induce IBD; Abbreviations are same as in Table 1.

Table 4: SCS analysis of different dietary subgroups in Groups 1 and 2 to assess the differences between individual dietary subgroups

Group 1*					Group 2			
Class	N	Accuracy	Regions (ppm)	Metabolites	N	Accuracy	Regions (ppm)	Metabolites
HFC	31	98.4%	4.18-4.29	Glyceryl moiety	35	97.0%	2.90-3.10	Creatine
HFF	30		3.20-3.24	Total choline	32		2.58-2.78	=HC- H_2C -HC=
			2.75-2.79	=HC- H_2C -HC=			1.95-1.98	- H_2C -HC=CH-
			1.49-1.54	- H_2C - H_2C -COO ⁻			0.94-0.98	-CH ₃
HFC	31	100%	3.73-3.80	Glutamic acid	35	98.4%	2.99-3.04	Creatine
HFB	34		2.58-2.71	=HC- H_2C -HC=	28		2.69-2.77	=HC- H_2C -CH=
			1.79-1.82	Unassigned			1.94-2.00	- H_2C -CH=CH-
HFF	30	100%	3.28	Glycerophosphoethanolamine	32	100%	2.88-3.05	Creatine
HFB	34		2.79-2.98	=HC- H_2C -HC=	28		2.57-2.68	=HC- H_2C -HC=
			2.31-2.35	Glutamic acid			1.59-1.61	- H_2C - H_2C -COO ⁻

*In Group 1, all animals were treated with 2% carrageenan to induce IBD; Abbreviations are same as in Table 1.

Table 5: Results of comparative SCS analysis between Group 1 and Group 2

Class	Accuracy	Regions (ppm)	Metabolites
LFC	100%	4.18-4.23	Phosphatidylcholine (-O-CH ₂ -)
		3.64-3.67	Phosphatidylcholine (-N-CH ₂ -)
		0.89-0.93	-CH ₃
HFC	97.0%	2.85-2.93	=HC-CH ₂ -CH=
		2.63-2.67	=HC-CH ₂ -CH=
		1.21-1.25	Leucine/isoleucine/valine
HFF	100%	3.20-3.24	Total cholines
		0.87-0.93	-CH ₃
HFB	100%	3.22-3.24	Total cholines
		2.95-3.04	Creatine
		0.85-0.92	-CH ₃

Abbreviations are same as in Table 1.