Effects of Dietary Fermentable Material on Innate and Adaptive Immune Measures in Male and Female Rats under Resting and Immunized Conditions

By

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Abstract

Sex-based differences in immune parameters are well recognized, and recent evidence suggests differences in microbiota composition between sexes, necessitating assessment of effects of dietary fermentable material (DFM) in both males and females. Effects of consumption of DFM of differing composition (Wheat Bran (WB), Oat Bran (OB), Resistant Starch (RS), Fructooligosaccharides (FOS)) on the immune system were compared under resting and immunized conditions between sexes. The gut microbiota composition and short chain fatty acid profiles differed between rats fed different DFM and also differed between sexes following FOS consumption. Immune parameters were analyzed for the effect of diet, differences between sexes or interactions (diet x sex), to detect the effect of DFM on male and female rats. The kinetics of primary (Day 0, 5, 10, 15) and secondary antibody responses (Day 21) were measured following immunization with a T cell-dependent antigen, Keyhole Limpet Hemocyanin (KLH). Under resting conditions, male rats had more FoxP3⁺ T_{reg} cells in the mesenteric lymph node (MLN) and spleen than did females. Levels of the regulatory cytokine TGF-β1 also increased in the MLN in a DFM-dependent manner. In contrast, percentages of MLN and splenic CD68⁺ macrophages and levels of gut tissue IL-10 were higher in females than males. These results suggest immune regulation at the mucosal level is controlled in a differential manner between sexes and was affected by DFM intake only in males. At the systemic level, DFM intake delayed the primary anti-KLH IgG antibody response kinetics in male (control>>WB>OB>RS>FOS) and female (control> FOS>WB>RS>OB) rats. The secondary anti-KLH IgG response in control-fed males was 10-fold higher than in controlfed females. DFM intake in males significantly decreased anti-KLH IgG levels relative to control-fed males. Collectively, these findings demonstrate DFM consumption in males potentially induces systemic immune regulation leading to a delayed response to immune challenge, an effect that was not observed in females.

"I long so much to make beautiful things. But beautiful things require effort and disappointment and perseverance."

-Vincent Van Gogh

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Table of Contents

Abstract	ii
Acknowledgement	iv
List of Figures	viii
List of Tables	xv
Abbreviations	xvi
1. Introduction	1
2. Background	5
Properties of DFM	5
Table 1: Structural properties of different DFM	7
DFM and gut microbiota	8
DFM and fermentation metabolites	10
The gut epithelium	12
Activation of epithelial innate mechanisms	13
Pathogen Recognition Receptors: The surveillance team	15
Mucin: Epithelial protector and microbiota fodder	18
Gut Associated Lymphoid Tissue (GALT): Where decisions are made	20
IgA: The adaptive immune response of the GALT in action	25
IgG: The systemic adaptive immune response	29
The gut-systemic axis	30
3. Study Objectives	32
<i>Objective 1:</i> To compare the effects of different types of DFM on mucosal and systemic immune parameters under steady state conditions	32
<i>Objective 2:</i> To compare the effects of DFM on mucosal and systemic immune measures steady state conditions between male and female rats	under 33
Objective 3: To compare the effects of DFM on systemic adaptive immune responses to immunization between male and female rats	34
Objective 4: To evaluate the time and dose-dependent effects of butyric acid on TLR-challenged IEC in the absence or presence of β -estradiol	34
<i>Objective 5:</i> To examine the effects of butyric acid and Pam ₃ CysK ₄ on TLR agonist-induc 8 production via the p38 pathway	<i>ed IL-</i> 35
4. Materials and Methods	37

	Rats and feeding trial under resting conditions	37
	Rats and feeding trial under immunized conditions	38
	Gas chromatography	40
	Serum biochemistry	40
	Flow cytometry	41
	Table 2: Surface or intracellular marker associated with each immune cell type	42
	Tissue preparation	43
	Cytokine and antibody analysis	43
	Anti-KLH antibody analysis	44
	Stimulation of whole blood cultures with TLR agonists and KLH	44
	End-point Chromogenic Limulus Amebocyte Lysate (LAL)	45
	Real Time PCR: Cecal tissue Mucin-2 gene expression	45
	Cell cultures	46
	Treatment of IEC-6 cells with butyric acid and TLR agonists	47
	p38 pathway inhibition in Poly (I:C) challenged HT-29 IEC treated with Pam ₃ CysK ₄ and/ butyric acid	′or 48
	β -estradiol treated IEC incubated with butyric acid and TLR agonists or TNF α	48
	Treatment of HT-29 cells with ATP and TLR agonists	49
	Tetrazolium dye reduction (MTT) Assay for cell viability	49
	Statistical analysis	50
5.	Results	51
	5.1 Effects of DFM ingestion on physiological and immune measures from male and fer rats under resting state conditions	<i>male</i> 51
	5.2 Effects of FOS consumption on physiological and immune parameters between mal female rats	e and 74
	5.3 Effects of DFM consumption on immune responses in male and female rats to immunization with KLH	88
	Table 18. Statistical analysis of the effect of DFM on the response of male and female ration immunization with KLH	s to 163
	5.4 Effects of butyric acid on chemokine production by TLR agonist-challenged IEC	C.169
6.	Discussion	188
	6.1a) RS-ingestion differentially impacted mucosal and systemic immunological parameter than did WB or OB-ingestion under resting conditions	ers 189

6 ti	5.1b) Physiological and immunological differences between male and female rats ingestime the WB supplement	ıg 193
6 n	5.1c) Differential metabolism of FOS resulted in altered systemic immune measures betwe nale and female rats	<i>en</i> 197
6 e	5.2 The immune modulatory effects of butyric acid on IEC are dependent upon timing of exposure.	200
6 fe	5.3 The kinetics of anti-KLH antibody production are more suppressed in males than in Temales following DFM consumption	204
7.	Conclusion	211
8.	Future Directions	214
9.	References	215
10.	Appendix	252

List of Figures

Figure IA. Gut microbiota and gut-derived metabolites interact with immune cells of the GALT

Figure A1. Change in the male and female faecal community in response to diet. Bar graphs indicate the distribution of phylotypes (%) at the Family level. (Appendix)

Figure 1. A schematic representing the immunization time line

Figure 2. Weight gain in male rats fed different DFM

Figure 3. Differences in weight gain between male and female rats fed the AIN-93G or WB-supplemented diet

Figure 4a). Cecal short chain fatty acid profiles from rats fed different DFM

Figure 4b). Fecal short chain fatty acid profiles from male rats fed different DFM

Figure 5. Differences in the fecal short chain fatty acid profiles between male and female rats fed the AIN-93G or WB-supplemented diet

Figure 6. Differences in the percentages of $Foxp3^+T_{reg}$ population in the MLN (A) and spleen (B) between male and female rats fed control or WB

Figure 7. Differences in the percentages of CD68⁺ population MLN (A) and spleen (B) between male and female rats fed control or WB

Figure 8. Cytokine concentrations (pg/g) in cecum (A) and MLN (B) of rats fed different DFM

Figure 9. Liver tissue cytokine concentrations (pg/g)

Figure 10. Differences in weight gain between male and female rats fed either Purina chow or the FOS supplement

Figure 11. Cecal SCFA profiles from male rats fed either Purina chow (Black bars) or FOS (Dark Grey bars)

Figure 12. Fecal SCFA profiles from male rats fed either Purina chow or FOS

Figure 13. IgA concentrations in the liver of male and female rats fed Purina chow or the FOS-supplemented diet $(\mu g/g)$

Figure 14. Serum LPS concentrations in male and female rats fed either the Purina chow or FOS-supplemented diet

Figure 15. Relative mRNA expression of the *MUC2* gene from cecal tissue of male and female rats fed either Purina chow or FOS

Figure 16. The percentage of $CD3^+$ total T cells in the PP of male and female rats fed different DFM

Figure 17a. Differences in the percentage of $CD3^+4^+T_H$ cells in the PP of rats fed different DFM

Figure 17b. The percentage of $CD3^+4^+$ T_H cells in the PP of male and female rats fed different DFM

Figure 18. The percentage of $CD3^+4^+25^+$ T_{reg} cells in the PP of male and female rats fed different DFM

Figure 19. The percentage $Foxp3^+ T_{reg}$ within the CD4⁺CD25⁺ T cell population in the PP of male and female rats fed different DFM

Figure 20. The percentage of $CD3^+8^+$ cytotoxic T cells in the PP of male and female rats fed different DFM

Figure 21. The percentage of CD62L⁺ within the CD3⁺ total T cell population in the PP of male and female rats fed different DFM

Figure 22. The percentage of $\gamma \delta TCR^+$ cells in the PP of male and female rats fed different DFM

Figure 23. The percentage of CD161a⁺ natural killer cells in the PP of male and female rats fed different DFM

Figure 24. The percentage of CD45RA⁺ B cells in the PP of male and female rats fed different DFM

Figure 25. The percentage of macrophages (HIS36⁺) in the PP of male and female rats fed different DFM

Figure 26. The percentage of CD103⁺ DC in the PP of male and female rats fed different DFM

Figure 27. Levels of IL-17A in the PP of male and female rats fed different DFM

Figure 28. Levels of TGF- β 1 in the PP of male and female rats fed different DFM

Figure 29. Levels of IL-10 in the PP of male and female rats fed different DFM

Figure 30. The percentage of CD3⁺ total T cells in the MLN of male and female rats fed different DFM

Figure 31. The percentage of $CD3^+4^+$ T_H cells in the MLN of male and female rats fed different DFM

Figure 32. The percentage of $CD3^+4^+25^+T_{reg}$ cells in the MLN of male and female rats fed different DFM

Figure 33. The percentage of $Foxp3^+ T_{reg}$ cells within the CD4⁺CD25⁺ T cell population in the MLN of male and female rats fed different DFM

Figure 34a. Differences in the percentage of $CD3^+8^+$ cytotoxic T cells in the MLN of rats fed different DFM

Figure 34b. The percentage of CD3⁺8⁺ cytotoxic T cells in the MLN of male and female rats fed different DFM

Figure 35. The percentage of CD62L⁺ within the CD3⁺ total T cell population in the MLN of male and female rats fed different DFM

Figure 36. The percentage of $\gamma \delta TCR^+$ in the MLN of male and female rats fed different DFM

Figure 37. The percentage of CD161a⁺ natural killer cells in the MLN of male and female rats fed different DFM

Figure 38. The percentage of B cells (CD45RA⁺) in the MLN of male and female rats fed different DFM

Figure 39. The percentage of CD103⁺ DC in the MLN of male and female rats fed different DFM

Figure 40. The percentage of HIS36⁺ macrophages in the MLN of male and female rats fed different DFM

Figure 41. The levels of CINC-1 in the MLN of male and female rats fed different DFM

Figure 42. The levels of IFN- γ in the MLN of male and female rats fed different DFM

Figure 43. The levels of IL-17A in the MLN of male and female rats fed different DFM

Figure 44. The levels of IL-4 in the MLN of male and female rats fed different DFM

Figure 45. The levels of IL-10 in the MLN of male and female rats fed different DFM

Figure 46a. Differences in the percentage of $CD3^+$ total T cells in the spleen of rats fed different DFM

Figure 46b. The percentage of CD3⁺ total T cells in the spleen of male and female rats fed different DFM

Figure 47. Effects of DFM on the percentage of $CD3^+4^+T_H$ in the spleen of male and female rats

Figure 48. The percentage of $CD3^+4^+25^+$ T_{reg} cells in the spleen of male and female rats fed different DFM

Figure 49. The percentage of $Foxp3^+ T_{reg}$ cells within the CD4⁺CD25⁺ T cell population in the spleen of male and female rats fed different DFM

Figure 50. Effects of DFM on the percentage of $CD3^+8^+$ cytotoxic T cells in the spleen of male and female rats

Figure 51. The percentages of $CD62L^+$ within the $CD3^+$ total T cell population in the spleen of males and females fed different DFM

Figure 52. Effects of DFM ingestion on the percentage of splenic $\gamma\delta$ TCR⁺ cells in male and female rats

Figure 53. The percentage of CD161a⁺ natural killer cells in the spleen of male and female rats fed different DFM

Figure 54a. Differences in the percentage of CD45RA⁺ B cells in the spleen of rats fed different DFM

Figure 54b. The percentage of splenic CD45RA⁺ B cells in male and female rats fed different DFM

Figure 55. The percentage of $CD103^+$ DC in the spleen of male and female rats fed different DFM

Figure 56. Effects of DFM ingestion on the percentage of splenic HIS36⁺ macrophages in male and female rats

Figure 57. Effects of DFM on splenic TGF- β 1 levels in male and female rats

Figure 58. Splenic IL-4 levels in the spleen of male and female rats fed different DFM

Figure 59. Splenic IFN-γ levels in male and female rats fed different DFM

Figure 60. The ratio of splenic IFN- γ /IL-4 in male and female rats fed different DFM

Figure 61. IL-10 levels following *ex-vivo* stimulation with TLR agonists and/or KLH in whole blood cultures from male rats fed different DFM

Figure 62. IL-10 levels following *ex-vivo* stimulation with TLR agonists and/or KLH in whole blood cultures from female rats fed different DFM

Figure 63. IFN- γ levels following *ex-vivo* stimulation with TLR agonists and/or KLH in whole blood cultures from male rats fed different DFM

Figure 64. IFN- γ levels following *ex-vivo* stimulation with TLR agonists and/or KLH in whole blood cultures from female rats fed different DFM

Figure 65. IL-4 levels following *ex-vivo* stimulation with TLR agonists and KLH stimulation in whole blood cultures from male rats fed different DFM

Figure 66. IL-4 levels following *ex-vivo* stimulation with TLR agonists and/or KLH in whole blood cultures from female rats fed different DFM

Figure 67. Concentrations of total IgM in the serum of KLH-immunized A) male and B) female rats fed different DFM

Figure 68. Concentrations of anti-KLH IgM in the serum of KLH-immunized A) male and B) female rats fed different DFM

Figure 69. Concentrations of total IgG in the serum of KLH-immunized A) male and B) female rats fed different DFM

Figure 70. Differences in the concentration of total IgG on day 0, in male and female rats fed different DFM

Figure 71. Concentrations of anti-KLH IgG in the serum of KLH-immunized A) male and B) female rats fed different DFM

Figure 72. Differences in the concentration of anti-KLH IgG, 5 days post-immunization, in rats fed different DFM

Figure 73. Differences in the concentration of anti-KLH IgG, 10 days post-immunization, in rats fed different DFM

Figure 74. Differences in the concentration of anti-KLH IgG on day 21, post-secondary immunization, in male and female rats fed different DFM

Figure 75. Concentrations of total IgG2a in the serum of KLH-immunized A) male and B) female rats fed different DFM were evaluated only on the 10^{th} and 15^{th} day post-immunization

Figure 76. Concentrations of anti-KLH IgG2a in the serum of KLH-immunized A) male and B) female rats fed different DFM were evaluated only on the 10th and 15th day post-immunization

Figure 77. Concentrations of total IgG2b in the serum of KLH-immunized A) male and B) female rats fed different DFM were evaluated only the 10th and 15th day post-immunization

Figure 78. Concentrations of anti-KLH IgG2b in the serum of KLH-immunized A) male and B) female rats fed different DFM were evaluated on the 10^{th} and 15^{th} day post-immunization

Figure 79. Differences in the concentration of anti-KLH IgG2b, 10 days postimmunization, in rats fed different DFM

Figure 80. Concentrations of total IgA in the serum of KLH-immunized A) male and B) female rats fed different DFM

Figure 81. Differences in the concentration of anti-KLH IgA, 15 days post-immunization, in female rats fed different DFM

Figure 82. Shift in the anti-KLH IgM kinetics in males fed different DFM

Figure 83. Shift in the anti-KLH IgM kinetics in females fed different DFM

Figure 84. Shift in the anti-KLH IgG kinetics in males fed different DFM

Figure 85. Shift in the anti-KLH IgG kinetics in males fed different DFM

Figure 86. Shift in the anti-KLH IgG kinetics in females fed different DFM

Figure 87. Shift in the anti-KLH IgG kinetics in females fed different DFM

Figure 88. CINC-1 production by IEC-6 cells challenged with LPS (20 ng/ml) in the presence of 10 mM or 25 mM butyric acid.

Figure 89. CINC-1 production by IEC-6 cells pretreated with 10 mM or 25 mM butyric acid for prior to challenge with LPS (20 ng/ml).

Figure 90. CINC-1 production by IEC-6 cells challenged with poly (I:C) (40 μ g/ml) in the presence of 10 mM or 25 mM butyric acid.

Figure 91. CINC-1 production by IEC-6 cells pretreated with 10 mM or 25 mM butyric acid prior to challenge with poly (I:C) (40 μ g/ml)

Figure 92. Viability of IEC-6 cells treated with 10 mM or 25 mM BA for 18 hrs

Figure 93. CINC-1 production by IEC-6 cells treated with varying concentrations of β -estradiol (0-10 ng/ml) prior to A) treatment with butyric acid alone or B) challenge with LPS (20 ng/ml) in the presence of 10 mM or 25 mM butyric acid

Figure 94. IL-8 production by HT-29 IEC treated with varying concentrations of β -estradiol (0-10 ng/ml) prior to treatment with TNF- α (50 or 100 ng/ml)

Figure 95. Effect of 10 mM BA on Pam3Cys (10 μ g/ml) and/or poly (I:C) (40 μ g/ml) - induced IL-8 and IP-10 production by HT-29 IEC after 6 hr

Figure 96. Viability of HT-29 IEC concurrently treated with 10 mM butyric acid and/or TLR-agonists following a 6 hr challenge

Figure 97. Effect of a p38 inhibitor on IL-8 production by HT-29 IEC concurrently treated with 10 mM butyric acid and/or TLR-agonists following a 4 hr challenge

Figure 98. IL-8 production by HT-29 IEC pre-treated with 10 mM butyric acid and/or Pam3Cys (10 μ g/ml) followed by challenge with poly (I:C) (40 μ g/ml)

Figure 99. Viability of HT-29 IEC pre-treated with 10 mM butyric acid and/or Pam3Cys followed by a 4 hr challenge with poly (I:C)

Figure 100. Effect of 25mM butyric acid on Pam3Cys (10 μ g/ml) and/or poly (I:C) (40 μ g/ml) -induced A) IL-8 and B) IP-10 production by HT-29 cells after a 6 hr challenge

Figure 101. Vability of HT-29 cells concurrently treated with 25 mM butyric acid and/or TLR-agonists following a 6 hr challenge

Figure 102. IL-8 production by HT-29 IEC pre-treated with 25 mM butyric acid and/or Pam3Cys (10 μ g/ml) followed by challenge with poly (I:C) (40 μ g/ml)

Figure 103. Viability of HT-29 IEC pretreated with 25 mM butyric acid and/or Pam3Cys followed by a 4 hr challenge with poly (I:C)

Figure 104. IL-8 production by HT-29 IEC stimulated with LPS and/or Flagellin for 6 hr in the presen $\$ of ATP (50 or 75nM)

Figure 105. IL-8 production by HT-29 IEC stimulated with Pam3Cys (10 μ g/ml) in combination with LPS (20ng/ml) or Flagellin (100 ng/ml) for 6 hr in the presence of ATP (50 nM)

List of Tables

Table 1: Structural properties of different dietary fermentable material

Table 2: Surface or intracellular marker associated with each immune cell type

Table 3. Diet composition of AIN-93G-supplemented with different sources of fibre

Table 4. Serum biochemical measures in male rats fed either AIN-93G, WB, OB or RS-supplemented diets

Table 5. Differences in serum biochemical measures between male and female rats fed
 either AIN-93G or WB-supplemented diets

Table 6: Relative ratios of SCFA in cecal and fecal contents from rats fed different DFM

Table 7: Relative ratios of SCFA in the fecal contents from male and female rats fed either the AIN-93G or the WB-supplemented diet

Table 8: Flow cytometric analysis of immune cell population percentages from the MLN and spleen in male rats fed different DFM

Table 9: Total IgA levels in cecal content and liver of males fed different DFM

Table 10: IgA concentrations in cecal content and liver tissue of male and female rats fed the AIN-93G or WB-supplement

Table 11: Cytokine levels in gut mucosal and systemic tissues from male and female rats fed AIN-93G or WB-supplemented diets.

Table 12: Relative ratios of SCFA in the cecal and fecal contents from rats fed the FOS-supplemented diet

Table 13: Relative ratios of SCFA in the fecal contents from male and female rats fed

 the FOS-supplemented diet

Table 14. Concentrations of IgA in cecal tissue and cecal content in male and female rats fed either Purina chow or the FOS-supplemented diet

Table 15: Comparison of serum biochemical measures obtained from male and female rats

 fed the Purina chow or the FOS-supplemented diet

Table 16. Total immune cell population percentages in the MLN and spleen of males and female rats consuming either Purina chow or the FOS-supplemented diet

Table 17. Cytokine levels in gastrointestinal and liver tissue in male and female rats fed

 Purina chow or the FOS-supplemented diet

Table 18. Statistical analysis of the effect of DFM on the response of male and female rats to immunization with KLH

Abbreviations

ALP: Alkaline phosphatase ALT: Alanine amino transferase AST: Aspartate amino transferase BA: Butyric acid BUN: Blood urea nitrogen CD: Cluster of Differentiation CINC: Chemokine induced neutrophil chemoattractant CLR: Carbohydrate lectin receptors CpG: Cystine phosphodiester guanine DC: Dendritic cells DC_{reg}: Regulatory dendritic cell DFM: Dietary fermentable material ELISA: Enzyme-Linked Immunosorbant Assay FFAR: Free fatty acid receptors FCRα1: Fragment, crystallisable receptor $\alpha 1$ FOS: Fructo-oligosaccharides GALT: Gut associated lymphoid tissue GI tract: Gastrointestinal tract HDAC: Histone deacetylase inhibitor IEC: Intestinal epithelial cells IEL: Intestinal epithelial lymphocytes IFN-γ: Interferon gamma IL: interleukin

IP-10: Interferon gamma induced protein-10 **IPEX:Immunodysregulation** polyendocrinopathy enteropathy Хlinked syndrome KLH: Kehole Limpet Hemocyanin LAL: Limulus amebocyte lysate LDH: Lactate dehydrogense LPS: Lipopolysaccharide MHC: Major histocompatibility complex MLN: Mesenteric lymph node MYD88: Myeloid differentiation primary response protein NF-KB: Nuclear factor kappa B NOD: Nucleotide oligomerization binding domain **OB:** Oat Bran PBS: Phosphate buffered saline PE: Phycoerithryn PP: Peyer's patch **PRR:** Pathogen Recognition Receptors **RS:** Resistant starch SCFA: Short chain fatty acids TGFβ: Transforming growth factor TLR: Toll like receptors TNFα: Tumor necrosis factor alpha T_{reg}: T regulatory cells WB: Wheat Bran

1. Introduction

According to a 2002 survey the mean dietary fibre intake for Canadians ranged from 16.5 to 19.4 g/day for men and 14.3 to 16.6 g/day for women, well below the recommended amounts of 38 g/day for men and 25 g/day for women by the Institute of Medicine (Health Canada, 2010). In contrast to the current intake, our ancestors ingested >100g/day of fibre, calculated based on a 50:50 animal-vegetable subsistence ratio (Eaton, 2006). The importance of dietary fibre to gut health was revived in the 1970s by Dennis Burkitt. Dr. Burkitt noted the incidence of colon cancer was the lowest in rural regions of underdeveloped countries (Africa, India and Iran) compared to developed Western nations. He hypothesized that the rise in gastrointestinal-related diseases was due to alterations in bowel behaviour, dictated by the increased fat, protein and sugar and reduced absorbable fibres in Western diets (Burkitt, 1973). Since then, interest in the role of dietary fibre in maintaining health has increased. Health Canada (2010) defines a dietary fibre as a "naturally occurring edible carbohydrate of plant origin that is not digested or absorbed by the small intestine" (Health Canada, 2010). The primary acceptance criteria for health claims associated with dietary fibres have been based upon beneficial physiological effects demonstrated in human subjects (Health Canada, 2010). Widely accepted physiological benefits from dietary fibre intake include stool laxation, lowering of serum low density lipoproteins (LDL) and glycemic control (Slavin, 2013). However, Health Canada (2010) has acknowledged that the benefits associated with dietary fibre intake may extend beyond physiological factors and has included the effects on the gut microbiota and immune

changes as parameters requiring more attention to thoroughly evaluate fibre-related healthclaims (Health Canada, 2010).

The gut microbiota-host interaction is complex and downstream immune changes from this interaction are dependent upon a myriad of immune cells that are evolutionarily conditioned to integrate multiple gut-derived signals and respond in a manner leading to mucosal immune homeostasis. Gut microbiota are critical to the development and maturation of the innate and adaptive immune system. In turn the microbiota diversity is regulated by the innate and adaptive arms of the immune system (Macpherson and Uhr, 2000; Peterson et al., 2007; Littman, 2010). Dietary fermentable material (DFM) consumption has been shown to change intestinal bacterial community composition (Abnous et al., 2009; Kalmokoff et al., 2013). DFM-mediated changes are often claimed to affect host immune parameters via a number of different mechanisms such as the prebiotic effect (Collins and Gibson, 1999), increases in short chain fatty acid (SCFA) outputs (Smith et al., 2013; Conlon et al., 2012) or by direct effects of the substrates themselves (Ortega-Gonzalez et al., 2014; Kovacs-Nolan et al., 2013). However, the extent to which dietary fibre-mediated effects on the gut microbiota in turn influence the immune system would be expected to vary with fibre structure and fermentability along the GI tract, potentially resulting in location-dependent effects on the underlying gut associated lymphoid tissue (GALT) (Roberfroid, 2000; Artis 2008; Hord, 2008). For example, dietary oat β -glucan acting via dectin-1 receptors on immune cells (Volman *et al.*, 2008) can increase expression of pro-inflammatory cytokines in both the cecum and colon of weanling pigs, an effect correlated to total SCFA production (Metzler-Zebli et al., 2012). In an *in vitro* system, oat β -glucan enhanced production of innate immune cytokines by

intestinal epithelial cells (IEC) (Ramkers *et al.*, 2007). In contrast, others have noted oat β glucan, depending on the immune cell type it acts upon, reduces the expression of proinflammatory cytokines (Chanput et al., 2012). Wheat protein ingestion led to an increase in B cell and dendritic cell percentages along with T_H1 (T helper 1) activation, in the mesenteric lymph node (MLN) of diabetes prone rats (Chakir *et al.*, 2005). Collectively these studies show that DFM-mediated alterations in immune parameters not only depend upon the physico-chemical and fermentation properties of a fermentable substrate, but that the immune cell type and location play an important role in the outcomes. Many studies have focused on immune changes in the colon in relation to DFM intake. However, intestinal tissues differ morphologically and immunologically from each other (Schultz and Pabst, 2013; Mowat and Agace, 2014) and fibre intake would thus be anticipated to demonstrate differential effects along the GI tract, especially considering the varied ways in which dietary fibre can engage the immune system.

Rodents are of particular significance in diet-related mechanistic studies (Brooks and Kalmokoff, 2012). However, in order to avoid confounding effects from the estrus cycle, these studies are typically carried out using males only, limiting insight into potential sex differences. Sexual dimorphism in immune parameters is well documented (Ahmed *et al.*, 1985; Ahmed *et al.*, 1990; Verthelyi, 2006). Recently, sex-based differences in certain genes associated with histone-modifying activity, and of genes associated with control of infection and inflammation, were observed in the small intestine and colon of prepubescent C47BL/6 mice (Steegenga *et al.*, 2014). Evidence also supports sex-based differences in gut community composition (Merkle *et al.*, 2013; Bernbom *et al.*, 2006; Li *et al.*, 2008), gut community metabolic activity (McOrist *et al.*, 2011) and in the effects induced by diet

on the gut microbiota across different species. While diet-associated changes in microbiota composition may correlate with the sex-diet interaction, the mechanism(s) underlying these compositional changes have yet to be elucidated (Bolnick, *et al.*, 2014). However, these differences demonstrate potential limitations in extrapolating from studies of effects of DFM on males to effects on females.

Immune events at the gut mucosa would be expected to induce systemic changes via the gut-liver axis, and the liver is an important location for systemic immune regulation. Relatively few studies have examined fibre-mediated immune effects at the systemic level (Nofrarias *et al.*, 2007; Ryz *et al.*, 2009; Buddington *et al.*, 2002), and even fewer have focused on the liver. Changes in immune parameters at the liver can thus serve as a downstream indicator of effects of DFM at the intestinal mucosal level.

The animal studies described here were designed in collaboration with researchers at Health Canada and Agriculture and Agri-Food Canada to examine the effects of different types of DFM on mucosal and systemic immune changes between male and female rodents under resting and immunized conditions, and are complemented by *in vitro* experiments focusing on effects of microbial products at the IEC level. Overall, the findings suggest DFM-mediated effects on immune parameters under resting and immunized conditions differ between sexes.

2. Background

Properties of DFM

DFM are classified as non-starch carbohydrates, consisting of monomeric sugar moieties. The variation in the linkage of monosaccharides and polymer chain length make DFM the most diverse and heterogenous group of molecules on earth (Hamaker and Tuncil, 2014). The physico-chemical aspects of DFM determine how readily it can breakdown into simple sugars by the gut microbiota. The degree of susceptibility of a carbohydrate to bacterial digestion is termed "fermentability" and varies between different types of fibres (Hamaker and Tuncil, 2014; Mugdil and Barak, 2013).

Dietary fibres are made up of hemicellulose, which maintains plant cell wall integrity by binding to cellulose and lignin and include arabinoxylans, xyloglucans, glucomannans, galactomannans and β -glucans. The present study evaluated the effects of different types of fibres, including wheat bran (WB) and oat bran (OB), which contain arabinoxylan and β -glucan, respectively. Arabinoxylan has a xylose backbone and arabinose side chain, configured in a β 1-4 linkage and is an insoluble complex structure (Lattimer and Haub, 2010; Hamaker and Tuncil, 2014). OB consists of β -glucans, made up of glucose molecules in a linear unbranched chain consisting of both β 1-4 and β 1-3 linkages in its backbone (Butt *et al.*, 2008). OB is a soluble fibre that forms a viscous gel. This facet of OB helps prolong its transit time in the gut and helps soften stool. In contrast, insoluble WB has a large water holding capacity which helps in fecal bulking but shortens its transit time in the small intestine. Soluble fibres are also completely fermented by the colonic microbiota, whereas insoluble fibres are slowly fermented along the gut and this contributes to the increase in fecal mass (James *et al.*, 2003; Lattimer and Haub 2010; Stevenson *et al.*, 2012; Mudgil and Barak, 2013).

Non-digestible fibres contain plant oligosaccharides which include fructooligosaccharides (FOS). FOS are fructose polymers linked by β (1-2) glycosidic bonds and often terminate in a glucose molecule (Singh and Singh, 2010). FOS are rapidly fermented in the proximal colon and due to their fermentation characteristics are classified as a prebiotic. Prebiotics are defined as "non-digestible food ingredients that beneficially affect the host by selectively promoting the growth and or activity of a limited number of colonic bacteria that promote health" (Roberfroid, 2007). Prebiotics are considered to be both soluble and fermentable and are unique in their solubility as they do not gel nor modify viscosity in the gut (Parnell and Reimer, 2009).

Resistant starch (RS), is a type of starch that is resistant to digestion and absorption in the small intestine, the bulk of which enters the large colon to be fermented. The large intestinal fermentation of resistant starch purportedly makes it beneficial to colonic health. Starch is mainly found in two forms: amylose and amylopectin. Amylose is a linear polymer consisting of glucose monomers linked by α (1–4) linkages, while amylopectin is a large glucose polymer that is branched with α (1-6) linkages. There are four types of resistant starches (RS1, RS2, RS3 and RS4), classified based on their amylose: amylopectin ratio and the manner in which they are processed. The current study evaluated the effect of RS2 or high amylose maize starch, which is found in green bananas and raw potato (Bird *et al.*, 2010; Hamaker and Tuncil, 2014).

Fibre	Main	Physico-chemical	Solubility	Degree & Location of	References
	Monomer	structure		Fermentation	
Wheat bran	Arabinoxylan	Branched chain polymer	Insoluble	Low fermentability	Stevenson et al.,
		β (1–4) linkage		Distal colon	2012; James <i>et</i>
					al., 2003
<u></u>	0.1	D 1 1 1 1 1	0 1 1 1	D (11	D. H. A. 1. 2010
Oat bran	β–glucan	Branched chain polymer	Soluble	Fermentable	Butt <i>et al.</i> , 2010;
		β (1-3) and (1-4)		proximal colon	James <i>et al.</i> ,
		linkages			2003
Fructo-	Fructose	Short length chains	Soluble	High fermentability	Singh & Singh,
oligosaccharide		β (1–2) glycosidic		Proximal colon	2010; Parnell &
		bonds			Reimer 2011
Type 2 resistant	Amylose	Linear polymer	Soluble	High fermentability	Bird et al., 2010
starch		α (1-4) linkages		Cecum	

Table 1: Structural properties of different DFM

DFM and gut microbiota

The microbial community is varied between different mammals, but similarities exist as the process of acquisition of the microbiota is comparable. Colonization, being time dependent (Topping and Clifton, 2001), is dictated by the sequential introduction of microbes that evolve into a stable trophic structure. Gut microbiota diversity is more evident at the strain level than the genus level, a factor that contributes to functional redundancy by colonizing microbes. This is believed to be an evolutionary attribute selected for by the host to help shape and maintain the stability of the microbial community (Ley et al., 2006). Differences in the structure of gut microbiota community profiles in homogenous animals occur because of changes over time at a functional level, which leads to metabolically diverse phenotypes (Li et al., 2008; Bernbom et al., 2006). Weaning, host genetics, microbiome (collective genome of the microbiota) and diet play important roles in the succession and shift towards the high density of colonization in the mammalian GI tract (Gaskins et al., 2008). A study involving 106 individual mammals representing 60 species, including Wistar rats, was conducted to assess the link between gut bacterial composition and diet. The results of the study showed mammalian microbiota varied across phylogeny and diet (Ley et al., 2008). More recently, findings by Carmody et al., (2015) revealed different types of diets consistently reshaped the gut microbiota community despite differences in host genotype (Carmody et al., 2015).

The selective stimulation of a specific genus or group of gut bacteria and/or a metabolic activity linked with these bacteria is often claimed to be a benefit associated with consumption of DFM. In rats, fructans appear to stimulate the growth of bifidobacteria, lactobacilli and other lactic acid bacteria (Howard *et al.*, 1995; Sghir *et al.*, 1998; Le Blay

et al., 2003), well known for their immuno-modulatory activities (Schley and Fields, 2008; Lebeer, 2010). The fecal microbiota composition of Wistar rats using comparative sequence analysis of 16s rDNA showed the percentage of total clones were divided amongst lactobacilli (most abundant at 23%), *Clostridium coccoides* group (18%), *Clostridium leptum* subgroup (9%), low GC content Gram positive bacteria (13%) and clones for Gram negative bacteria aligned within the *Bacteroides-Cytophaga* lineage (36%) (Brooks *et al.*, 2003). However, the composition of the mammalian gut microbiota remains largely unknown as the majority of bacterial sequences are that of uncultivable and or novel bacteria (Quigley, 2010) and analysis is further complicated because microbial ecology varies along regions in the gastrointestinal tract of most mammals (Zoentendal *et al.*, 2004). Correlating DFM-mediated effects on select bacteria to downstream immune changes can be misleading for two reasons 1) the immune changes would be regionally biased 2) immune cell function is diverse and location dependent in the gut (explained in detail further below).

Our collaborators examined the shift in gut bacterial community structure as a whole in rats fed different dietary fibres (Abnous *et al.*, 2009; Kalmokoff *et al.*, 2013). They noted that major changes in fecal community richness in OB and WB-fed male rats occurred within the *Clostridium coccoides* and *Clostridium leptum* groups relative to rats fed the control diet (Abnous *et al.*, 2009). In contrast, high amylose maize starch (RS2) reduced species richness in both the cecum and feces, resulting in a gut community dominated by *Bacteroidetes* (Kalmokoff *et al.*, 2013). FOS supplementation not only affected the fecal community differently from the other DFMs, but also changed the microbiota structure differently between sexes. In males FOS intake increased the abundance of faecal *Lachnospiraceae*, while in female rats FOS intake altered the community structure by increasing abundance in the Phylum *Bacteroidetes* and reducing abundance of the Family *Lachnospiraceae* (manuscript in preparation). Others have noted microbiota compositional differences between male and female mice fed a basal diet (Markle *et al.*, 2014). Bolnick *et al.* (2014) were the first to show that diet-induced microbiota changes among vertebrate populations including fish in the wild, mice housed in labs and humans differed between the sexes (Bolnick *et al.*, 2014). These findings illustrate DFM with different physicochemical characteristics can impact fecal microbiota composition and that the compositional changes induced by the same diet can be different between sexes, leading to potential differences in secreted microbial metabolites.

DFM and fermentation metabolites

Microbial fermentation of carbohydrates in the intestine results in production of large quantities of metabolites, including short chain fatty acids (SCFA). SCFA production is dependent upon the microbiota composition, transit time and fermentation characteristics of the carbohydrate (Flint *et al.*, 2007). SCFA reach concentrations >80 millimolar in the colon of humans and their quantity is dependent upon the balance between production and absorption rates and the transit time through the gut (Miller, 2004). Organic acids reduce the intra-luminal pH and increase the concentration of ionized minerals (Ca⁺² and Mg⁺²) which favours the passive diffusion of SCFA into IEC (Roberfroid, 2007; Perez de la Cruz, 2006). SCFA stimulate the absorption of water and salt, which increases the moisture of the cecal digesta, leading to an increase in intestinal motility (Sabater-Molina *et al.*, 2009). Acetate, butyrate and propionate, the main types of SCFA, are usually present in molar concentrations of 3:1:1 and have been shown to be important to colonic health (Miller,

2004). RS intake in humans can induce butyrate production, however this was shown to vary between individuals, leading to a higher rate of production and excretion of butyrate in males compared to females (McOrist *et al.*, 2011). Rats fed the RS-supplemented diet used in this study also had high concentrations of total SCFA and butyrate in their cecal and fecal matter (Kalmokoff *et al.*, 2013). WB, being highly lignified, would be anticipated to support only low amounts of SCFA production, however wheat bran intake can increase the levels of total SCFA and butyrate in the cecum of rats (Chen *et al.*, 2011).

Butyrate is preferred over glucose to undergo β -oxidation by colonocytes for energy, influencing IEC growth, differentiation and apoptosis (Marteau *et al.*, 2004, Miller, 2004; Singh *et al.*, 1997; Barnard and Warwick, 1993; Bailon *et al.*, 2010). Cummings *et al.*, (1987) noted that the molar ratios of acetate, butyrate and propionate changed from colonic contents to portal blood to hepatic vein, and that butyrate levels were the lowest outside of the gut (Cummings *et al.*, 1987). Butyrate's straight chain 4-carbon monocarboxylic configuration may play a role in its quick absorption and metabolism by gut epithelial cells (Miller, 2004). Butyrate is also well characterized as a histone-deacetylase (HDAC) inhibitor. It can increase the acetylation of histone and non-histone proteins allowing it control over gene expression. However, butyrate's HDAC property induces a paradoxical effect on neoplastic versus normal colonocytes (Vinolo *et al.*, 2011; Wu *et al.*, 2012; Schilderink *et al.*, 2013).

The effect of butyrate on colonocytes has been shown to vary depending upon the type of fibre serving as the substrate for butyrate production. For example, Zoran *et al.* (1997) compared the effect of oat bran and wheat bran ingestion on azoxymethane-induced colonic tumor growth in rats. Wheat bran feeding induced higher butyrate levels over

propionate levels, but it was the significant increase in distal butyrate concentrations from oat bran-intake that induced the development of more tumors in the rat colon (Zoran *et al.*, 1997). In contrast, high butyrate concentrations in rats fed a high amylose starch diet correlated negatively to colonocyte DNA damage (measured as single strand breaks) relative to rats fed a Western-type diet (Conlon *et al.*, 2012). These results illustrate how different types of fermentable substrates modulate host biology differently and that DFMmediated effects may potentially extend beyond those induced by SCFA alone.

The gut epithelium

The intestinal lumen has the highest burden of microbes and secreted microbialmetabolites and as such, defences are required by the host to protect internal tissue from invasion and subsequent sepsis. Innate responses start with tight barrier protection provided by IEC. IEC join together through the action of tight junction proteins (occludins, claudins and zona occludins) to form a monolayer that is usually impenetrable by both commensal and pathogenic microbes (Kunisawa and Kiyono, 2005; Neish, 2002; Ismail and Hooper, 2005). The epithelium not only acts as a physical barrier but employs surveillance tactics that allow it to interact with the luminal environment. The duality in epithelium activity helps to cement the central idea of gut immunity: immune tolerance.

Immune responses in the gut lead to neutralizing a foreign presence rather than clearing the intruder via an inflammatory process. Inflammation is counter-productive in the gut unless required for dealing with infection, as the antigenic load from the microbiota and food particles would otherwise constantly trigger inflammatory responses, as the innate arm of the immune system lacks memory. Instead IEC interactions with luminal microbes induces a state of "physiological inflammation" that is both transient and self-limiting, and is modulated by immune cells in the underlying gut associated lymphoid tissues (GALT), such as macrophages and dendritic cells. The microbial composition helps determine whether the epithelium responds in an inflammatory manner or non-inflammatory manner (Schiffrin and Blum, 2002). A loss in specific gut microbial populations and/or loss of immune regulation at the host level, leads to intestinal disorders and disease and is termed dysbiosis. For example, one of the most abundant bacterium within the *Clostridium leptum* group, *Faecalibacterium prausnitzii*, has been found to be defective in the microbiota of patients suffering from Crohn's disease (Sokol *et al.*, 2008). The host-microbiota relationship is bi-directional and the communication is maintained by a dynamic epithelial barrier that also acts as the first line of defense.

Activation of epithelial innate mechanisms

IECs can detect microbe-associated molecular patterns (MAMPs) through various types of innate immune sensors that are collectively known as pattern recognition receptors (PRR). Upon sensing either a commensal or pathogenic microbe, PRRs start a signalling cascade, activating MAP kinases and downstream transcription factors, including nuclear factor- κ B (NF- κ B) (Neish, 2002; Parhar *et al.*, 2003). NF- κ B is held in an inactive form in the cytosol, by the inhibitory protein I κ B α . The events leading to transcription of proinflammatory mediators are preceded by the phosphorylation of I κ B α , as a result of PRR activation. Following phosphorylation, I κ B is ubiquitinated, which tags it for rapid proteosome degradation, and leads to the release of NF- κ B. Free NF- κ B translocates into the nucleus and binds to DNA to start transcription of genes encoding several proinflammatory mediators (Neish, 2002; Ismail and Hooper, 2005). The expression of rat chemokine cytokine induced neutrophil chemoattractant (CINC)-1, a homologue of human IL-8, is controlled by NF- κ B (Reed, 2005). The transcription of chemokines, such as CINC-1, leads to the recruitment of cytokine-releasing T cells, dendritic cells, natural killer cells, neutrophils and basophils that mount an inflammatory response that helps clear the microbe (Hooper and Macpherson, 2010; De Palma *et al.*, 2009). CINC-1 can be secreted by macrophages or IECs (Alonso, 1996; Yoshida, 2002) and CINC-1 concentrations form a gradient for neutrophil infiltration in areas of inflammation (Takaishi, 2000).

NF- κ B also mediates the transcription of several other pro-inflammatory cytokines. Cytokines are soluble proteins that are by far the most important orchestrators of immune activity and are secreted, by a variety of different immune cell types. They can be generally classified on the basis of their activity as lymphocyte growth factors, pro-inflammatory, anti-inflammatory or regulatory mediators. However, these effector molecules tend to be pleiotropic in nature, examples of which are detailed below. Cytokines evolved from early intracellular signalling molecules before the appearance of receptors and were first detected as a "soluble factor" in pus exudates. Their mode of action is comparable to that of hormones, in that they perform in an autocrine or paracrine manner, however their key role is in directing immune activity (Dinarello, 2007).

SCFA can act on IEC through G-coupled protein receptors GPR43 and GPR41 to induce cytokine and chemokine production through mitogen-activated protein kinase (MAPK) signalling (Kim *et al.*, 2013). Butyrate down-regulates NF-κB mediated proinflammatory cytokine production in IECs, potentially via apical GRPR109A (Kumar *et al.*, 2009; Cox *et al.*, 2009; Thangaraju et al., 2009). Kumar *et al.*, (2009) demonstrated physiological

concentrations of butyrate de-neddylated Cullin-1, a subunit of the ubiquitin ligase, thus suppressing the release of NF- κ B. They noted the de-neddylation was linked to butyrate-induced increase of reactive oxygen species (ROS) by IEC (Kumar *et al.*, 2009). However an earlier study by Nenci *et al.*, (2007) showed NEMO (NF- κ B essential modulator) knockout mice spontaneously developed chronic intestinal inflammation. NEMO^{-/-} mice displayed a compromised epithelial barrier, allowing tumor necrosis factor (TNF), a potent immune stimulant, to evoke epithelial apoptosis (Nenci *et al.*, 2007). Collectively these findings establish that epithelial barrier integrity is linked to NF- κ B activation and point to a role for butyrate in influencing barrier integrity through down-regulatory effects on NF- κ B activation. Rats fed different types of DFM would have a differential SCFA profiles and levels of butyric acid production, which may in turn produce DFM-associated alterations to gut epithelial integrity

Pathogen Recognition Receptors: The surveillance team

The most important PRRs are the Toll Like receptors (TLRs) that were first discovered in *Drosophila* (Medzhitov, 2007) and were later shown to play an important role in epithelial homeostasis through interactions with commensal bacteria (Rakoff-Nahoum *et al.*, 2004). TLRs are present on both the apical and basolateral side of IECs and other immune cells, especially dendritic cells and macrophages, and their expression varies along the gut (Abreu, 2010). Thirteen TLR have been identified in mammals, of which TLR 1, 2, 4, 5, 6 and 9 recognize bacterial constituents (Schiffrin and Bloom, 2002). TLR4 detects the presence of lipopolysaccharides (specifically hexa-acylated LPS) of Gram-negative bacteria and TLR5 recognizes bacterial flagellin (Ismail and Hooper, 2005). TLR2 recognizes lipotechoic acids within the peptidoglycan of Gram-positive bacteria (Abreu, 2010). TLR2 is also known to hetrodimerize with TLR1 and TLR6, when activated by triacylated or diacylated lipoproteins, respectively (Takeuchi *et al.*, 2002). A recent study by Hayashi *et al.* (2013) demonstrated that *Clostridium butyricum* induced IL-10 production in intestinal macrophages via TLR2 in mice with experimental colitis (Hayashi *et al.*, 2013). Resident microbiota also participate in maintaining basal level inflammation through TLR2 interaction, which leads to the transcription of antimicrobials and defensin peptides, resulting in homeostasis (Mazmanian, 2005). TLR9 is expressed in the endosome membrane and recognizes particular unmethylated CpG DNA motifs of native bacterial cells (Verthelyi and Zeuner, 2003; Courthesy and Gaskins, 2007) and thus is a critical player in the downregulation of inflammatory responses.

The highly conserved Toll/Interleukin-1 domains of TLRs, when bound to their ligands, interact with the adaptor protein MYD88 (myeloid differentiation primary-response protein) and start the signalling cascade for nuclear factor- κ B (NF- κ B) activation (Lebeer *et al.*, 2010; Ismail and Hooper, 2005). While most TLRs primarily act through the MYD88 pathway, TLR3, which detects viral double stranded RNA, instead triggers the MYD88-independent pathway. This was ascertained from studies utilizing MYD88^{-/-} knockout mice, where TLR3 stimulation was noted to still activate NF- κ B. TLR3 activation does not involve the Toll/ Interleukin 1-MYD88 interaction, but instead utilizes the adaptor protein TRIF (Toll/Interleukin1 domain containing adaptor inducing IFN- β). TRIF in turn induces the transcription factor IRF-3 which associates with I κ B kinases leading to NF- κ B activation. The MYD88 independent pathway also leads to the transcription of IFN- β inducible genes such as IP-10 (Takeda and Akira, 2004).

Another means of NF- κ B activation is through the nucleotide binding oligomerization domains (NOD1 and NOD2), pattern recognition receptors found in the cytoplasm which also recognize peptidoglycans (Tlaskalova-Hogenova, 2005). NOD1 and NOD2 are expressed by IEC and antigen presenting cells (APC), such as dendritic cells, but are not expressed by lymphocytes. NOD 1 binds to Gram negative bacterial ligands and NOD2 recognize ligands of both Gram positive and Gram negative bacteria. Their activation through ligand binding leads to NF- κ B activation and pro-inflammatory gene upregulation (Strober *et al.*, 2006).

Carbohydrate-specific receptors, a separate category of PRRs, recognize carbohydrate domains. These receptors are a reflection of convergent evolution, as they have been identified in animals, yeast and bacteria and are dependent on Ca^{2+} binding for their activation (Taylor and Drickamer, 2014). PRRs such as C-type lectin receptors (CLR), including dectin-1, can recognize carbohydrate sugar moieties. Dietary oat β -glucan acting via dectin-1 receptors on immune cells (Volman et al., 2010) has been shown to reduce the expression of pro-inflammatory cytokines (Chanput et al., 2012). Many dietary components demonstrate direct immune-modulatory effects via different types of CLRs (Ortega-Gonzalez et al., 2014; Kovacs-Nolan et al., 2013). For example, fructooligosaccharides (FOS) can act through Peptidoglycan Recognition Proteins on IEC, affecting cytokine production (Zenhom et al., 2011). However, MYD88 and TLR4 gene knock down in IEC-18 cells was shown to decrease the production of MIP-2 (macrophage inflammatory protein-2) and GRO- α (growth-related oncogene) when stimulated with oligosaccharides. These findings suggest oligosaccharides could induce proinflammatory cytokine production directly via interactions with TLR4 (Ortega et al., 2014). Taken

together these studies show that DFM also can directly influence immune parameters independent of microbiota changes or fermentation-related activity and that these effects are based on the ability of DFM components to engage PRR.

Sex hormone-mediated effects on cells of the immune system are well documented (Fish, 2008; Verthelyi, 2006) although few studies have examined their effects on TLRmediated immune activity. Testosterone has been shown to decrease TLR4 expression, and this is speculated to be a possible mechanism for immune suppression in males (Fish, 2008). Expression of peripheral TLR-induced pro-inflammatory cytokines has been shown to be dependent upon the menstrual cycle (Dennison *et al.*, 2012). Recently the expression of TLR 2 and 4 has been reported to be sex-biased in mice affected with Coxsackie virus (Roberts *et al.*, 2013), with male mice being more susceptible to virus-induced myocarditis due to a suppression in regulatory T cells via TLR signaling (Roberts *et al.*, 2013). In contrast, rodent astrocytes stimulated with LPS did not demonstrate a difference in TLR4 expression between sexes. Taken together DFM intake may have differential effects on TLR activation between sexes at the level of the gut epithelium.

Mucin: Epithelial protector and microbiota fodder

Mucin is a gelatinous glycoprotein secreted by goblet cells of the intestine. It is a major barrier that separates the microbiota in the lumen from the intestinal epithelial surface (Deplancke and Gaskins, 2001; Neish, 2002; Hooper and Macpherson, 2010). Early studies of the actions of dietary fibres and resistant starch used changes in intestinal mucin as a physiological measure to understand the effects of fibre. For example, Satchitanandam *et al.* (1990) compared the effect of feeding two types of fibres (citrus fibre and guar gum)

on tissue versus luminal mucin. They measured mucin from the stomach, small intestine and colon of male rats. Rats fed 5% citrus fibre had the highest amount of luminal mucin in the small intestine relative to rats fed either the fibre free diet or the 5% guar gum diet; no changes in tissue mucin levels were noted. The authors hypothesized that the change in luminal mucin may result in alterations to nutrient absorption (Satchitanandam et al., 1989). Years later Morita et al. (2008) followed up by confirming that adding an insoluble fibre (polystyrene foam) to the diet increased luminal mucin content in male rats independent of nutrient absorption and that consumption of insoluble fibres stimulated continuous mucin secretion even after food depletion (Morita et al., 2008). Besides providing additional barrier protection, mucin is a source of nutrition provided by the host to maintain the local gut microbiota populations. A regional bias to the pattern of mucin glycosyl modifications has been suggested as a strategic response to accommodate the nutritional needs of resident microbiota (Van Tassell, 2011). Studies have focused on understanding the regional bias in mucin secretion with particular fibre diets, as a way to evaluate host-microbiota interactions, and to correlate mucin production to potential health benefits to the host. For example, long chain arabinoxylan and inulin feeding in germ free mice inoculated with human gut microbiota led to an increase in cecal mucin relative to control fed mice, and shifted mucin degradation to the distal colon. The authors noted the prebiotic-supplemented diets changed the microbiota structure by inducing the growth of butyrate and propionate producers and altering the population of Akkermansia muciniphila, a mucin degrader found primarily in the human gut. The shift in mucin degradation to the distal colon was speculated to be due to a lessening in the utilization of host-derived nutrients by local microbiota following prebiotic-intake, allowing for a more balanced

metabolism of mucin along the entire length of the gut (van den Abbelee *et al.*, 2011). Insoluble and soluble fibres have also been noted to alter the type of mucin produced by the host. Mucin glycoproteins terminate in either sialic acid or sulfate sugar residues and the terminal moiety dictates their utilization by bacteria. The presence of a sulfate group makes the mucin less accessible to bacterial mucinases. Rats fed different types of insoluble and soluble fibres demonstrated an increase in goblet cell proliferation in the ileum, accompanied by sialylated mucin production (Hino *et al.*, 2012), while pigs fed RS2 demonstrated an increase in hindgut mucin sulfation (Nofrarias *et al.*, 2007), illustrating the potential for different substrates to influence mucin sulfation profiles and accessibility of this substrate to gut microbes.

Besides its relevance to gut physiology, mucin plays a role in gut immune regulation. Secreted mucin (MUC2) can act as a molecular signal that circulating dendritic cells in the underlying gut-associated lymphoid tissue (GALT) actively detect. MUC2 can assemble a signal transducing complex on dendritic cells that can block NF- κ B activation and induce the secretion of IL-10 (a regulatory cytokine), effectively suppressing the transcription of pro-inflammatory mediators (Shan *et al.*, 2013). Thus it is reasonable to expect changes in local mucin production from fibre intake could induce measurable alterations to local tissue cytokines under resting conditions.

Gut Associated Lymphoid Tissue (GALT): Where decisions are made

To understand the potential effect fibre consumption may have on the gut mucosal immune system it is necessary to understand the architecture of the GALT. The GALT lies underneath the gut epithelium and can be partitioned based on inductive and effector
immune functions. The inductive sites of the GALT include the Peyer's patches (PP) and the intestinal lymphoid follicles (ILF), while the lamina propria is the main effector site (Tsuji *et al.*, 2008). The population density and organization of immune cells varies between the organized inductive sites of the Peyer's Patches in the ileum and the morphologically heterogenous intestinal lymphoid follicles (ILF) of the colon, to the more diffuse effector site of the lamina propria located beneath the intestinal mucosa (Brandtzeg, 2010).

Adaptive immune responses in the GALT are carried out by T and B lymphocytes. For an effective adaptive immune response to occur, APC need to be activated. APC bearing the marker CX_3CR1^+ sample pathogenic and non-pathogenic bacteria from the lumen by expressing receptors for tight junction proteins that allows them to extend their dendrites between enterocytes (Smith and Nagler-Anderson, 2005). However, recent evidence demonstrates that CX₃CR1⁺ APC, previously thought to be dendritic cells (DC), are actually gut tissue macrophages, as these cells do not migrate to peripheral lymph nodes and are poor at priming naïve T cells to differentiate into effector T cells (Denning et al., 2007; Mann et al., 2013). APC present processed antigen to T cells via the major histocompatibility complex (MHC). There are two classes of MHC molecules: MHC class I and MHC class II. Expression of MHC I on APC initiates CD8⁺T cell engagement, while MHC II presents antigen to CD4⁺ T cells. The differentiation and proliferation of naïve T cells is also dependent upon the cytokine milieu and expression of co-stimulatory surface molecules on APC (CD40, CD80 and CD86). The alterations in spatial distribution and the phenotypic character of DC and lymphocytes in the follicular epithelium of conventionalized rats versus germ-free rats was shown to reflect location-specific interactions of the gut microbiota with the underlying GALT (Yamanaka *et al.*, 2003).

The gut T cell population includes intestinal intraepithelial lymphoid (IEL) cells and naïve T cells that migrate from the periphery. IELs are considered extra-thymic as their presence was first discovered in athymic mice and they were noted to be different from thymus selected T cells, which make up a substantial cohort of peripheral T cells. Thymicderived T cells predominantly express $\alpha\beta$ T cell receptors (TCR) and are deleted in the thymus if they respond to self-antigens through a process of negative selection. In contrast, IELs are a mixed population of T cells that contain a sub-population that expresses the $\gamma\delta$ TCR and require self-antigen presentation to be selected. Recognition of self-antigens by $\gamma\delta$ T cells help maintain oral tolerance in gut mucosal tissues (Rocha *et al.*, 1992; Kapp *et al.*, 2004). Resident microbiota, sloughed epithelial cells (renewed every 4-5 days) and mucin proteins are a sources of "self-antigens". These "self-antigens" can be captured directly by circulating DCs in the Peyer's patches or can be passed on to the DCs by microfold (M) cells present in the follicular epithelium.

DC can induce the differentiation of CD4⁺ T cells into effector regulatory T cells (T_{reg}) and this T cell phenotype is key to modulating gut mucosal immune responses. Under homeostatic conditions, the milieu in the gut associated lymphoid tissue has a continuous background activation of T_{reg} cells, leading to the release of IL-10 and transforming growth factor (TGF)- β , which help maintain an environment of immune tolerance (Christensen, 2002; Mowat, 2003; Rook and Burnet, 2004). The T_{reg} subtype CD4⁺ CD25⁺ is regulated by a critical lineage-specific transcription factor: FoxP3 (Kang *et al.*, 2007). DCs positive for the surface receptor CD103 migrate from the PP to the mesenteric lymph node (MLN) and present antigen from commensal bacteria to naïve T cells, differentiating them into FoxP3⁺ T_{reg} cells. In contrast, macrophage populations support the maintenance of FoxP3⁺ T_{reg} cells in the lamina propria, where macrophage numbers are higher (Coombes and Powrie, 2008). IL-10, an important regulatory cytokine, is not only secreted by T_{regs} (Atarashi *et al.*, 2011) but also by intestinal macrophages under steady state conditions (Mantovani *et al.*, 2004; Takada *et al.*, 2010). FOXP3 is constitutively expressed by T_{reg} cells and mutations in the FOXP3 gene cause an X-linked autoimmune condition (immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome), whereby T_{reg} cells cannot produce the cytokine environment necessary for immune regulation in affected male infants (Fish, 2008). FOXP3 stimulation is dependent upon TGF- β (Izcue *et al.*, 2006); for instance, *in vitro* stimulation of CD4⁺CD25⁺FoxP3⁻ T effector cells in the presence of TGF- β resulted in FOXP3 upregulation, generating CD4⁺CD25⁺FoxP3⁺ T cells with regulatory properties (Kang *et al.*, 2007).

Both the microbiota and microbial-derived antigens help maintain the regulatory milieu in the gut. For example, both Clostridia-related segmented filamentous bacteria and polysaccharide A of *Bacteroides* have been shown to induce T_{reg} populations essential for mediating tolerance in the intestine (Izcue *et al.*, 2006; Hooper and Macpherson, 2010). *Bacteroides fragilis* release zwitterionic polysaccharides that induce the production of CD4⁺ T cells via TLR2 and can correct certain immune defects (Mazmanian *et al.*, 2005). *F. prausnitzii* when administered orally can increase IL-10 production by peripheral blood mononuclear cells (Round and Mazmanian, 2009), however culture supernatants from *F. prausnitzii* have also demonstrated this effect, suggesting a secreted component is involved in increasing IL-10 production (Sokol *et al.*, 2008). Another key study showed that indigenous gut populations of *Clostridium* clusters IV and XIVa promoted the accumulation of TGF- β -secreting FoxP3⁺ T_{reg} cells in the colonic lamina propria (Atarashi *et al.*, 2011). The study demonstrated specific pathogen free (SPF) mice treated with the antibiotic vancomycin, which targets Gram-positive bacteria, showed lower T_{reg} frequencies in the colon compared to the control mice. They also noted a significant increase in TGF- β levels in the colon and IEC of germ-free mice inoculated with *Clostridium* (Atarshi *et al.*, 2011).

Although TGF- β is an integral T_{reg} regulator, IL-6 is known to synergize with low concentrations of TGF- β to induce naïve T cells into pro-inflammatory TH₁₇ cells, illustrating the versatile function of TGF- β in the gut (Veldhoen, 2006; Hu, 2011). TH₁₇ cells are rare in the GALT; their numbers decline from duodenum to ileum and they are barely found in the healthy colon (Denning *et al.*, 2011). Although there is as yet no conclusive evidence about the type of APC that induces TH₁₇ differentiation, recent evidence suggests the T cell/APC ratio in the lamina propria dictates DCs to efficiently promote the TH₁₇ lineage in a location-specific manner (Denning *et al.*, 2011). Microbial ATP has also been shown to activate CX₃CR1⁺ APCs to promote TH₁₇ differentiation (Atarashi *et al.*, 2008). In addition, ATP can induce IECs to secrete IL-6 by influencing their response to TLR ligands (Yao *et al.*, 2012), which may help facilitate T_H17 differentiation *in vivo*. The TH₁₇ lineage can also be induced by segmented filamentous bacteria, which adhere to the small intestinal epithelium of mice (Ivanov *et al.*, 2009).

Mucosal immune changes resulting from DFM ingestion can influence different elements of the GALT. For example, *in vivo*, inulin or FOS consumption can increase lymphocyte numbers in the Peyer's patches of female rodents under healthy and endotoxemic conditions, impacting the main inductive portion of GALT in the small intestine (Manhart *et al.*, 2003; Ryz *et al.*, 2009). In contrast, Smith *et al.* (2013) recently demonstrated that the microbiota-derived SCFA propionate induced an increase in colonic T_{reg} numbers, along with increased expression of FoxP3⁺ and IL-10, but not TGF- β . T_{reg} frequency or number in the MLN of SPF mice were unaffected by SCFA administration. The authors determined SCFAs acted on free fatty acid receptor (FFAR) 2 to affect colonic T_{reg} activity by HDAC inhibition (Smith *et al.*, 2013).

Taken together these studies illustrate the GALT responses are a) constantly evoked by multiple gut-derived factors b) driven by the co-ordinated activity of various cell types and cytokines in a location-dependent manner c) geared towards maintaining an environment that supports immune regulation and d) possibly different between sexes.

IgA: The adaptive immune response of the GALT in action

It has been suggested that adaptive immunity has been evolutionarily selected in vertebrates to manage varied microbial symbionts (Neish, 2009). The gut microbiota is crucial for the induction of 75% of secreted IgA (Macpherson and Slack, 2007). Secretory IgA, besides forming a barrier against invasion, neutralizes pathogens through non-inflammatory high affinity binding (Cerutti, 2008). Gut microbiota signaling drives IgA synthesis by engaging the innate immune system at the epithelial interface (Macpherson and Uhr, 2004). Microbiota-driven IgA production occurs through a recurrent process in

which the innate immune system is activated, initiating an inflammatory response, following IgA production that dampens the inflammatory response by neutralizing the microbe. Successful commensal microbes maintain the cyclical process by constantly changing their antigenic determinants so as to engage the adaptive immune system of the gut to induce tolerance and evade the memory response against them (Peterson et al., 2007). However under TLR deficient conditions the adaptive system has been shown to effectively clear translocated commensal bacteria at the systemic level, illustrating the host immune system is capable of maintaining regulation against resident gut microbes in the absence of innate input (Slack et al., 2009). In addition, using a reverse germ free colonization model it was discovered that microbiota-mediated IgA synthesis is dependent upon a population threshold of 10^8 - 10^9 bacteria and that the concentration of secreted IgA constantly adapts to the microbial population (Hapfelmeier *et al.*, 2010). These studies establish that mucosal adaptive immune responses are very susceptible to changes in the microbiota, which would in turn vary depending on diet and host genotype, potentially resulting in differential outcomes between male and female rats fed different types of DFM.

A number of studies have shown FOS intake up-regulates secretion of IgA in cecal and fecal contents of rodents (Hosono *et al.*, 2003; Nakamura, 2004; Manhart, 2009). Fecal IgA levels increased in rats fed a WB supplement relative to their control fed counterparts (Gannon, 2009). In contrast, Stillie *et al.*, (2005) noted rats weaned on a diet with a fermentable substrate (inulin) showed no change in the percentage of IgA⁺ B cells in the lamina propria (Stillie *et al.*, 2005). A recent fibre feeding trial in pigs which included wheat bran also showed no diet-induced changes to either ileal or colonic secretory IgA levels (Chen *et al.*, 2015). These findings underscore the variation in mucosal IgA secretion depending on type of DFM, but also highlight location-based differences in mucosal IgA levels. Sex-associated variations in secretory IgA levels have also been noted in specific mucosal locations (Dahlgren *et al.*, 1991).

Secretory IgA production can occur through either a T cell-dependent or a T cellindependent pathway (Mora *et al.*, 2006; Artis, 2008; Cerutti, 2008) and is directed by a suite of different cytokines including IL-4, TGF- β , IL-10 and IL-6 (Bradtzaeg, 2009). In the T cell-dependent pathway, APC present antigen to CD4⁺ T cells and activate them to express CD40 ligand (CD40L). Mature antigen-specific B cells, expressing CD40, in the germinal centers of the Peyer's patch undergo class switching in response to T cell signals delivered through CD40L-CD40 interaction (He *et al.*, 2007).

The T cell-independent pathway can be activated under steady state conditions (Kunisawa and Kiyono, 2005). The T cell-independent pathway helps produce polyclonal IgA quickly against commensal microbiota and is facilitated by IEC. Bacterial ligands bind to TLR expressed on IECs, initiating the secretion of retinoic acid, which stimulates lamina propria DC. The lamina propria DCs are stimulated to secrete B cell Activating Factor (BAFF) and A Proliferation Inducing Ligand (APRIL), two B cell stimulating factors of the TNF family that are similar in structure and function to CD40L. IL-4, TGF- β , IL-10 and IL-6 trigger DNA-editing enzyme activation induced cytidine-deaminase (AID) induces somatic hypermutation and class switch recombination in B cells at the lamina propria. AID then directs a class switch from the IgM isotype to the IgA isotype (He *et al.*, 2007; Cerutti, 2008; Macpherson and Slack, 2007). Macpherson *et al.* (2000) showed IgA synthesis against commensal bacteria was mainly induced via the T cell-independent

pathway, and that T cell-independent IgA synthesis did not need to occur in organized follicular regions such as the PP, which has germinal centres with B and T cell regions. In their study, anti-commensal IgA produced in mice deficient in the T cell receptor (TCR^{-/-}) and normal control mice were identical, highlighting the lack of T-B cell interaction in T cell-independent IgA production (Macpherson *et al.*, 2000).



Figure IA, adapted from Macpherson and Harris (2004). Gut microbiota and gut-derived metabolites interact with immune cells of the GALT to induce the production of IgA-secreting plasma B cells. IgA-bearing plasma B cells drain from the MLN into the circulation via the thoracic duct. Plasma B cells, home to the lamina propria to secrete IgA. Secreted IgA is transcytosed across the epithelium and adheres to the mucin lining, allowing IgA to form a protective layer while neutralizing invading microbes.

IgG: The systemic adaptive immune response

A major difference between the adaptive immune response in the mucosal compartment versus the systemic compartment is the isotype (class) of antibody produced. Immune tolerance in mucosal sites is facilitated by IgA, which neutralizes invading microbes without additional inflammation. In contrast, a systemic adaptive response leads to pathogen clearance by IgG, an antibody that fixes complement via the classical pathway for effective clearing by macrophages (Janeway, 2001).

Keyhole limpet hemocyanin (KLH), a copper-containing respiratory protein from the marine gastropod *Megathura crenulata*, is a potent T cell-dependent antigen that has been extensively used in immune studies due to its robust induction of cellular and humoral immune responses in different mammals including humans and rodents. KLH is made up of two subunits (KLH1 (390 KDa), KLH2 (360 KDa) and 4% oligosaccharides. The carbohydrate portion has been thought to contribute to the protein's antigenic properties (Harris and Markl, 1999; Kawai *et al.*, 2013; Fujihara *et al.*, 2014). Parenteral administration of KLH initiates a primary response in the spleen, a peripheral lymphoid organ that filters circulating antigen directly from the blood.

In addition to B and T cells that enter the spleen from the periphery in search of cognate antigen, the splenic environment is rich in APCs. Antigen from the blood is captured and processed by APCs and presented to T cells via MHC. The differentiation and proliferation of naïve CD4⁺ T cells is dependent upon the cytokine milieu: IL-12 and IL-4 induce T_{H1} and T_{H2} effector T cells respectively. T_{H1} cells produce IFN γ and are activated during infections with intracellular pathogens, while T_{H2} cells produce IL-4, a cytokine that is upregulated during helminth infections and allergic reactions, but is also

necessary for B cell class switching. T_H cells provide help to B cells through co-stimulatory molecules and cytokines, inducing antibody-secreting plasma B cells. A successful immunization response is achieved when there is balance between T_H1 and T_H2 activity, leading to IgG secreting plasma B cells (Janeway, 2001; Bronte and Pillet, 2014).

The gut-systemic axis

It is generally understood that commensal bacteria and gut-derived antigens that cross the epithelial barrier and enter underlying gut tissues, to a large extent, are compartmentalized from systemic sites by the mesenteric lymph node (MLN). The MLN resides outside of the GALT and acts as a sieve for GALT drainage. The MLN has been characterized as the "firewall" of the GALT as it provides an important location for T cell tolerization, through T_{reg} activation, leading to the production of regulatory cytokines TGF- β and IL-10. IgA production in the MLN is another means by which translocation of commensal bacteria into underlying systemic tissues is blocked (Macpherson and Uhr, 2004). Macrophages in the MLN phagocytose stray microbes, preserving systemic ignorance to commensals, and preventing systemic tolerance which would prove fatal in case of commensal-induced sepsis (Macpherson and Uhr, 2004).

However, recent studies have shown that some microbes and microbiota-derived components do enter into the circulation and affect systemic immune activity. For example, the bacterial cell wall component peptidoglycan primes the innate immune system systemically under basal conditions, acting through NOD1 receptors to enhance neutrophil activity (Clarke *et al.*, 2010). Recent evidence also shows the liver serves as the vascular "firewall" at the systemic level to help capture gut bacteria that have entered into venous blood circulation during pathological conditions (Balmer *et al.*, 2014). These findings

suggest that the gut is not completely compartmentalized from the systemic system as initially thought, and that DFM-intake induced alterations to the gut microbiota community could in turn effect changes to systemic immune parameters. Rats fed an inulinsupplemented diet demonstrated a decrease in splenic $\alpha\beta$ CD8⁺ T cells and $\gamma\delta$ T cells numbers relative to control-fed rats (Ryz *et al.*, 2009). Splenic T helper lymphocyte numbers were also observed to decrease in pigs fed resistant starch (Nofrarias *et al.*, 2007). However, the number of DFM-related studies that have focused on the liver as a site of systemic immune change are limited.

Kupffer cells (liver macrophages), hepatocytes and endothelial cells also help maintain immune regulation at the liver by inducing tolerance to gut-derived antigens (Crispe, 2008; Yu *et al.*, 2008; Thomson and Knolle, 2010). Dahlgren *et al.* (1991) demonstrated in oophorectomized rat's treated with estradiol, immunization in the PP with *E.coli* O6 led to an increase in levels of biliary total IgM and anti-LPS IgM relative to sham-operated control rats. These findings illustrate the effect of sex-hormones on gutderived antigen stimulated liver B cells (Dahlgren *et al.*, 1991). In mice, liver immune defense-related gene expression was shown to differ between sexes (Yang *et al.*, 2006). Sexual dimorphism has also been noted in rat liver gene expression (Ahluwalia *et al.*, 2004). Therefore, changes in liver immune markers could serve as a downstream indicator of effects of DFM intake at the mucosal level between sexes.

3. Study Objectives

This study examined changes in mucosal and systemic immune measures arising from gut microbiota community and SCFA profile alterations in rats fed different DFM. For some diets the gut microbiota community analysis was conducted by our collaborators at Health Canada and Agriculture and Agri-Food Canada, as another aspect of this study but these data are not included here. *In vitro* approaches were also utilized to examine timing and dose-dependent effects of the SCFA butyric acid on chemokine production by TLR agonist-challenged IEC to gain insight into potential mechanisms of action.

Objective 1: To compare the effects of different types of DFM on mucosal and systemic immune parameters under steady state conditions

Although several studies have demonstrated the effects of DFM on immune parameters, these have mainly focused on the anti-inflammatory immune effects mediated by fibre (Cavaglieri *et al.*, 2000; Cavaglieri *et al.*, 2003; Qu *et al.*, 2005; Bassaganya-Reiera *et al.*, 2011; Fan *et al.*, 2012). In addition, many of these observations pertained to changes observed in immune-stimulated rodent models or in animals exposed to pro-inflammatory or carcinogenic stimuli (Zoran *et al.*, 1997; Perrin *et al.*, 2000; Clarke *et al.*, 2008; Cao *et al.*, 2011). In contrast, a variety of carbohydrates are routinely consumed by healthy subjects, who would not necessarily be subject to these types of immune challenges. The initial aim of this work was to assess the impact of fermentable substrates (wheat bran (WB), oat bran (OB) and resistant starch (RS)), with distinct physico-chemical structures, fermentative characteristics and effects on gut community composition and mucosal and systemic immune cell population percentages and tissue cytokine levels under unperturbed

immune conditions. Analysis of serum biochemical measures and short-chain fatty acid profiles was also conducted.

Objective 2: To compare the effects of DFM on mucosal and systemic immune measures under steady state conditions between male and female rats

The effects of dietary fibre intake have predominantly been examined in male animal models, while health claims are often extrapolated to both sexes. The inclusion of females in this animal study was initiated by the animal care committee at Health Canada. However, recently the National Institutes of Heath also mandated the inclusion of females in animal studies to redress the over-reliance on male animals in pre-clinical trials (McCullough et al., 2014). Considering that sex-based differences in immune parameters are well recognized (Ahmed et al., 1985; Klein, 2004; Verthelyi, 2006), and that recent evidence suggests differences in microbiota composition between sexes are dependent upon interactions between sex and diet (Markle et al., 2014; Bolnick et al., 2014), we anticipated sex-based differences would also affect gut microbiota composition and SCFA profiles, leading to differential downstream immune outcomes. We thus examined the impact of a highly fermentable prebiotic, fructo-oligosaccharide (FOS), and the slowly fermented fibre WB, on serum biochemical measures including blood urea nitrogen, serum lipopolysaccharide concentration and fecal short chain fatty acid profiles, along with gut mucosal and systemic immune parameters, between male and female rats.

Objective 3: To compare the effects of *DFM* on systemic adaptive immune responses to immunization between male and female rats

In the work described in trials 1 and 2, liver IgA and cytokine levels were altered in male rats fed either FOS, RS or OB supplemented-diets, while WB consumption affected splenic immune measures in both sexes. This suggested that gut-derived components are capable of extending their influence on immune parameters to systemic sites. The immune profile generated within local mucosal and systemic microenvironments under steady state conditions would be better understood in the context of overall health by comprehensively examining the influence of fibre on the host response to immunogenic stimuli. We explored this idea by immunizing male and female rats, two weeks into their feeding trial with the aforementioned DFM. A standard T cell-dependent antigen, KLH, was administered parenterally to evaluate primary (Day 0) and secondary (Day 15) antibody responses to KLH immunization over a three-week time course. Mucosal and systemic tissue immune changes were measured at the end of the 10 week trial in order to thoroughly understand the effects of DFM on immune status.

Objective 4: To evaluate the time and dose-dependent effects of butyric acid on TLRchallenged IEC in the absence or presence of β -estradiol

Butyric acid, a microbial fermentation metabolite, is the preferred energy source for colonocytes and suppresses intestinal epithelial-induced chemokine production (Vinolo *et al.*, 2011; Kumar *et al.*, 2009). However in our *in vivo* findings, rats fed DFM demonstrated an increase in the levels of pro-inflammatory cytokines in mucosal tissues, concurrent with high cecal and fecal butyric acid concentrations. We were interested in understanding whether the concentration of, and time of contact with, butyric acid affected the intestinal

epithelial response to immune stimulants. An *in vitro* approach was designed to test the effect of varying concentrations of butyric acid (10 and 25 mM) administered at different times (co or pre-incubation) in the presence of different TLR agonists on chemokine production using IEC-6, a non-transformed rat IEC line. Production of the chemokine cytokine-induced chemoattractant (CINC)-1 by IEC-6 was induced by treatment with either the TLR4 agonist lipopolysaccharide (LPS), a strong pro-inflammatory stimulus frequently encountered in the gut, or the TLR3 agonist poly (I:C), a synthetic variant of viral dsRNA. The methodology was also applied to examine the effects on the production of the chemokine IL-8 by HT-29, a commonly used human IEC line model. The influence of β -estradiol on the effects of butyric acid was also examined using TLR agonist challenged IEC-6 and HT-29 IEC to gain insight into potential effects of this sex hormone on SCFA activity, at the IEC level.

Objective 5: To examine the effects of butyric acid and Pam₃CysK₄ on TLR agonistinduced IL-8 production via the p38 pathway

In vivo, rats fed the WB-supplemented diet demonstrated a significant increase in the percentage of butyric acid, however rats fed the RS-supplemented diet had increased percentage of propionic acid in both cecal and fecal contents. However, the mucosal immune effects were different between rats fed either DFM. We speculated this could be due to WB intake inducing a fecal microbiota community enriched with Clostridial phylotypes (Gram-positive bacteria) (Abnous *et al.*, 2009). TLR2 is an innate immune receptor present on gut epithelial cells that recognizes Gram positive microbial-derived cell wall component peptidoglycan and/or lipoproteins, frequently encountered in the gut along with other microbial components. We therefore wanted to investigate whether butyric acid

effects on chemokine production by HT-29 IEC-challenged with a TLR agonist altered in the presence of TLR2. The effect of butyric acid was examined on IL-8 and IP-10 production by HT-29 IEC challenged with the combination of TLR2 agonist (Pam3Cystriacetylated lipoprotein) and TLR3 agonist poly (I:C). The chemokine IP-10 was included in our measures because TLR3, unlike TLR2, acts through a MYD88-independent pathway inducing interferon β–inducible genes such as IP-10, allowing us to also examine whether butyric acid modulation is limited to MYD88-dependent pathways. Ligand binding to TLR3 induces the downstream activation of transcription factors such as NF- κ B and AP-1. In IEC, IL-1β can activate p38-induced IL-8 production, independent of NF- κ B activation (Parhar *et al.*, 2003). However, few studies have examined the effect of butyric acid on TLR-induced p38 activation in HT-29 IEC. For these reasons, the production of IL-8 by HT-29 IEC stimulated with TLR2 and/or TLR3 in the presence of butyric acid was evaluated following p38 inhibition.

These *in vitro* approaches complemented our *in vivo* studies and aimed to further our understanding of the effects of DFM intake on both mucosal and systemic immune measures between male and female rats.

4. Materials and Methods

Rats and feeding trial under resting conditions

A total of 132 weanling (28-42 day old) BioBreeding rats, a Wistar-derived breed, were obtained from the Animal Resource Division of Health Canada. Rats had free access to reverse-osmosis treated water, were separately housed within individual mesh bottomed-stainless steel cages and subjected to a 12 hr light/dark cycle. A two-week acclimatization period (control diet) preceded the 6-week experimental feeding trial, after which the rats were euthanized and tissues were collected and flash frozen for subsequent cytokine analysis. This study was approved by the Health Canada Animal Care Committee and the University of Ontario Institute of Technology Animal Care Committee. Six animals from each treatment group were randomly selected for cytokine analysis, and the remaining six used for immunophenotyping. The first feeding trial was set up to examine three experimental outcome: as explained below.

- a) Male rats (n=12/feed) were divided into four separate feeding groups: control diet (AIN-93-G), 5% wheat bran (WB), 5% oat bran (OB), or 5% resistant starch (RS) for 6 weeks, to evaluate effects of different DFM on SCFA profiles and on mucosal and systemic immune parameters in male rats.
- b) Female rats (n=12/diet) were fed either the AIN-93G or the 5% WB supplemented diet, to compare the effects of WB consumption on SCFA profiles and immune parameters between sexes.

c) The effects of fructo-oligosaccharide (FOS) intake were also compared between male (n=12/diet) and female (n=12/diet) rats. Rats were fed a control diet (Purina 5001 rodent chow) or rodent chow supplemented with 5% (w/w) FOS (dp 2-8, Orafti, Pemuco, Chile) for 6 weeks and mucosal and systemic immune parameters, along with SCFA profiles were evaluated.

Rats and feeding trial under immunized conditions

A total of 100 weanling (40 day old) Wistar rats were obtained from the Animal Resource Division of Health Canada. Rats were pair housed in wire bottomed cages with metal plates covering a portion of the bottom. Rats had free access to reverse-osmosis treated water and subjected to a 12 hr light/dark cycle. Rats were acclimatized on the AIN-93G diet for 1 week before separating them into different feeding groups (AIN-93G, RS, WB, OB or FOS) for 6 weeks. There were 10 animals per diet and sex in each feeding group. Animals were weighed at the end of the 6 weeks to ensure sufficient weight for blood sampling (\geq 200 g). Rats were immunized intravenously with 300 µg/kg soluble KLH in 0.5 mL of sterile saline. The dosage is based on several studies examining the kinetics of anti-KLH response (Smith *et al.*, 2003; Kawai *et al.*, 2013).

A preliminary trial was conducted to confirm the kinetics of the response, examining anti-KLH production over 21 days. Tail vein blood samples (300 μ L) were collected to provide baseline parameters. On the day of immunization serum was collected (via tail vein) was then carried out on day 5, 10 and 15 to determine the effects of dietary fibre on the primary immune response to KLH challenge. Secondary immunization was carried out two weeks after primary immunization, and the experiment ended 7 days later with exsanguination for total serum collection at the end of the study. Six animals from each treatment group were randomly selected for tissue cytokine analysis, and the remaining six used for immunophenotyping. Mucosal and systemic tissues were also collected and flash frozen for subsequent cytokine analysis and immunophenotyping by flow cytometry was conducted in spleen and MLN. Rats were anaesthetized using inhaled isofluorane during immunization and blood collection procedures. This study was approved by the Health Canada Animal Care Committee and the University of Ontario Institute of Technology Animal Care Committee (AUP 12-003).



Fig 1. Schematic of the immunization time line

Gas chromatography

Cecal and fecal short chain fatty acids (SCFAs) were determined as previously described (Weaver et al., 1997) and the analysis was conducted at Health Canada. Chromatographic analysis was performed on an Agilent 6890A gas chromatograph, which utilized a flame ionization detector (FID) and a 7683 automatic liquid sampler (Agilent, USA). A NUKOL 24108 fused silica capillary column (60m x 0.25mm) with a coated thickness of 0.25 μ m (SUPLECO, Sigma Aldrich) was used. Helium supplied at a flow rate of 0.75 ml/min acted as the gas carrier. The oven temperature was ramped from 50°C to 200°C at 8°C/min. GC analysis on each sample was conducted on 1µl of injected sample volume with a run time of 17.5 min. Data analysis was performed using the MSD Chemstation software (Agilent).

Serum biochemistry

Individual serum samples were analyzed for blood urea nitrogen (BUN), alanine amino transferase (ALT), alkaline phosphatase (ALP), aspartate amino transferase (AST) and lactate dehydrogenase (LDH). Serum biochemical analysis was conducted by technical personnel at Health Canada. BUN levels were measured using the urease-glutamate dehydrogenase method (Talke and Schubert, 1965). ALP and LDH levels were analyzed using the method outlined by IFCC (International Federation of Clinical Chemistry), while the modified IFCC method (1985) without pyridoxal phosphate was used to analyze serum ALT and AST levels. Serum enzyme levels were determined using the ABX Pentra 400Automated clinical chemistry analyzer and ABX Pentra test kits for each analyte (Horiba Canada Inc., Burlington, Ontario).

Flow cytometry

Immunophenotyping analysis was performed by direct immunofluorescence and Flow cytometry on cells from the MLN, spleen and Peyer's patches. PP were isolated from 10-12 cm piece of ileum. The tissues were placed into a 70 μ m cell strainer in a 50 ml Falcon tube, and tissues were dispersed in 5 ml ice cold RPMI-1640 medium + 10% FBS using a 3ml syringe plunger. Cell suspensions were centrifuged at 340 x *g* for 6 min. The pellets were re-suspended in 1ml D-PBS (PBS w/o calcium and magnesium) and diluted to obtain 1 x10⁷ cells/ml with D-PBS. FC was done at Health Canada using a standard configuration Becton Dickinson FACSCalibur flow cytometer (San Jose, California), equipped with 488 nm and 635 nm lasers. Gating techniques were standard and similar to those used by Shingadia *et al.* (2001). Fluorescent antibodies against standard cell surface markers were initially titrated to determine concentrations for optimal detection.

Antibodies used were anti-CD45 (PE-Cy5, Clone OX-1) (Leukocyte Common Antigen), anti-CD3 (APC, Clone 1F4) (total T cell), anti-CD62L (PE, Clone HRL1) (Lselectin), anti-CD4 (PE, Clone OX-35) (T helper), anti-CD25 (FITC, Clone OX-39) (T_{reg}), anti-Foxp3 (PE, Clone FJK-16s) (T_{reg} transcription factor) was analyzed by intracellular staining, anti-CD8a (FITC, Clone OX-8) (cytotoxic T cell), anti- $\gamma\delta$ TCR (FITC, Clone V65), anti-CD103 (Alexa Fluor 647, Clone OX-62) (DC marker), anti-CD45RA (FITC, Clone OX-33) (B cell), anti-CD161a (PE, Clone 10/78) (Natural Killer cell). Anti-HIS36 (PE, Clone HIS36) (macrophage cell surface marker) and intracellular staining for anti-CD68 (Alexa Fluor 488, ED1 clone) were used to enumerate macrophage populations. Intensity of macrophage CD68 expression was analyzed as an indicator of macrophage activation (Onodera *et al.*, 1997). Fluorochrome-matched isotype controls were used to assess non-specific binding. All antibodies were obtained from BD Biosciences, except for anti-CD68, purchased from AbD Serotec, anti-CD25 and anti-Foxp3 from eBioscience, and anti-CD103 from BioLegend. Table 2 (below) summarizes the surface markers associated with each immune cell phenotype examined.

Surface/Intracellular	Cell type			
Marker				
CD3 ⁺	Total T cell			
CD4 ⁺	T helper (T _H) cell			
CD25 ⁺	T _{reg} cells			
Foxp3 ⁺	Treg Transcriptional factor			
	(intracellular)			
CD62L	Lymphocyte homing receptor			
CD8a ⁺	Cytotoxic T cell			
γδ TCR	Athymic T cell			
CD45RA ⁺	B cell			
CD103 ⁺	Dendritic cell (DC)			
CD161a ⁺	Natural Killer cell			
HIS36 ⁺	Macrophage			
CD68 ⁺	Macrophage intensity			
	(intracellular)			

Table 2: Surface or intracellular marker associated with each immune cell type

Tissue preparation

Ileum, cecum, colon, Peyer's patches, MLN, spleen and liver were collected at the end of the feeding trial, snap frozen and stored at -80°C. Blood was collected during necropsy from the portal vein of the liver in order to obtain serum and plasma. Tissue homogenization was performed according to the method of Hoentjen *et al.* (2005). Tissues were suspended in 1 g/5ml phosphate buffered saline containing 1% protease inhibitor cocktail (Sigma-Aldrich, USA), and homogenized using a Tissuemiser (Fisher Scientific, USA). Homogenized tissues were centrifuged for 30 minutes at 16,100 x g at 4° C. The liver and spleen were centrifuged twice to remove fat and tissue debris. Supernatants were then removed and stored at -80°C for cytokine and immunoglobulin analysis.

Cytokine and antibody analysis

IL-10, TGF- β 1, IL-6, CINC-1, IL-4, IFN- γ , and IL-17A concentrations were measured from tissues and serum using enzyme-linked immunosorbent assay (ELISA) and following manufacturer's protocols (R&D Systems, USA). Immunoglobulin A (IgA) was also measured by ELISA (Bethyl Laboratories, USA). ELISAs were performed in 96 well clear plates (Biogreiner) using streptavidin conjugated to horseradish-peroxidase and 3,3',5,5' Tetramethylbenzadine as the substrate and the reactions were stopped with 2N H₂SO₄. Plates were read at a wavelength of 450 nm on a Synergy HTTR microplate reader (Bio-Tek, USA). Results for cytokines and IgA were expressed as pg/g or μ g/g of tissue, respectively, unless otherwise stated.

Anti-KLH antibody analysis

96 well clear high-binding ELISA plates (Biogreiner) were coated with KLH (Sigma) at a final concentration of 10 µg/ml in coating buffer (carbonate-bicarbonate, pH 9.6) and incubated overnight at 4°C prior to antibody analysis. Serum samples (day 0, 5, 10, 15 and 21) were diluted in the assay diluent (50 mM Tris, 0.14 M NaCl, 1% bovine serum albumin, 0.05% Tween 20, pH 8) to obtain an analyte concentration within the range of the standard curve for each immunoglobulin isotype. A rat Ig standard curve (4-parameter logistic model) was used to determine Ig concentrations. The anti-KLH (IgG, IgM, IgA, IgG2a and IgG2b) and total antibody concentrations for each time point were measured by ELISA, following the use of horseradish peroxidase conjugated goat anti-rat detection antibodies specific for each immunoglobulin isotype (Bethyl laboratories, USA). ELISAs were read at a wavelength of 450 nm on a Synergy HTTR microplate reader (Bio-Tek, USA).

Stimulation of whole blood cultures with TLR agonists and KLH

An *ex-vivo* measure was utilized to evaluate responses in whole blood cultures to TLRagonist and/or KLH stimulation (Ida *et al.*, 2006). The TLR 4 agonist lipopolysaccharide (LPS), purified from *E.coli* (Sigma), or the TLR2 agonist Pam₃CysK₄ (Calbiochem) were used as innate stimulants and KLH (Sigma) was used to assess the KLH recall response. An initial test was conducted to find optimal stimulant concentrations prior to conducting the whole blood culture-stimulation assay. For the assay 900 μ L of whole blood was stimulated with 10 μ g/ml of either LPS or Pam₃CysK₄, and/or 150 μ g/ml of KLH (final concentrations) in a 5 ml polypropylene round-bottom tube (BD Bioscience) and incubated at 37°C, 5% CO₂ humidified incubator for 20 hours. Following incubation, the tubes were centrifuged at 1300 x g for 5min at 4°C, culture supernatants were collected and stored at -80°C and were later measured for cytokines IL-10, IFN- γ and IL-4.

End-point Chromogenic Limulus Amebocyte Lysate (LAL)

Serum LPS concentrations were analyzed from males and females fed either Purina chow or FOS diets only, using the endpoint chromogenic LAL assay (Lonza, Switzerland). A 1:1 ratio of serum and LAL reagent were mixed in a 96 well plate and incubated for 10 min in a block heater maintained at 37°C. 100 μ l of pre-warmed chromogenic substrate, at a concentration of 2 mM, was then pipetted into each well and incubated for 6 min. The reaction was stopped by the addition of 100 μ l of 25% acetic acid to each well. The plate was read at an absorbance wavelength of 405 nm. LPS (EU/ml) levels were calculated based on the standard curve (range 0.1 -1.0 EU/ml) with purified LPS from *E.coli*.

Real Time PCR: Cecal tissue Mucin-2 gene expression

Cecal tissues stored in RNA Later (Qiagen) at -80°C were used for mucin 2 (MUC2) gene expression analysis. Total RNA from thawed cecal tissue was extracted using Qiagen RNeasy kit as per manufacturer's instructions. Total RNA was quantified (ng/µl) using a NanoDrop spectrophotometer (at Health Canada, Ottawa). A260/280 ratios and gel electrophoresis analysis of total RNA were performed to determine RNA quality. Total RNA was DNase treated (Qiagen) to remove genomic DNA contamination prior to cDNA synthesis and treated total RNA was re-quantified using the Nanodrop spectrophotometer. 350ng of RNA was reverse transcribed using the First strand cDNA synthesis kit (Thermo Fisher).

For real time PCR, cDNA was diluted to obtain a concentration of 1200 pg/µl in RNase-and DNase free water, and 1µl of diluted cDNA was added to a PCR mixture (10 µl of Bio-Rad SYBR green supermix, primers at a final concentration of 300nM, final reaction volume, 15 µl). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) and Ribosomal Protein 10A (RPL10A) were used as housekeeping genes (Martinez-Beamonte *et al.*, 2011). Real time PCR was carried out using a Bio-Rad CFX-manager software. Sequences of the primers, created using MacVector, are as follows:

MUC2 Forward: 5' TGTGGTAAAGCAGTTGGAAGAGG 3'

MUC2 Reverse: 5' CCAGATAACAATGATGCCAGAGC 3'

HPRT Forward: 5[°] TTGCTCGAGATGTCATGAAGGA 3[°]

HPRT Reverse: 5' AGCAGGTCAGCAAAGAACTTATAG 3'

RPL10A Reverse: 5' AGAAGCGTTTGTCCTTCTGAGGGT 3'

RPL10A Forward: 5' AGCCATGAGCAGCAAAGTTTCACG 3'

Cell cultures

The non-transformed small intestinal rat cell line IEC-6 and the human colorectal adenocarcinoma cell line HT-29 were used *in vitro* to examine the effects of butyric acid on TLR agonist-induced responses. Both cell lines were purchased from the ATCC (USA)

and maintained in 75cm^2 tissue culture flasks (CellStar, Greiner-Bio-One). IEC-6 cells were grown in Dulbecco's modified Eagle's medium (Sigma) with 4 mL-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 50 µg/ml gentamycin (Sigma) and 10% FBS (Sigma). HT-29 IEC cells were grown in RPMI-1640 medium (Sigma) with 0.3 g/L Lglutamine, 2 g/L sodium bicarbonate, 50 µg/ml gentamycin, phenol red and 10% FBS. Cells were maintained in a 5% CO₂ incubator (Thermo Fisher) at 37°C and 95% humidity. All assays to examine effects of butyric acid and TLR agonists were performed with cells seeded at a density of 5x 10⁵ cells/ml and in serum-free media.

Treatment of IEC-6 cells with butyric acid and TLR agonists

The effects of varying timing of contact and concentration of butyric acid on TLR agonistinduced chemokine production were examined using the IEC-6 cell line. Production of the chemokine CINC-1 by IEC-6 was induced by treatment with either 20 ng/ml of TLR 4 agonist LPS or 40 μ g/ml of TLR 3 agonist poly I:C. Effects on CINC-1 production were evaluated by co-incubating IEC-6 cells (passage 11-19) with 10 or 25 mM butyric acid (Sigma) in the presence of either TLR agonist (Mirmonsef *et al.*, 2012). The effect of preincubation was also tested by pre-treating IEC-6 cells with either 10 or 25 mM butyric acid for 18 hrs, followed by 6 hr challenge with either TLR agonist. Media was removed prior to TLR-agonist challenge, which was performed with fresh media. CINC-1 (pg/ml) levels were measured using ELISA and manufacturer's protocol (R&D Systems, USA) as previously described.

p38 pathway inhibition in Poly (I:C) challenged HT-29 IEC treated with Pam₃CysK₄ and/or butyric acid

The effect of Pam₃CysK₄ (TLR2 agonist) and butyric acid on chemokine (IL-8 and IP-10) production was evaluated from HT-29 cells stimulated with TLR 3 agonist poly I:C. HT-29 cells (passage 15-19) were incubated with Pam₃CysK₄ (10 μ g/ml) and 10 or 25 mM butyric acid and challenged with poly I:C (40 μ g/ml) for 6 hrs. HT-29 cells were also pretreated with Pam₃CysK₄ and/or butyric acid (10 or 25 mM) for 2 hrs prior to a challenge with poly I:C for 4 hrs. TLR-agonist challenge was performed with fresh media. Supernatants were collected after challenge and stored at -80°C and later measured for IL-8 and IP-10 production by ELISA (R&D Systems, USA). HT-29 IEC (passage 28-30) were incubated with 20 μ M of the p38 inhibitor, SB203580 (Invivogen) in the presence of 10mM butyric acid and 10 μ g/ml Pam₃CysK₄. The assay was performed to test the effect of butyric acid on IL-8 production induced by poly I:C alone or with Pam₃CysK₄ via the p38 pathway. Following 4 hrs of incubation, supernatants were collected and IL-8 production was evaluated by ELISA (R&D Systems, USA).

β -estradiol treated IEC incubated with butyric acid and TLR agonists or TNF α

The influence of β -estradiol on the effects of butyric acid were examined using IEC-6 and HT-29 IEC. IEC were treated overnight with varying concentrations of β -estradiol (Sigma) (1ng/ml, 5ng/ml and 10ng/ml) (Looijer-van Langen *et al.*, 2011). IEC-6 cells (passage 19-22) were then treated for 6 hours with LPS (20 ng/ml) or poly I:C (40µg/ml) along with butyric acid (Sigma) at concentrations of 10 mM or 25 mM in fresh serum free media. The

effect of β -estradiol on HT-29 IEC (passage 25-30) were also examined with 50 or 100 ng/ml of TNF- α (Pepro-Tech). Cell culture supernatants were assayed for chemokine production by ELISA (R&D systems, USA).

Treatment of HT-29 cells with ATP and TLR agonists

The effects of ATP on HT-29 IEC chemokine production by TLR agonists were also evaluated. HT-29 cells (passage 29-32) were incubated with ATP (Sigma), at concentrations of 50 μ M and 75 μ M, and challenged with TLR agonists Pam₃CysK₄ (10ng/ml), LPS (20 ng/ml) or flagellin (100 ng/ml; TLR5 agonist) (Invivogen) for 6 hrs at 37°C. Cell culture supernatants were collected and analyzed for chemokine production by ELISA (R&D Systems, USA).

Tetrazolium dye reduction (MTT) Assay for cell viability

Following collection of supernatants for cytokine measurements, cell viability of IEC-6 and HT-29 IECs was performed using the tetrazolium dye reduction assay. The assay was developed by Mosmann (1983) to detect cellular growth and survival. Actively respiring cells soluble (3-[4,5-dimethylthiazol-2-yl]-2,5, can convert the MTT dye Diphenyltetrazolium) (Sigma) to insoluble formazan. The MTT dye at 0.5 mg/ml (final concentration) was added to each well with IECs and incubated at 37°C for 2 hrs. Following incubation supernatants were removed and 100 µL of dimethyl sulfoxide (DMSO) (Sigma) solution was added to the each well. Plates were shaken at room temperature for 10 min to dissolve the purple formazan crystals, and then read at 600 nm using a Synergy HTTR microplate reader (Bio-Tek, USA). Cell viability was expressed as the % of control (untreated cells).

Statistical analysis

Immunological data was analyzed using a two-way of variance (ANOVA) to examine overall effects of diet, differences between sexes and interactions (diet x sex). Differences were further analyzed using the Tukey's multiple range test when ANOVA p<0.050. If the two-way ANOVA detected an interaction, differences based on diet were further delineated using one-way ANOVA and a Tukey's multiple range test when ANOVA results yielded a *P*-value less than 0.05. The nonparametric Kruskal-Wallis (KW) test was applied in conditions when a significant result was obtained with Bartlett's test for homogeneity of variance. If the KW test result was significant, a Dunn's test was used to detect significant differences between diet groups. Results are presented as the mean \pm SEM.

5. Results

5.1 Effects of DFM ingestion on physiological and immune measures from male and female rats under resting state conditions

The following section encompasses the results obtained from male rats fed WB, OB or RSsupplemented diets relative to AIN-93G (control diet) fed rats. Data on the comparative effects of the WB supplement between sexes are also presented in this section. Gut microbiota analysis was conducted by our collaborators and is not included here (Abnous *et al.*, 2009; Kalmokoff *et al.*, 2013).

Diet Ingredient	AIN-93G	\mathbf{WB}^2	OB ³	\mathbf{RS}^4
Vitamin-free casein, g/kg	200.0	181.8	166.7	200.0
Corn starch, g/kg	397.5	382.9	368.3	372.5
Dextrinized corn starch, g/kg	132.0	117.4	102.8	107.0
Sucrose, g/kg	100.0	85.4	70.8	75.0
Soybean oil, g/kg	70.0	65.0	56.0	70.0
AIN-93G minerals, g/kg	35.0	35.0	35.0	35.0
AIN-93G vitamins, g/kg	10.0	10.0	10.0	10.0
choline bitartrate, g/kg	2.5	2.5	2.5	2.5
α-methionine, g/kg	3.0	3.0	3.0	3.0
Tert-butylhydroquinone	0.014	0.014	0.014	0.014
Wood cellulose, g/kg	50.0	20.0	0.0	0.0
Bran, g/kg	0	164.8	117.0	125
⁵ DF from bran source, %	0	3.0	5.0	0
Total ⁵ DF, %	5.0	5.0	5.0	5.0
Protein, %	20.0	20.0	20.0	20.0
Total carbohydrate, %	62.9	61.1	61.8	62.9
Fat, %	7.0	7.0	7.0	7.0
Energy density, kJ/kg	16.52	16.30	16.38	16.92

Table 3. Diet composition of AIN-93G-supplemented with different sources of fibre

²Oat bran composition: 20.2% protein, 8.5% fat, 41.1% carbohydrate (nonfibre), 18.2% dietary fibre, 7% moisture, 5% ash.

³Hard red wheat bran composition: 15.6% protein, 4.3% fat, 21.8% carbohydrate (nonfibre), 42.7% dietary fibre, 9.9% moisture, 5.8% ash.

⁴High amylose maize starch, 40% resistant starch type II (measured using Megazyme Resistant Starch Assay kit following the AOAC 2002.2 approved method), 60% available carbohydrate.

⁵DF- Dietary Fibre



Fig 2. Weight gain in male rats fed different DFM. Bars indicate mean weight gain (g) \pm SEM (n=18). Significant effects of diet were analyzed by one-way ANOVA. Differing letters represent significant differences in weight gain as determined by the Tukey's multiple range test.



Fig 3. Differences in weight gain between male and female rats fed the control or WBsupplemented diet. Bars indicate mean weight gain (g) \pm SEM (n=18 for males; n=12 for females per diet). (*) indicates significant effect of sex was analyzed by two-way ANOVA.

Effects of DFM feeding on weight gain between male and female rats

The percentage of protein, non-fibre carbohydrate and fat were equal between the AIN-93G formulated control diet and DFM-supplemented diets (Table 3). The energy density across all diets was equal, ensuring the intake of an equivalent amount of nutrients except for the fibre composition. DFM supplementation did affect the amount of weight gain over the course of the feeding trial. Overall weight gain was lower in male rats fed the WBsupplement compared to RS-fed rats over the course of the 6 week feeding trial (Fig 2). In contrast, the amount of weight gain was higher in males relative to females fed either diet (Fig 3).

					p value
Measure	AIN-93G	WB	OB	RS	Diet ²
LDH (U/L)	220 ± 44^1	156 ± 41	177 ± 29	175 ± 38	NS^4
ALP (U/L)	145 ± 3	138 ± 2	157 ± 7	201 ± 7	NS
ALT (U/L)	30 ± 1^{a}	47 ± 3^{c}	149 ± 8^{b}	31 ± 2^{a}	< 0.0001
AST (U/L)	64 ± 3	67 ± 2	62 ± 1	61 ± 1	NS
BUN (mg/dL)	19 ± 1^{ab}	21 ± 1^{b}	20 ± 1^{ab}	17 ± 1^{a}	0.009 ³

Table 4. Serum biochemical measures in male rats fed either the AIN-93G, WB, OB or RS-supplemented diets

¹Data indicates mean \pm SEM (n=6).

²Significant effects of diet were analyzed by one-way ANOVA.

³Differences in BUN levels in rats fed different DFM were analyzed by Kruskal-Wallis. Differing letters represent significant differences in serum biochemical levels as determined by the Tukey's multiple range test or the Dunn's test.

⁴NS-not significant.

Measure _	Males		Females		p value		
	AIN-93G	WB	AIN-93G	WB	^a Diet	^b Sex	^c D x S
LDH (U/L)	220 ± 44^1	156 ± 41	116 ± 12	106 ± 4	NS ²	0.03	NS
ALP (U/L)	146 ± 3^{b}	138 ± 2^{a}	201 ± 7^{b}	118 ± 4^{a}	< 0.001	< 0.001	0.003
ALT (U/L)	30 ± 1	47 ± 3	31 ± 2	40 ± 1	< 0.001	NS	NS
AST (U/L)	64 ± 3	67 ± 2	59 ± 1	61 ± 2	NS	NS	NS
BUN (mg/dL)	19 ± 1	21 ± 1	23 ± 1	24 ± 1	NS	NS	NS

Table 5. Differences in serum biochemical measures between male and female rats fed either the AIN-93G or WB-supplemented diet

¹Data indicates mean \pm SEM (n=6).

Significant effects for diet^a (D), differences between the sexes^b (S) or an interaction $(D \times S)^c$ were analyzed using two-way ANOVA.

Differing letters indicate significant differences based on diets as determined by Tukey's multiple range test.

²NS-not significant.
Effects of DFM ingestion on serum biochemical measures between male and female rats

Effect of DFM consumption on standard serum biochemical measures for liver (LDH, ALP, AST and ALT) and kidney function (BUN) were evaluated. Serum BUN levels also reflect host/endogenous nitrogen utilization by the gut microbiota (Kalmokoff *et al.*, 2013). Diet affected certain serum biochemical measures; however, the values for each biochemical parameter fell within the reference range for laboratory rats (Wolford *et al.*, 1986). Major changes to levels would indicate DFM intake was detrimental to host health, which was not the case. Levels of LDH were unaffected by diet but were different between sexes (Table 4 and 5, respectively). Males had higher levels of LDH compared to females (Table 5) (p=0.03). Basal levels of ALP were higher in female rats relative to male rats, in addition to which WB feeding reduced ALP levels in both sexes, however the decrease was greater in females compared to males (Table 5). WB and OB-fed rats showed a decrease (p<0.001) in ALT levels relative to control-fed rats (Table 4). BUN levels were lower in RS-fed rats compared to WB-fed rats (Table 4). AST levels were unaffected by diet and did not differ between sexes (Table 5).



Fig 4a). Cecal short chain fatty acid (SCFA) profiles from rats fed either control or the WB, OB or RS diets. Bars indicates mean (% of total) \pm SEM (n=8). Significant effects of diet were analyzed by one-way ANOVA. Differing letters denote significance based on diet determined by Tukey's multiple range test.



Figure 4b). Fecal short chain fatty acid (SCFA) profiles from male rats fed either the control or the WB, OB or RS diets. Data indicates mean (% of total) \pm SEM (n=6-8). Significant effects of diet were analyzed by one-way ANOVA. Differing letters indicate significance based on diet determined by Tukey's multiple range test.

	Cecal (Wet wt) Acetic:Propionic:Butyric	Fecal (Dry wt) Acetic:Propionic:Butyric
AIN-93G	$4:1:1^1$	7:1:1 ²
WB	3:1:1	8:1:1
OB	2:1:1	5:1:1
RS	5:1:1	5:2:1

Table 6: Relative ratios of SCFA in cecal and fecal contents from rats fed different DFM

^{1,2}Ratios of SCFA from cecal (wet wt) and fecal (dry wt) contents of rats fed different

DFM.



Figure 5. Differences in the fecal short chain fatty acid (SCFA) profiles between male and female rats fed the control or WB-supplemented diet. Bars indicate mean (% of total) \pm SEM (n=5-6). No significant effects of diet, differences between sexes or an interaction (diet x sex) were detected by two-way ANOVA.

	Male Fecal Acetic:Propionic:Butyric	Female Fecal Acetic:Propionic:Butyric
AIN-93G	$7:1:1^1$	$10:2:1^2$
WB	8:1:1	7:1:2

Table 7: Relative ratios of SCFA in the fecal contents from male and female rats fed
 either the control or the WB-supplemented diet

^{1,2}Differences in the ratios of SCFA from the fecal contents of male and female rats fed the WB-supplemented diet

Effects of DFM ingestion on cecal and fecal SCFA profiles between male and female rats

Cecal and fecal SCFA levels are shown as a percentage of their respective total SCFA amounts for male rats fed the different DFM (Fig 4 a) and b) respectively). Table 6 depicts the relative ratios of SCFA from cecal and fecal contents. The SCFA ratios were lower in both cecal and fecal contents from OB-fed rats relative to control-fed rats. In contrast, RS fermentation doubled the proportion of propionic acid in the fecal content (Table 6). Cecal contents from female rats were not analyzed for SCFA concentrations. However, the basal ratios of fecal SCFA showed females had higher proportions of acetic and propionic acid relative to males, while butyric acid proportions were similar between the sexes. However the proportion of fecal butyric acid in females fed the WB- supplement was twice as much as that in males (Table 7).



Fig 6. Differences in the percentages of $Foxp3^+T_{reg}$ population within the $CD3^+4^+25^+T$ cell population in the MLN (A) and spleen (B) between male and female rats fed control or WB. Data are shown as % mean \pm SEM (n=6). (*) indicates higher $Foxp3^+T$ cell population percentages in both the MLN and spleen of males relative to females as detected by two-way ANOVA.



Fig 7. Differences in the percentages of $CD68^+$ population MLN (A) and spleen (B) between male and female rats fed control or WB. Data are shown as % mean \pm SEM (n=4-6). (*) indicates higher $CD68^+$ population percentages in both the MLN and spleen of females compared to males as detected by two-way ANOVA.

	AIN93G	WB	OB	RS		AIN93G	WB	OB	RS
MLN:					Spleen:	-			
%CD3+	79±0.4 ^b	73±0.6 ^a	76±0.7 ^{a,b}	79±1.3 ^b		71±0.4 ^{a,b}	73±0.08 ^b	69±0.4ª	$71{\pm}0.8^{ab}$
%CD3 ⁺ CD4 ⁺	57±1.0 ^b	50.5±1.0 ^a	55.8±0.7 ^b	58.7±1.2 ^b		44.7±0.8	46.0±1.0	44±1.0	45±1.5
%CD3 ⁺ CD8 ⁺	20±0.6	20±0.8	19±0.6	20±0.8		24.2±0.4	24±0.6	23±0.40	24±1.1
%CD45RA ⁺	19±0.5 ^a	25±0.3 ^b	21±0.8 ^a	18±0.8 ^a		18.8±0.7 ^b	17±0.5 ^a	22±1.0 ^b	20±0.9 ^{a,b}
%CD3 ⁻ 161a ⁺	0.0±0.0	0.1±0.0	0.1±0.0	0.1±0.0		0.70±0.1	0.90±0.1	1.1±0.4	0.70±0.1
%CD68 ⁺	7.2±0.9	5.2±0.7	6.6±0.8	6.4±0.8		47±2.2	45±1.5	42±3.9	47±2.3
%Foxp3 ⁺ *	14±2.6	12±1.4	13±2.6	20±4.0		7.7±1.2	8.6±1.5	6.9±1.1	13±2.8

Table 8: Flow cytometric analysis of immune cell population percentages from the MLN and spleen in male rats fed different DFM

Data are shown as the mean $\% \pm \text{SEM}$ (n=4-6) for each diet and tissue. (*) indicates the percentage of Foxp3⁺ T_{reg} population within the CD3⁺4⁺25⁺ in the MLN and spleen of male rats. Significant effects of diet were detected by one-way ANOVA. Means with different letters are significantly different as determined by Tukey's multiple range test.

Effects of DFM consumption on immune cell population percentages in the MLN and spleen of male and female rats

Flow cytometric analysis of immune cell population percentages were mainly performed on male spleen and MLN tissues. Analysis of immune cells percentages from female rats was limited due to inadvertent sample loss.

Male rats consuming the WB-supplemented diet showed the largest change in the percentage of T and B cell populations (Table 8). In the MLN, total T cell (CD3⁺) and T helper cell (CD3⁺CD4⁺) percentages were lower (p<0.05) , while B cell (CD45RA⁺) percentages were higher (p<0.05) in WB-fed rats than in rats consuming the control AIN 93G diet. In the spleen, feeding WB lowered the percentage of splenic B cells (CD3⁻ $45RA^+$) and slightly increased the percentage of T cells (CD3⁺), although the latter effect was only significant compared to rats fed OB. Diet had no effect on macrophage populations in the MLN or spleen.

Sex-based differences were noted for MLN and splenic regulatory T cell and macrophage population percentages. Regulatory T cell ($CD3^+4^+Foxp3^+$) population percentages were higher (p<0.05) in the MLN and spleen of males (Fig 6), while macrophage ($CD68^+$) population percentages were significantly (p<0.001) higher in both tissues of females (Fig 7).

				I	o value
	AIN-93G	WB	OB	RS	Diet
Cecal Content					
IgA(µg/g)	8.6 ± 0.6^{1}	11 ± 2.7	7.6 ± 1.9	6.5 ± 0.8	NS
Liver IgA					
IgA(µg/g)	3.2 ± 0.2^{2}	3.5 ± 0.2	3.7 ± 0.2	3.0 ± 0.4	NS

Table 9: Total IgA levels in cecal content and liver of males fed different DFM

^{1,2}Total IgA concentrations were shown as mean ($\mu g/g$) ± SEM for cecal content (n=5-7)

and liver tissue (n=9-10).

No significant effect of diet was determined by one-way ANOVA.

Table 10: IgA	concentrations in ceca	l content and	l liver tissue	e of male an	d female	rats fed
the AIN-93G of	or WB-supplement					

	MAI	<u>LE</u>	FEM	ALE		p va	lue ³
	AIN-93G	WB	AIN-93G	WB	Diet ^a	Sex ^b	Diet x Sex ^c
Cecal Conter	nt						
IgA(µg/g)	$8.6\pm0.6^{\rm 1}$	11 ± 2.7	9.0 ± 1.7	16 ± 3.6	NS	NS	NS
Liver IgA							
IgA(µg/g)	3.2 ± 0.2^2	3.5 ± 0.2	3.1 ± 0.5	3.0 ± 0.3	NS	NS	NS

^{1,2}Total IgA concentrations were shown as mean ($\mu g/g$) \pm SEM for cecal content (n=5-7) and liver tissue (Male n=9-10; Female n=5-9).

³No significant effects of diet^a, differences between sexes^b, or an interaction^c (diet x sex) were detected by two-way ANOVA.

Effects of DFM-supplementation on IgA concentration in cecal contents and liver tissue

Total IgA levels were determined in cecal contents and in liver tissue. Cecal and liver IgA levels were unaffected by DFM (Table 9) and did not differ between the sexes (Table 10).



Fig 8. Cytokine concentrations (pg/g) in cecum (A) and MLN (B) of rats fed different DFM. MLN IL-10 and IL-6 were below detectable levels for all rats. Data are shown as mean (pg/g) \pm SEM (n=3-6). Significant effects of diet were detected by one-way ANOVA. Means with different letters are significantly different as determined by Tukey's multiple range test.



Fig 9. Liver tissue cytokine concentrations (pg/g). Data are shown as mean (pg/g) \pm SEM (n=6). Significant effects of diet were detected by one-way ANOVA. Means with different letters are significantly different as determined by Tukey's multiple range test.

		М	ale	Fen	nale	Diet ^a	Sex ^b	Diet x Sex ^c
Tissue	Cytokine	AIN-93G	WB	AIN-93G	WB	(P value)	(P value)	(P value)
	(ng/g)							
	IL-6	0.1 ± 0.1	0.5 ± 0.1	1.5 ± 0.3	1.1 ± 0.1	NS	< 0.01	NS
¹ Ileum	IL-4	0.9 ± 0.2	1.4 ± 0.2	0.3 ± 0.1	0.7 ± 0.1	0.05	< 0.01	NS
	IL-10	0.3 ± 0.2	0.9 ± 0.2	1.9 ± 0.5	0.7 ± 0.2	NS	0.06	0.02
	TGF-β1	0.3 ± 0.1	0.5 ± 0.1	BLQ	BLQ	-	-	-
	CINC-1	0.9 ± 0.4	2.1 ± 0.2	0.3 ± 0.1	0.2 ± 0.06	NS	NS	NS
	IL-6	8.0 ± 0.5	5.7 ± 0.8	10.1 ± 0.8	9.1 ± 1.5	NS	< 0.02	NS
Cecum	IL-4	2.8 ± 0.3	2.5 ± 0.1	3.3 ± 1.7	2.8 ± 1.1	NS	NS	NS
	IL-10	7.2 ± 0.3	6.1 ± 0.6	7.9 ± 0.2	7.0 ± 0.8	NS	0.03	NS
	TGF-β1	1.3 ± 0.5	1.5 ± 0.4	nd	Nd	-	-	-
	CINC-1	0.9 ± 0.1	0.5 ± 0.2	nd	Nd	-	-	-
	IL-6	2.1 ± 0.2	2.6 ± 0.2	7.8 ± 0.9	6.3 ± 0.4	NS	< 0.01	NS
¹ Colon	IL-4	1.6 ± 0.1	1.6 ± 0.3	5.0 ± 0.5	3.7 ± 0.5	NS	NS	NS
	IL-10	1.5 ± 0.2	1.4 ± 0.2	8.1 ± 0.6	9.7 ± 1.1	NS	< 0.01	NS
	TGF-β1	0.5 ± 0.0	0.6 ± 0.1	0.4 ± 0.3	0.5 ± 0.1	NS	NS	NS
	CINC-1	1.3 ± 0.4	1.3 ± 0.1	1.8 ± 0.2	2.2 ± 0.3	NS	NS	NS
¹ Spleen	IL-6	0.2 ± 0.02	0.2 ± 0.02	BLQ	BLQ	-	-	-
	IL-4	0.03 ± 0.01	0.05 ± 0.01	0.5 ± 0.2	0.8 ± 0.1	NS	< 0.001	NS
	IL-10	nd	nd	2.7 ± 0.1^{a}	$4.7 \pm 0.3^{\circ}$	-	-	-
	TGF-β1	0.1 ± 0.0	0.1 ± 0.0	1.3 ± 0.2	1.5 ± 0.2	NS	< 0.001	NS
	CINC-1	0.2 ± 0.02	0.3 ± 0.04	1.1 ± 0.02^{5}	0.7 ± 0.1^{a}	0.03	<0.001	0.02
	II -6	151 + 10 5	148 + 7 1	126*	106 + 11.4			
Liver	IL-0	151 ± 10.5	140 ± 7.1	62*	100 ± 11.4	-	-	-
	IL-4	39.3 ± 2.2	39.3 ± 0.9	03	00.0 ± 7.5	-	-	-
	IL-10	8/.3 ± 4.4	/3.3 ± 8.9	91.2	100 ± 11.2	-	-	-
	TGF-β1	45.1 ± 8.5	43.3 ± 4.8	nd	Nd	-	-	-
	CINC-1	8.6 ± 1.4	11.8 ± 2.0	10.0^{*}	8.4 ± 5.9	-	-	-

Table 11: Cytokine levels in gut mucosal and systemic tissues from male and female rats fed control or the WB-supplemented diet

Values indicate the mean $(ng/g) \pm SEM$ (n=3-6) for each tissue. Significance for diet^a, sex^b and diet x sex^c was detected by Two-way ANOVA. Differing letters denote significance based on diet alone as determined by the Unpaired t-test (ileal and splenic IL-10) or the Mann-Whitney non-parametric test (splenic CINC-1). (¹)Cytokine measures from male ileum, colon and spleen were performed by J McCarville (MSc. Thesis, 2012). *denotes the mean of 2 rats. BLQ denotes values that were below levels of quantification; nd-not done; NS-not significant

Impact of DFM ingestion on gut and systemic tissue cytokines

Tissue cytokines were measured to gain an understanding of localized (gut) and distal (systemic) immune impacts arising from DFM consumption. Tissue cytokines were most affected in RS-fed rats. Cecal IL-6 (p=0.08) and IL-4 (p=0.06) trended upwards in RS-fed rats but only relative to rats consuming the WB diet (Fig 8A). Effects arising from feeding the RS-containing diet were most evident in the MLN. Levels of CINC-1, IL-4 and TGF- β 1 in the MLN increased in rats fed RS relative to control-fed rats (Figure 8B). In the MLN, levels of IL-6 and IL-10 were below the level of detection for all rats, regardless of diet. However in the liver, levels of the regulatory cytokine TGF- β 1 significantly increased in rats fed RS and OB compared to rats fed the control diet. Liver IL-10 levels were also significantly higher in rats fed either RS or OB but only relative to those fed WB (Fig 9).

The effects of WB intake on gut mucosal and systemic tissue cytokines were compared between male and female rats (Table 11). Females consistently demonstrated higher levels of IL-6 in the ileum, cecum, and colon relative to males. Levels of ileal IL-10 were also higher in females relative to males. In addition, WB feeding induced an increase in ileal IL-10 levels in males while significantly decreasing levels in females (diet x sex interaction p=0.02). In contrast, IL-6 levels were below the level of detection in the spleen of female rats. While levels of splenic IL-4 and TGF- β 1 were 10-fold higher in females than in males. IL-10 levels significantly increased in WB-fed females compared to control-fed females (p<0.01). Splenic CINC-1 levels were 5-fold higher in females compared to males and WB consumption slightly increased CINC-1 levels in males while significantly decreasing levels in females (diet x sex interaction p=0.02).

5.2 Effects of FOS consumption on physiological and immune parameters between male and female rats

This trial assessed the effect of FOS ingestion on tissue immune cell population percentages and cytokine levels between male and female rats. In addition, gut microbiota compositional changes, SCFA profiles and serum biochemical measures including serum LPS were examined. The gut microbiota analysis was conducted by our collaborators and the distribution of phylotypes (%) at the Family level is included in the Appendix (Fig A1).



Fig 10. Differences in weight gain between male and female rats fed either Purina chow or the FOS supplement. Data are shown as mean weight gain (g) \pm SEM (n=18 for males; n=12 for females). (*) indicates a significant difference in weight gain between male and female rats as detected by two-way ANOVA.

Effects of FOS feeding on weight gain in male and female rats

Rats were fed either Purina chow 5001 formulated for rodents or the 5% FOSsupplemented diet consisting of 5g of FOS mixed with 950g of Purina chow. FOS ingestion did not affect the amount of weight gain in males or females. Weight gain over the 6 week feeding trial, was higher in males (p<0.001) relative to females on either diet (Fig 10).



Fig 11. Cecal SCFA profiles from male rats fed either Purina chow (Black bars) or FOS (Dark Grey bars). Bars indicate mean (% SCFA) \pm SEM (n=8). Significant effects of diet were detected by an Unpaired T-test.

Table 12: Relative ratios of SCFA in the cecal contents from rats fed Purina chow or the FOS-supplemented diet

Cecal (A	cetic:Propionic:Butyric)	
Purina Chow	5:1:2 ¹	
FOS	4:1:2	
¹ Differences in the ratios of SCE	A from the cecal contents of	rate fed either Puring chow

¹ Differences in the ratios of SCFA from the cecal contents of rats fed either Purina chow

or the FOS-supplemented diet



Fig 12. Fecal SCFA profiles from male rats fed either Purina chow or FOS. Data are shown as mean (% of total) \pm SEM (n=8). (*) indicates the increase in the percentages of fecal butyric acid in FOS-fed male rats relative to control fed male rats as detected by an Unpaired T-test.

Table 13: Relative ratios of SCFA in the fecal contents from male and female rats either

 Purina chow or the FOS-supplement

	Male Fecal Acetic:Propionic:Butyric	Female Fecal Acetic:Propionic:Butyric
Purina Chow	$5:1:1^1$	$4:1:1^2$
FOS	5:1:2	4:1:1
^{1,2} Differences	in the ratios of SCEA from the fecal	contents from Purina chow or

FOS-fed male and female rats

Effects of FOS ingestion on percent total cecal and fecal SCFA between male and female rats

FOS consumption increased the percent total cecal butyric acid (p=0.03) in rats relative to control-fed counterparts, while no significant changes in the percentages of acetic or propionic acid were observed (Fig 11). An increase in percent total fecal butyric acid production was also seen in male rats fed FOS relative to those fed chow (P=0.002) (Fig 12). The proportions of fecal butyric acid in FOS-fed males were double that of FOS-fed females (Table 13).

Table 14. Concentrations of IgA in cecal tissue and cecal content in male and female rats fed either Purina chow or the FOS-supplemented diet

	MALE		FEM		p value ³		
	Purina chow	FOS	Purina chow	w FOS	Diet ^a	Sex ^b	Diet x Sex ^c
Cecal Tissue							
$IgA(\mu g/g)$	9.4 ± 1.4^{1}	9.4 ± 0.1	6.6 ± 0.6	6.7 ± 0.04	NS	NS	NS
Cecal Content	t						
$IgA(\mu g/g)$	15 ± 1.3^2	17 ± 4.3	20 ± 7.7	13 ± 2.8	NS	NS	NS

 1,2 IgA concentrations are shown as mean (µg/g) \pm SEM for cecal tissue (n=3-5) and cecal

content (n=4-5).

³No significance for the effects of diet^a, differences between sexes^b or an interaction^c

(diet x sex) were detected by two-way ANOVA.



Fig 13. IgA concentrations in the liver of male and female rats fed Purina chow or the FOS-supplemented diet (μ g/g). Bars indicate the mean (μ g/g) ± SEM (n=5-6). (*) indicates a significant increase in the levels of liver IgA in FOS-fed males relative to FOS-fed females as detected by two-way ANOVA.

Effects of FOS intake on IgA concentration

Total IgA levels were evaluated from cecal contents and cecal and liver tissue. Cecal content and tissue IgA levels were unaffected by diet and did not differ between the sexes (Table 14). In contrast, liver IgA concentrations were higher in males compared to females (p=0.01) and concentrations increased in FOS-fed males relative to FOS-fed females (interaction p=0.04) (Fig 13).

	Ma	ales	Ferr	nales			
Measure	Control	FOS	Control	FOS	Diet ^a	Sex ^b	D x S ^c
LDH (U/L)	386 ± 163^1	221 ± 54	92.8 ± 18	90.8 ± 13.9	NS^2	0.002	NS
ALP (U/L)	208 ± 13	$144 \pm 28.2^{*}$	207.4 ± 7.7	216 ± 15.8	0.06	NS	0.06
ALT (U/L)	62.2 ± 4.7	53.9 ± 2.2	61.2 ± 3	51.5 ± 2.4	0.008	NS	NS
AST (U/L)	71.3 ± 6.4	62.4 ± 2.2	63.4 ± 2.4	65.9 ± 2.5	NS	NS	NS
BUN (mg/dL)	18.6 ± 0.9	17.9 ± 1.5	18.9 ± 1.3	17.6 ± 0.9	NS	NS	NS

Table 15: Comparison of serum biochemical measures obtained from male and female rats fed the Purina chow or the FOS-supplemented diet

¹Data are shown as mean \pm SEM (n=5-6).

two-way ANOVA was performed to determine the main effects of diet^a, sex^b and interaction^c.

(*) significance based on diet alone for ALP was further determined by the Mann-Whitney test.

²NS-not significant.



Fig 14. Serum LPS concentrations in male and female rats fed either the Purina chow or FOS-supplemented diet. Bars indicate mean (EU/ml) \pm SEM (n=4-5). (**) significant differences between the sexes were detected by two-way ANOVA.

Effects of FOS feeding on serum biochemical measures between male and female rats

ALT levels decreased in rats following FOS consumption relative to Purina chowfed counterparts (p=0.008). Levels of ALP demonstrated a downward trend in FOS-fed males, while slightly elevating in FOS-fed females (diet*sex interaction p=0.06). In contrast, levels of LDH were significantly higher in males relative to females, regardless of diet. Levels of AST and BUN were unaffected by diet and did not differ between the sexes (Table 15). In addition to serum biochemical measures, LPS concentrations in the serum of male and female rats fed either diet were determined to assess systemic dissemination of gut-derived LPS. Diet had no effect on serum LPS concentrations; however males had higher circulating LPS concentrations relative to females (p=0.004) (Figure 14).



Fig 15. Expression of *MUC2* from cecal tissue of male and female rats fed either Purina chow or FOS. Gene expression relative to housekeeping genes (*RPL10A* and *HPRT*) is shown as mean \pm SEM (n=3-4 for males; n=2 for females).

MUC2 gene expression in FOS-fed male and female rats

MUC2 gene expression was evaluated to determine whether the rapid fermentation of FOS in the cecum affected the secretion of gel-forming mucin between sexes. Overall, *MUC2* mRNA expression from cecal tissue was low (Fig 15). Although the expression of *MUC2* was lower in FOS-fed male rats, this was not significantly different from Purina chow-fed males. The limitation in the availability of female cecal tissue restricted the comparison of cecal tissue *MUC2* expression between sexes.

		Male		Fen	Female		Sex ^b	Diet x Sex ^c
		Control	FOS	Control	FOS	(p value)	(p value)	(p value)
	CD3 ⁺	73 ± 0.9^{1}	73±1.5	77±0.2	77±1.3	NS	0.002	NS
MLN	$CD3^{+}4^{+}$	52±0.7	53±0.7	55±0.8	53±1.4	NS	NS	NS
	CD3+ 8+	19±1.2	18±1.0	20±0.7	22±1.5	NS	NS	NS
	CD45RA ⁺	23±0.8	23±1.6	19±0.6	19±1.3	NS	0.001	NS
	CD3-161+	0.30±0.1	0.30±0.1	0.40±0.1	0.30±0.04	NS	NS	NS
	CD68 ⁺ Total	5.9±0.8	5.5±0.5	8.8±0.8	8.1±0.5	NS	0.0005	NS
	*Foxp3+	11±2.2	9.7±2.1	6.8±1.8	4.9±1.1	NS	0.02	NS
	CD3 ⁺	72±1.2	69±2.0	73±0.9	69±1.1	0.03	NS	NS
Spleen	$CD3^{+}4^{+}$	46±1.5	44±1.4	46±0.90	43±1.2	NS	NS	NS
	CD3+ 8+	26±0.9	25±0.5	26±0.7	25±0.5	NS	NS	NS
	CD45RA ⁺	19±1.1	21±1.4	15±1.2	19±1.4	NS	NS	NS
	CD3+161-	1.2±0.2	1.6±0.2	1.8±0.2	$1.4{\pm}0.1$	NS	NS	NS
	CD68 ⁺ Total	42±1.7	41±1.4	46±2.4	49±1.3	NS	0.002	NS
	*Foxp3+	5.9±1.4	5.6±1.6	2.1±0.4	1.3±0.6	NS	0.001	NS

Table 16. Total immune cell population percentages in the MLN and spleen of males and female rats consuming either Purina chow or the FOS-supplemented diet

¹Data are shown as the percent mean \pm SEM (n=5-6). * indicates the percent Foxp3⁺ T cell population with the CD3⁺4⁺25⁺ T cells in the MLN and spleen of male and female rats. Significance (p<0.05) was analyzed for diet^a, sex^b and interaction^c for each immune cell percentage using two-way ANOVA.

Effects of FOS ingestion on MLN and splenic immune cell population percentages between male and female rats

In the MLN, supplementing the diet with FOS had no major impact on the population profiles of immune cells including lymphocytes and macrophages. However, significant differences were noted in immune cell phenotype distributions between sexes (Table 16). MLN B lymphocyte (CD45RA⁺) population percentages were higher in males compared to females, while MLN total T cell (CD3⁺) percentages were significantly higher in females. Although T helper (CD3⁺4⁺) population percentages remained unchanged, percentages of regulatory T cell (Foxp3⁺) were significantly higher in males compared to females in the MLN and spleen. In contrast, macrophage (CD68⁺) population percentages were higher in the MLN and spleen of females relative to males. Diet affected splenic total T cell (CD3⁺) population percentages, which were observed to decrease in both sexes upon FOS intake.

	•	Male		Female		Diet ^a	Sex ^b	Diet x Sex ^c
Tissue	Cytokine	Control	FOS	Control	FOS	(P value)	(P value)	(P value)
	(ng/g)							
	IL-6	BLQ	BLQ	$1.3\pm0.5^{\mathrm{a}}$	2.4 ± 0.2^{b}	-	-	-
Ileum ¹	IL-4	0.1 ± 0.02	0.1 ± 0.03	0.9 ± 0.2	1.1 ± 0.2	NS	< 0.001	NS
	IL-10	3.9 ± 0.3	4.9 ± 0.7	1.3 ± 0.2	1.2 ± 0.2	NS	< 0.001	NS
	TGF–β1	0.5 ± 0.03	0.5 ± 0.04	BLQ	BLQ	-	-	-
	CINC-1	0.2 ± 0.01	0.3 ± 0.02	0.2 ± 0.05	0.2 ± 0.06	NS	NS	NS
	IL-6	7.9 ± 0.9	7.7 ± 0.9	5.8 ± 0.7	5.1 ± 0.5	NS	< 0.01	NS
Cecum	IL-4	2.9 ± 0.5	2.3 ± 0.4	2.0 ± 0.1	2.3 ± 0.1	NS	NS	NS
	IL-10	5.3 ± 0.2	6.5 ± 1.3	8.2 ± 0.8	6.7 ± 0.3	NS	0.03	NS
	TGF-β1	2.5 ± 0.9	2.2 ± 0.9	2.1 ± 1	2.5 ± 1.3	NS	NS	NS
	CINC-1	0.9 ± 0.2	0.7 ± 0.1	0.1 ± 0.1	0.5 ± 0.2	NS	0.03	NS
	IL-6	1.9 ± 0.2	2.5 ± 0.4	6.2 ± 1.2	4.6 ± 0.6	NS	< 0.001	NS
Colon ¹	IL-4	1.1 ± 0.1	0.8 ± 0.1	$3.9\pm0.3^{\rm a}$	$4.7\pm0.1^{\text{b}}$	NS	< 0.001	0.008
	IL-10	1.0 ± 0.1	0.8 ± 0.1	10.0 ± 0.7	9.8 ± 0.6	NS	< 0.001	NS
	TGF-β1	0.4 ± 0.01	0.5 ± 0.03	0.7 ± 0.1	0.7 ± 0.04	0.04	< 0.01	NS
	CINC-1	0.7 ± 0.1	0.5 ± 0.1	1.6 ± 0.6	1.8 ± 0.1	NS	< 0.01	NS
	IL-6	1.1 ± 0.6	1.7 ± 0.4	0.7 ± 0.2	0.6 ± 0.1	NS	0.01	NS
MLN	IL-4	0.1 ± 0.02	0.3 ± 0.04	0.4 ± 0.1	0.4 ± 0.08	NS	NS	NS
	IL-10	1.0 ± 0.1	0.7 ± 0.2	1.5 ± 0.1	1.8 ± 0.2	NS	< 0.001	NS
	TGF-β1	nd	nd	0.1 ± 0.02	0.2 ± 0.06	-	-	-
	CINC-1	0.3 ± 0.0	0.2 ± 0.05	nd	nd	-	-	-
	IL-6	0.1 ± 0.04	0.1 ± 0.1	2.4 ± 0.2	1.5*	NS	-	-
Spleen ¹	IL-4	1.9 ± 0.3	1.8 ± 0.1	0.6 ± 0.1	0.50 ± 0.1	NS	< 0.01	NS
	IL-10	nd	nd	5.4 ± 0.6	4.9 ± 0.4	NS	NS	NS
	TGF-β1	0.4 ± 0.04	0.3 ± 0.02	1.0 ± 0.4	1.0 ± 0.6	NS	0.07	NS
	CINC-1	0.4 ± 0.05	0.2 ± 0.02	0.8 ± 0.1	0.8 ± 0.06	NS	< 0.001	NS
	IL-6	159 ± 12	173 ± 19	108 ± 9.4	93.8 ± 12	NS	< 0.0001	NS
Liver	IL-4	32.6 ± 0.8	41.0 ± 6.1	49.0 ± 6.2	45.2 ± 6.5	NS	NS	NS
	IL-10	73.2 ± 4.2	75.4 ± 7.1	108 ± 11.1	111 ± 10	NS	0.0005	NS
	TGF-β1	37.0 ± 1.5	41.5 ± 2.2	39.4 ± 3.2	37.5 ± 4.7	NS	NS	NS
	CINC-1	10.9 ± 0.9	9.1 ± 1.0	7.1 ± 1.5	5.3 ± 0.4	NS	0.001	NS

Table 17. Cytokine levels in gastrointestinal and liver tissue in male and female rats fed either Purina chow or the FOS-supplemented diet.

Values indicate the mean $(ng/g) \pm SEM$ (n=3-6) for each tissue. Significance for diet^a, sex^b and diet x sex^c was obtained by two-way ANOVA. Differing letters denote significance based on diet alone as determined by the Unpaired t-test or Mann-Whitney test. (¹)Cytokine measures from male ileum, colon and spleen were performed by J McCarville (MSc. Thesis, 2012). *denotes the mean of 2 rats. BLQ denotes values that were below levels of quantification; nd-not done; NS-not significant.

Effects of FOS ingestion on gut mucosal and systemic tissue cytokine profiles in male and female rats

Cytokine concentrations in gut mucosal and systemic tissues were compared between male and female rats fed FOS (Table 17). Basal cytokine levels varied significantly between sexes. In general, colonic tissue cytokine concentrations were higher in females than in males. Pro-inflammatory cytokines IL-6 and CINC-1 were higher in the cecum and liver of males compared to females. In contrast, the regulatory cytokine IL-10 was higher in the cecum and liver of females relative to males. Although diet had no major effect on tissue cytokine levels, interactions for colonic IL-4 (diet × sex; p=0.008) and a main effect based on diet for colonic TGF- β 1 (p=0.04) were noted. In the MLN males had higher IL-6 levels, while IL-10 levels were higher in females, regardless of diet. Levels of splenic IL-4 and CINC-1 were higher in females compared to males, while splenic IL-6 and IL-10 levels did not differ between sexes.

5.3 Effects of DFM consumption on immune responses in male and female rats to immunization with KLH

This trial assessed the effect of DFM-intake on immune parameters from male and female rats immunized with KLH. Tissue immune cell population percentages, tissue cytokine levels, cytokine levels following ex-vivo stimulation with TLR and KLH recall from whole blood were analyzed for the effect of diet, differences between sexes or an interaction (diet x sex), to detect the effect of DFM intake on male and female rats to immunization with KLH. The kinetics of primary antibody responses were measured at time intervals over a 2 week period (Day 0, 5, 10, 15) following immunization. A secondary response was also measured from male and female rats a week after (day 21) the KLH booster was administered.



Fig 16. The percentage of CD3⁺ total T cells in the PP of male and female rats fed different DFM. Symbols indicate the % mean \pm SEM (n=5). (*) indicates a significant difference between sexes was detected by two-way ANOVA and Tukey's multiple range test.



Fig 17a. Differences in the percentage of $CD3^+4^+$ T_H cells in the PP of rats fed different DFM. Bars indicate the % mean (male and female rats pooled) \pm SEM (n=10). Significant effects of diet were detected by two-way ANOVA. Bars with differing letters are significantly different from each other as determined by Tukey's multiple range test.



Fig 17b. The percentage of CD3⁺4⁺ T_H cells in the PP of male and female rats fed different DFM. Results separated by sex is shown here for consistency. Symbols indicate the % mean ± SEM (n=5). (*) indicates a significant difference between sexes as detected by two-way ANOVA and Tukey's multiple range test.



Fig 18. The percentage of $CD3^+4^+25^+$ T_{reg} cells in the PP of male and female rats fed different DFM. Symbols indicate the % mean \pm SEM (n=5). No significant effects of diet, differences between sexes or an interaction (diet x sex) were detected by two-way ANOVA.


Fig 19. The percentage $Foxp3^+ T_{reg}$ within the CD4⁺CD25⁺ T cell population in the PP of male and female rats fed different DFM. Symbols indicate the % mean ± SEM (n=5). No significant effects of diet, differences between sexes or an interaction (diet x sex) were detected by two-way ANOVA.



Fig 20. The percentage of CD3⁺8⁺ cytotoxic T cells in the PP of male and female rats fed different DFM. Symbols indicate the % mean \pm SEM (n=5). (*) indicates a significant difference between the sexes as detected by two-way ANOVA and Tukey's multiple range test.



Fig 21. The percentage of CD62L⁺ within the CD3⁺ total T cell population in the PP of male and female rats fed different DFM. Symbols indicate the % mean \pm SEM (n=5). (*) indicates significant differences between the sexes as detected by two-way ANOVA and Tukey's multiple range test.



Fig 22. The percentage of $\gamma\delta TCR^+$ cells in the PP of male and female rats fed different DFM. Symbols indicate the % mean ± SEM (n=5). No significant effects of diet, differences between the sexes or an interaction (diet x sex) were detected by two-way ANOVA.



Fig 23. The percentage of CD161a⁺ natural killer cells in the PP of male and female rats fed different DFM. Symbols indicate the % mean \pm SEM (n=5). (*) indicates significant differences between sexes as detected by two-way ANOVA and Tukey's multiple range test.



Fig 24. The percentage of CD45RA⁺ B cells in the PP of male and female rats fed different DFM. Symbols indicate the % mean \pm SEM (n=5). (*) indicates significant differences between sexes as detected by two-way ANOVA and Tukey's multiple range test.



Fig 25. The percentage of macrophages (HIS36⁺) in the PP of male and female rats fed different DFM. Symbols indicate the % mean \pm SEM (n=5). No significant effects of diet, differences between the sexes or an interaction (diet x sex) were detected by two-way ANOVA.



Fig 26. The percentage of CD103⁺ DC in the PP of male and female rats fed different DFM. Symbols indicate the % mean \pm SEM (n=5). A significant difference between sexes (p<0.0001) and a diet x sex interaction (p=0.043) were detected by two-way ANOVA. A trend towards significance (p=0.07) in females was detected by one-way ANOVA upon further analysis. No further differences were determined by Tukey's multiple range test.



Fig 27. Levels of IL-17A in the PP of male and female rats fed different DFM. The thin black line separates FOS-fed females (n=2) from females fed the other diets. Symbols indicate the mean (pg/g) \pm SEM (n=2-5). No significant effects of diet, differences between the sexes or an interaction (diet x sex) were detected by two-way ANOVA.



Fig 28. Levels of TGF- β 1 in the PP of male and female rats fed different DFM. The thin black line separates FOS-fed females (n=2) from females fed the other diets. Data are shown as the mean (pg/g) ± SEM (n=3-5). Significance for the effect of diet on females was analyzed by one-way ANOVA. Symbols with differing letters are significantly different from each other as detected by Tukey's multiple range test.



Fig 29. Levels of IL-10 in the PP of male and female rats fed different DFM. The thin black line separates FOS-fed females (n=2) from females fed the other diets. Symbols indicate the mean (pg/g) \pm SEM (n=2-5). No significant effects of diet, differences between the sexes or an interaction (diet x sex) were detected by two-way ANOVA.

Effects of DFM consumption on immune cell percentages and cytokine levels in the PP of male and female rats

The percentage of certain immune cell populations differed significantly between sexes. The population percentages of CD3⁺ total T cells, CD3⁺4⁺ T helper cells, CD3⁺8⁺ cytotoxic T cells and CD62L⁺ lymphocytes were significantly higher (p<0.001) in the PP of males relative to females (Fig 16, 17b, 20 and 21, respectively). Rats ingesting the DFM-supplemented diets had significantly reduced (p=0.02) percentages of CD3⁺4⁺ T helper cells in the PP relative to control-fed rats (Fig 17a). The percentage of CD161a⁺ Natural Killer cells (Fig 23) and CD45RA⁺ B cells were significantly higher (p<0.0001) in the PP of females compared to males (Fig 24). The percentage of CD103⁺ DC was not only higher (p<0.0001) in the PP of females relative to males but also showed significance for an interaction (diet x sex; p=0.043) (Fig 26). The limited amount of PP tissue restricted the range of cytokine analysis, especially in FOS-fed females (n=2). Although diet did not alter IL-17A levels (Fig 27), TGF- β 1 levels were significantly lower (p=0.02) in RS-fed females relative to control-fed females (Fig 28).



*

Fig 30. The percentage of CD3⁺ total T cells in the MLN of male and female rats fed different DFM. Symbols indicate the % mean \pm SEM (n=5). (*) indicates significant differences between sexes as detected by two-way ANOVA and Tukey's multiple range test.



Fig 31. The percentage of $CD3^+4^+T_H$ cells in the MLN of male and female rats fed different DFM. Symbols indicate the % mean \pm SEM (n=5). No significant effects of diet, differences between sexes or an interaction were detected by Two-way ANOVA.



Fig 32. The percentage of CD3⁺4⁺25⁺ T_{reg} cells in the MLN of male and female rats fed different DFM. Symbols indicate the % mean ± SEM (n=5). No significant effects of diet, differences between the sexes or an interaction were detected by two-way ANOVA.



Fig 33. The percentage of Foxp3⁺ T_{reg} cells within the CD4⁺CD25⁺ T cell population in the MLN of male and female rats fed different DFM. Symbols indicate the % mean ± SEM (n=5). No significant effects of diet, differences between the sexes or an interaction (diet x sex) were detected by two-way ANOVA.



Fig 34a. Differences in the percentage of CD3⁺8⁺ cytotoxic T cells in the MLN of rats fed different DFM. Bars indicate the % mean (male and female rats pooled) \pm SEM (n=10). Significant effects of diet were detected by two-way ANOVA. Bars with differing letters are significantly different from each other as determined by Tukey's multiple range test.



Fig 34b. The percentage of $CD3^+8^+$ cytotoxic T cells in the MLN of male and female rats fed different DFM. Results separated by sex is shown here for consistency. Symbols indicate the % mean \pm SEM (n=5). No significant differences between sexes or an interaction (diet x sex) were detected by two-way ANOVA.



Fig 35. The percentage of CD62L⁺ within the CD3⁺ total T cell population in the MLN of male and female rats fed different DFM. Symbols indicate the % mean \pm SEM (n=5). No significant effects of diet, differences between the sexes or an interaction were detected by two-way ANOVA.



Fig 36. The percentage of $\gamma\delta$ TCR⁺ in the MLN of male and female rats fed different DFM. Symbols indicate the % mean ± SEM (n=5). (*) indicates significant differences between sexes as detected by two-way ANOVA and Tukey's multiple range test.



Fig 37. The percentage of CD161a⁺ natural killer cells in the MLN of male and female rats fed different DFM. Symbols indicate the % mean \pm SEM (n=5). No significant effects of diet, differences between sexes or an interaction (diet x sex) were detected by two-way ANOVA.



Fig 38. The percentage of CD45RA⁺ B cells in the MLN of male and female rats fed different DFM. Symbols indicate the % mean \pm SEM (n=5). (*) indicates significant (p=0.02) differences between the sexes as detected by two-way ANOVA and Tukey's multiple range test.



Fig 39. The percentage of CD103⁺ DC in the MLN of male and female rats fed different DFM. Symbols indicate the % mean \pm SEM (n=5). No significant effects of diet, differences between the sexes or an interaction (diet x sex) were detected by two-way ANOVA.



*

Fig 40. The percentage of HIS36⁺ macrophages in the MLN of male and female rats fed different DFM. Symbols indicate the % mean \pm SEM (n=5). (*) indicates significant differences between the sexes as detected by two-way ANOVA and Tukey's multiple range test.



Fig 41. The levels of CINC-1 in the MLN of male and female rats fed different DFM. Symbols indicate mean $(pg/g) \pm SEM$ (n=4). No significant effects of diet, differences between the sexes or an interaction (diet x sex) were detected by two-way ANOVA.



Fig 42. The levels of IFN- γ in the MLN of male and female rats fed different DFM. Symbols indicate mean (pg/g) ± SEM (n=3-4). (*) indicates significant differences between the sexes as detected by two-way ANOVA and Tukey's multiple range test.



Fig 43. The levels of IL-17A in the MLN of male and female rats fed different DFM. Symbols indicate mean $(pg/g) \pm SEM$ (n=4). No significant effects of diet, differences between the sexes or an interaction (diet x sex) were detected by two-way ANOVA.



Fig 44. The levels of IL-4 in the MLN of male and female rats fed different DFM. Symbols indicate mean $(pg/g) \pm SEM$ (n=4). No significant effects of diet, differences between the sexes or an interaction were detected by two-way ANOVA.



Fig 45. The levels of IL-10 in the MLN of male and female rats fed different DFM. Symbols indicate mean $(pg/g) \pm SEM$ (n=4). (*) indicates significant differences between sexes as detected by two-way ANOVA and Tukey's multiple range test.

Effects of DFM ingestion on immune cell population percentages and cytokine levels in the MLN of male and female rats

Overall the effects of diet and differences between sexes in immune cell population percentages in the MLN were limited. Percentages of CD3⁺ (total T cells) in females were higher (p=0.02) compared to males (Fig 30), as were percentages of $\gamma\delta TCR^+$ cells in females (p<0.001) (Fig 36). In contrast, control and FOS-fed males had higher percentages of CD45RA⁺ B cells compared to females fed the same diets (p=0.02) (Fig 38). MLN HIS36⁺ macrophages were also higher in males (p<0.001) compared to females (Fig 40). The percentages of CD103⁺ DC in the MLN were unaffected by diet and did not differ between sexes (Fig 39). The difference in the percentage of CD3⁺8⁺ cytotoxic T cells between RS-fed and control-fed females was significantly large (Fig 34b). No significant differences between sexes were detected and the lowest percentages of CD3⁺8⁺ cytotoxic T cells were observed in RS and FOS-fed rats compared to control-fed rats (Fig 34a). Levels of MLN IL-10 (Fig 45) were generally higher in males (p<0.0001) compared to females, possibly reflecting the higher percentage of macrophages (HIS36⁺) observed in the MLN of males. In contrast, levels of IFN- γ (Fig 42) were significantly (p<0.0001) lower in males relative to females, concurrent with lower MLN total T cell (CD3⁺) percentages in males. Levels of MLN CINC-1, IL-17A and IL-4 were unaffected by diet and did not differ between sexes (Figs 41, 43 and 44).



Fig 46a. Differences in the percentage of $CD3^+$ total T cells in the spleen of rats fed different DFM. Bars indicate the % mean (male and female rats pooled) \pm SEM (n=10). Significance for the effects of diet were detected by two-way ANOVA. Bars with differing letters are significantly different from each other as determined by Tukey's multiple range test.



Fig 46b. The percentage of CD3⁺ total T cells in the spleen of male and female rats fed different DFM. Results separated by sex is shown here for consistency. Symbols indicate the % mean \pm SEM (n=5). No significant differences between sexes or an interaction (diet x sex) were detected by two-way ANOVA.



Fig 47. Effects of DFM on the percentage of $CD3^+4^+T_H$ in the spleen of male and female rats fed. Symbols indicate the % mean ± SEM (n=5). An effect of diet (p=0.02) and a diet x sex interaction (p=0.008) were detected by two-way ANOVA. (*) indicates control-fed males had lower splenic $CD3^+4^+$ percentages compared to control-fed females. Symbols with differing letters are significant differences based on diet as detected by one-way ANOVA and Tukey's multiple range test.



Fig 48. The percentage of $CD3^+4^+25^+$ T_{reg} cells in the spleen of male and female rats fed different DFM. Symbols indicate the % mean \pm SEM (n=5). No significant effects of diet, differences between the sexes or an interaction (diet x sex) were detected by two-way ANOVA.



Fig 49. The percentage of $Foxp3^+T_{reg}$ cells within the CD4⁺CD25⁺ T cell population in the spleen of male and female rats fed different DFM. Symbols indicate the % mean ± SEM (n=5). No significant effects of diet, differences between the sexes or an interaction were detected by two-way ANOVA.



Fig 50. Effects of DFM on the percentage of $CD3^+8^+$ cytotoxic T cells in the spleen of male and female rats. Symbols indicate the % mean ± SEM (n=5). An effect of diet (p=0.01), sex (p=0.004) and a diet x sex interaction (p=0.03) were detected by two-way ANOVA. (*) indicates significantly different percentages of splenic $CD3^+8^+$ T cells between males fed RS, WB or FOS and females fed the same diets as determined by the Tukey's multiple range test. Symbols with differing letters indicate significant differences based on diet as detected by one-way ANOVA and Tukey's multiple range test conducted on females alone.


Fig 51. The percentages of CD62L⁺ within the CD3⁺ total T cell population in the spleen of males and females fed different DFM. Results are shown as % mean \pm SEM (n=5). No significant effects of diet, differences between sexes or an interaction (diet x sex) were detected by two-way ANOVA.



Fig 52. Effects of DFM ingestion on the percentage of splenic $\gamma\delta$ TCR⁺ cells in male and female rats. Symbols indicate the % mean ± SEM (n=5). Differences between sexes (p=0.002) and a diet x sex interaction (p=0.01) were detected by two-way ANOVA. (*) indicates significantly lower percentages of splenic $\gamma\delta$ TCR⁺ cells in males fed OB or FOS relative to females fed the same diets as detected by the Tukey's test. Symbols with differing letters indicate significant differences based on diet as determined by one-way ANOVA and Tukey's multiple range test conducted on males alone.



Fig 53. The percentage of CD161a⁺ natural killer cells in the spleen of male and female rats fed different DFM. Symbols indicate the % mean \pm SEM (n=5). No significant effects of diet, or differences between sexes or an interaction (diet x sex) were detected by two-way ANOVA.



Fig 54a. Differences in the percentage of CD45RA⁺ B cells in the spleen of rats fed different DFM. Bars indicate the % mean (male and female rats pooled) \pm SEM (n=10). Significance for the effects of diet were detected by two-way ANOVA. Bars with differing letters are significantly different from each other as determined by Tukey's multiple range test.



Fig 54b. The percentage of splenic CD45RA⁺ B cells in male and female rats fed different DFM. Results separated by sex is shown here for consistency. Symbols indicate the % mean \pm SEM (n=5). (*) indicates significant differences between sexes fed DFM as detected by two-way ANOVA and Tukey's multiple range test.



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Fig 55. The percentage of CD103⁺ DC in the spleen of male and female rats fed different DFM. Symbols indicates the % mean \pm SEM (n=5). (*) indicates significant differences between sexes as detected by two-way ANOVA and Tukey's multiple range test.



Fig 56. Effects of DFM ingestion on the percentage of splenic HIS36⁺ macrophages in male and female rats. Symbols indicate the % mean \pm SEM (n=5). An effect of diet (p<0.05), sex (p<0.05) and a diet x sex interaction (p<0.001) were detected by two-way ANOVA. (*) indicates RS-fed males had significantly lower percentages of splenic HIS36⁺ macrophages compared to RS-fed females. Symbols with differing letters indicate significant differences based on diet as detected by one-way ANOVA and Tukey's multiple range test conducted on females alone.



Fig 57. Effects of DFM on splenic TGF- β 1 levels in male and female rats. Symbols indicate the mean (pg/g) ± SEM (n=4-5). A trend toward a significant interaction (diet x sex p=0.074) was detected by two-way ANOVA. Symbols with differing letters indicate significant differences based on diet as detected by one-way ANOVA and Tukey's multiple range test conducted on males alone.



Fig 58. Splenic IL-4 levels in the spleen of male and female rats fed different DFM. Symbols indicate mean $(pg/g) \pm$ SEM (n=4-5). A significant difference between sexes (p=0.002) was detected by two-way ANOVA. Tukey's multiple range test did not detect further differences.



Fig 59. Splenic IFN- γ levels in male and female rats fed different DFM. Symbols indicate mean (pg/g) ± SEM (n=4-5). No significant effects of diet, or differences between sexes or an interaction (diet x sex) were detected by two-way ANOVA.



Fig 60. The ratio of splenic IFN- γ /IL-4 in male and female rats fed different DFM. Symbols indicate mean ratio \pm SEM (n=4-5). A significant difference between sexes (p<0.0001) was detected by two-way ANOVA.

Effect of DFM on immune cell population percentages and cytokine levels in the spleen of male and female rats

Splenic immune cell percentages were affected by diet, were different between sexes and for certain immune cell types a significance for an interaction (diet x sex) was also observed. While differences in the percentages of CD3⁺ total T cells between FOS-fed and control fed males and RS-fed and control-fed females were the largest (Fig 46b), there was no significant difference between sexes or an interaction (diet x sex). Analysis of combined male and female data indicated the lowest percentages of CD3⁺ total T cells were in RS and FOS-fed rats compared to control-fed rats (Fig 46a). The percentage of $CD3^{+}4^{+}$ T helper and $CD3^+ 8^+$ cytotoxic T cells were significantly lower in RS, WB or FOS-fed females relative to control-fed females (Fig 47 and 48, respectively), as DFM intake in females reduced the percentages of these two immune cell types to the levels observed in males. In contrast, splenic $\gamma\delta TCR^+$ population percentages not only significantly decreased in OB-fed males (p<0.05) relative to control-fed males, but were also lower in OB and FOS-fed males compared to females fed the same diets (Fig 52). The percentage of $CD45RA^+$ B cells significantly increased (p=0.02) in RS and FOS-fed rats relative to control fed rats (Fig 54a). Overall, males had a higher percentage (p<0.05) of CD45RA⁺B cells relative to females (Fig 54b). DFM intake influenced the differences in splenic CD103⁺ DC percentages between the sexes. CD103⁺ DC percentages were the same between male and female rats fed the control diet, however DFM intake induced higher percentages of splenic CD103⁺ DC in females relative to males fed the DFM supplements (Fig 55). RS intake increased (p<0.0001) splenic macrophage (HIS36⁺) percentages

relative to all other diets in females, and RS-fed females also had significantly higher percentages of HIS36⁺ macrophages than RS-fed males (p<0.001) (Fig 56). WB and OB intake decreased (p=0.001) splenic TGF- β 1 levels (Fig 57). Although females had lower splenic ratios of IFN- γ /IL-4 compared to males, RS intake in females elevated the ratio to levels similar to those observed in RS-fed males (p<0.001) (Fig 60).



Fig 61. IL-10 levels following *ex-vivo* stimulation with TLR agonists and/or KLH in whole blood cultures from male rats fed different DFM. Bars indicate mean $(pg/ml) \pm SEM$ (n=2-3). Co-incubation with KLH and LPS induced higher IL-10 levels in *ex vivo* cultures from FOS-fed males, reflected in a trend towards significance relative to unstimulated negative control cultures. Data was analyzed by Kruskal-Wallis test.



Fig 62. IL-10 levels following *ex-vivo* stimulation with TLR agonists and/or KLH in whole blood cultures from female rats fed different DFM. Bars indicate mean (pg/ml) \pm SEM (n=2-3). Diet did not affect IL-10 (pg/ml) levels produced by stimulation with TLR agonists and/or KLH.



Fig 63. IFN- γ levels following *ex-vivo* stimulation with TLR agonists and/or KLH in whole blood cultures from male rats fed different DFM. Bars indicate mean (pg/ml) ± SEM (n=2-3). Diet did not affect IFN- γ (pg/ml) levels produced by stimulation with TLR agonists and/or KLH.



□ AIN-93G □ RS ■ WB ■ OB ■ FOS

Fig 64. IFN- γ levels following *ex-vivo* stimulation with TLR agonists and/or KLH in whole blood cultures from female rats fed different DFM. Bars indicate mean (pg/ml) ± SEM (n=2-3). Diet did not affect IFN- γ (pg/ml) levels produced by stimulation with TLR agonists and/or KLH.



Fig 65. IL-4 levels following *ex-vivo* stimulation with TLR agonists and KLH stimulation in whole blood cultures from male rats fed different DFM. Bars indicate mean (pg/ml) \pm SEM (n=2-5). Diet did not affect IL-4 (pg/ml) levels produced by stimulation with TLR agonists and/or KLH.



Fig 66. IL-4 levels following *ex-vivo* stimulation with TLR agonists and/or KLH in whole blood cultures from female rats fed different DFM. Bars indicate mean (pg/ml) \pm SEM (n=2-5). Diet did not affect IL-4 (pg/ml) levels produced by stimulation with TLR agonists and/or KLH.

Effects of DFM ingestion on ex-vivo cytokine production in whole blood cultures stimulated with TLR agonists and/or KLH

The whole blood culture stimulation assay was performed to evaluate TLR agonistinduced innate responses at the systemic level in rats fed different fermentable substrates. Whole blood cultures from rats fed different DFM were also stimulated with KLH to determine recall responses. The recall response to KLH was also assessed in the presence of TLR agonists as this approach has been reported to enhance the production of T cell cytokines in response to recall antigen (Dammerman *et al.*, 2013). Overall, stimulantinduced whole blood cultures had higher levels of IL-10 relative to IFN- γ and IL-4, regardless of diet or sex. In whole blood cultures from FOS-fed males, the combination of KLH and LPS induced IL-10 levels that showed a trend towards significance (p=0.07) relative to unstimulated control (Fig 61). In general, whole blood cultures from OB-fed females appeared to be the least responsive to exogenous stimulation.



B)



Fig 67. Concentrations of total IgM in the serum of KLH-immunized A) male and B) female rats fed different DFM. Bars indicate mean (μ g/ml) \pm SEM (n=4-5). (*) indicates significantly higher concentrations of total IgM on day 0 in males compared to females, while on days 5, 10 and 21 females showed higher concentrations relative to males as detected by two-way ANOVA and Tukey's multiple range test. Please refer to Table 15 (below) for a summary of the statistical analysis of the effects of diet, differences between sexes or an interaction (diet x sex).



Fig 68. Concentrations of anti-KLH IgM in the serum of KLH-immunized A) male and B) female rats fed different DFM. Bars indicate mean (μ g/ml) \pm SEM (n=3-5). (*) indicates significantly higher concentrations of anti-KLH IgM in females compared to males on Day 10, while on day 15 males showed higher concentrations relative to females as detected by two-way ANOVA and Tukey's multiple range test. Please refer to Table 15 (below) for a summary of the statistical analysis of the effects of diet, differences between sexes or an interaction (diet x sex).



Fig 69. Concentrations of total IgG in the serum of KLH-immunized A) male and B) female rats fed different DFM. Bars indicate mean (μ g/ml) \pm SEM (n=4-5). (*) indicates significantly higher concentrations of total IgG on days 0 and 21 in males compared to females as detected by two-way ANOVA and Tukey's multiple range test. Please refer to Table 15 (below) for a summary of the statistical analysis of the effects of diet, differences between sexes or an interaction (diet x sex).



Fig 70. Differences in the concentration of total IgG on day 0 between male and female rats fed different DFM. Bars indicate the mean (μ g/ml) ± SEM (n=4-5). A trend towards significance (p=0.07) for the effects of diet and significance (p=0.01) for an interaction (diet x sex) were detected by two-way ANOVA. (*) indicates OB-fed males had significantly higher total IgG concentrations on day 0 compared to OB-fed females as determined by Tukey's multiple range test. Bars with differing letters are significantly different from each other as detected by Tukey's multiple range test.



Fig 71. Concentrations of anti-KLH IgG in the serum of KLH-immunized A) male and B) female rats fed different DFM. Bars indicate mean (μ g/ml) \pm SEM (n=3-5). (*) indicates significantly higher concentrations of anti-KLH IgG on days 10 and 15 in females relative to males, while concentrations were significantly higher on day 21 in males compared to females as detected by two-way ANOVA and Tukey's multiple range test. Please refer to Table 15 (below) for a summary of the statistical analysis of the effects of diet, differences between sexes or an interaction (diet x sex).



Fig 72. Differences in the concentration of anti-KLH IgG, 5 days post-immunization, in rats fed different DFM. Bars indicate the mean (male and female rats pooled) (μ g/ml) \pm SEM (n=9-10). Significance for the effect of diet was detected by two-way ANOVA. Bars with differing letters are significantly different from each other as determined by Tukey's multiple range test.



Fig 73. Differences in the concentration of anti-KLH IgG, 10 days post-immunization, in rats fed different DFM. Bars indicate the mean (male and female rats pooled) (μ g/ml) \pm SEM (n=7-10). Significance for the effect of diet was detected by two-way ANOVA. Bars with differing letters are significantly different from each other as detected by Tukey's multiple range test.



Fig 74. Differences in the concentration of anti-KLH IgG on day 21, post-secondary immunization, in male and female rats fed different DFM. Bars indicate the mean (μ g/ml) \pm SEM (n=4-5). Significance for the effects of diet (p<0.01), (*) differences between sexes (p<0.0001) and an interaction (diet x sex) (p=0.01) were detected by two-way ANOVA. Bars with differing letters are significantly different from each other as detected by one-way ANOVA and Tukey's multiple range test conducted in males alone.



Fig 75. Concentrations of total IgG2a in the serum of KLH-immunized A) male and B) female rats fed different DFM were evaluated only on the 10th and 15th day post-immunization. Bars indicate mean (μ g/ml) \pm SEM (n=4-5). (*) indicates significantly higher concentrations of total IgG2a on day 10 in males than females, while on day 15 females had higher concentrations over males as detected by two-way ANOVA and Tukey's multiple range test. Please refer to Table 15 (below) for a summary of the statistical analysis of the effects of diet, differences between sexes or an interaction (diet x sex).



B)



Fig 76. Concentrations of anti-KLH IgG2a in the serum of KLH-immunized A) male and B) female rats fed different DFM were evaluated only on the 10^{th} and 15^{th} day post-immunization. Bars indicate mean (μ g/ml) \pm SEM (n=4-5). (*) indicates significantly higher concentrations of anti-KLH IgG2a on day 10 in males than females. Females showed a trend towards significance for anti-KLH IgG2a concentration on day 15 as detected by two-way ANOVA and Tukey's multiple range test. Please refer to Table 15 (below) for a summary of the statistical analysis of the effects of diet, differences between sexes or an interaction (diet x sex).



Fig 77. Concentrations of total IgG2b in the serum of KLH-immunized A) male and B) female rats fed different DFM were evaluated only the 10^{th} and 15^{th} day post-immunization. Bars indicate mean (µg/ml) ± SEM (n=4-5). No differences between sexes were detected by two-way ANOVA. Please refer to Table 15 (below) for a summary of the statistical analysis of the effects of diet or an interaction (diet x sex).



Fig 78. Concentrations of anti-KLH IgG2b in the serum of KLH-immunized A) male and B) female rats fed different DFM were evaluated on the 10th and 15th day post-immunization. Bars indicate mean (μ g/ml) \pm SEM (n=4-5). (*) indicate significantly higher concentrations of anti-KLH IgG2a on day 10 in males than females as detected by two-way ANOVA and Tukey's multiple range test. Please refer to Table 15 (below) for a summary of the statistical analysis of the effects of diet, differences between sexes or an interaction (diet x sex).



Fig 79. Differences in the concentration of anti-KLH IgG2b, 10 days post-immunization, in rats fed different DFM. Bars indicate the mean (male and female rats pooled) (μ g/ml) \pm SEM (n=9-10). Significance for the effect of diet was detected by two-way ANOVA. Bars with differing letters are significantly different from each other as determined by Tukey's multiple range test.



Fig 80. Concentrations of total IgA in the serum of KLH-immunized A) male and B) female rats fed different DFM. Bars indicate mean (μ g/ml) ± SEM (n=4-5). (*) indicates significantly higher concentrations of total IgA on days 0, 5 and 10 in males than females as detected by two-way ANOVA and Tukey's test. Total IgA concentrations could not be examined from males on day 15. Please refer to Table 15 (below) for a summary of the statistical analysis of the effects of diet, differences between sexes or an interaction (diet x sex).



Fig 81. Differences in the concentration of anti-KLH IgA, 15 days post-immunization, in female rats fed different DFM. Bars indicate the mean (μ g/ml) \pm SEM (n=4-5). Significance for the effect of diet was detected by one-way ANOVA. Bars with differing letters are significantly different from each other as detected by Tukey's multiple range test.

Table 18. Statistical analysis of the effect of DFM on the response of male and female rats to immunization
with KLH.

	Day 0	Day 5	Day 10	Day 15	Day 21
Total IgM	Diet – NS ¹	Diet – NS	Diet - NS	Diet –NS	Diet - NS
	Sex - p <0.001 ² ;	Sex - p <0.001;	Sex - p <0.001;	Sex- NS	Sex - p <0.001;
Fig 67	(M > F)	(F > M)	(F > M)	Diet x Sex –NS	(F > M)
	Diet x Sex – NS ³	Diet x Sex – NS	Diet x Sex – NS		Diet x Sex – NS
And VIIII	n/a	Dist NS	Dist $n=0.07$	Dist NC	Dist NC
Anti-KLH IgM	II/a	Diet – NS	Diet $p=0.07$	Diet - NS	Diet – NS
E:~ (9		Sex-INS Dist y Say MS	Sex - $p < 0.001$;	Sex - $p=0.05$;	Dist y Say MS
F1g 00		Diet x Sex -NS	$(\Gamma > M)$	$(M > \Gamma)$ Dist v Sev. NS	Diet x Sex -NS
Total IcC	Dist 0.07	Dist NG	Diet NG	Diet NG	Dist NC
Total IgG	Diet = 0.07	Diet – NS	Diet – NS	Diet – NS	Diet $-NS$
\mathbf{E}_{α}^{*} (0) \mathbf{E}_{α}^{*} 70	Dist v Sov	Dist v Cov. NC	Dist v Cov. NC	Dist y Say MC	Sex - p < 0.001
rig 09, rig 70	p = 0.01	Diet x Sex -INS	Diet x Sex -INS	Diet x Sex -NS	p=0.004
	p = 0.01				p=0.004
	Tukov's MDT.				Tukov's MDT.
	$OB_{fed} M(\uparrow)$				FOS-fed M(1)
	OB-fed $F(1)$				FOS-fed $F()$
	C > WB(F)				FOS > RS(M)
	C > OB(F)				FOS > WB (M)
Anti-KLH IgG	C > OD (I)	Diet - n=0.01	Diet - n=0.023	Diet – NS	Diet - $p=0.01$
Anti-KEII 1g0		Sex $-$ NS	Sex - $p=0.023$	Sex - $n < 0.001$	Sex - $p < 0.001$
Fig 71 Fig 72		Diet x Sex – NS	(F > M)	(F > M)	Diet x Sex -
Fig 73 Fig 74	n/a ⁴	Diet A Ben Tib	Diet x Sex $-$ NS	Diet x Sex $-$ NS	p=0.01
119 /0,119 / 1					Tukev's MRT:
		Tukey's MRT:	Tukey's MRT:		C > RS
		C > RS	C > RS		C > WB
		C > OB	C > WB		C > OB
		C > FOS	C > OB		C > FOS
Total IgG2a			Diet - NS	Diet – NS	
8	n/a	n/a	Sex - p < 0.001;	Sex - p < 0.001;	nd ⁵
Fig 75			(M > F)	(F > M)	
8			Diet x Sex – NS	Diet x Sex – NS	
Anti-KLH			Diet - NS	Diet – NS	
IgG2a	n/a	n/a	Sex - p <0.001	Sex - p=0.06	nd
-			Diet x Sex – NS	Diet x Sex – NS	
Fig 76			(M > F)		
Total IgG2b			Diet -NS	Diet -NS	
_	n/a	n/a	Sex - NS	Sex - NS	nd
Fig 77			Diet x Sex - NS	Diet x Sex – NS	
Anti-KLH			Diet - p=0.01	Diet – NS	
IgG2b	n/a	n/a	Sex - NS	Sex - p <0.001;	nd
			Diet x Sex –NS	(F > M)	
Fig 78, Fig 79			Tukey's MRT:	Diet x Sex – NS	
			C > RS		
			C >OB		

^{1,2,3}Effects of diet, differences between sexes or an interaction. ⁴n/a- not applicable; nd⁵- not done; Male-M; Female-F



Fig 82. Shift in the anti-KLH IgM kinetics in males fed different DFM. The plot indicates the effect of DFM intake on the mean concentration of anti-KLH IgM (μ g/ml) primary and secondary responses of males to immunization with KLH. (The plot summarizes the mean (μ g/ml) ± SEM at each time interval shown in Fig 68 A).



Fig 83. Shift in the anti-KLH IgM kinetics in females fed different DFM. The plot indicates the effect of DFM intake on the mean concentration of anti-KLH IgM (μ g/ml) primary and secondary responses of females to immunization with KLH. (The plot summarizes the mean (μ g/ml) ± SEM at each time interval shown in Fig 68 B).


Fig 84. Shift in the anti-KLH IgG kinetics in males fed different DFM. The plot indicates the effect of DFM intake on the mean concentration of anti-KLH IgG (μ g/ml) primary responses of males to immunization with KLH. (The plot summarizes the mean (μ g/ml) ± SEM at each time interval shown in Fig 71 A).



Fig 85. Shift in the anti-KLH IgG kinetics in males fed different DFM. The plot indicates the effect of DFM intake on the mean concentration of anti-KLH IgG (μ g/ml) primary and secondary responses of males to immunization with KLH. (The plot summarizes the mean (μ g/ml) ± SEM at each time interval shown in Fig 71 A).



Fig 86. Shift in the anti-KLH IgG kinetics in females fed different DFM. The plot indicates the effect of DFM intake on the mean concentration of anti-KLH IgG (μ g/ml) primary responses of females to immunization with KLH. (The plot summarizes the mean (μ g/ml) ± SEM at each time interval shown in Fig 71 B).



Fig 87. Shift in the anti-KLH IgG kinetics in females fed different DFM. Plot indicates the effect of DFM intake on the mean concentration of anti-KLH IgG (μ g/ml) primary and secondary responses of females to immunization with KLH. (The plot summarizes the mean (μ g/ml) ± SEM at each time interval shown in Fig 71 B).

Effect of DFM consumption on systemic immune responses of male and female rats to immunization with KLH

The adaptive immune response in rats to KLH immunization differed significantly between sexes (Table 15). Prior to immunization male rats had higher serum concentrations of total IgM, however post-immunization, total IgM levels were significantly higher in females relative to males. Total IgG concentrations did not differ between sexes, but an interaction (diet x sex) for total IgG concentrations was detected on the day of immunization (day 0) and on day 21, seven days following the KLH booster (Table 15). Significantly lower concentrations of total IgG were observed on day 0 in females fed WB and OB relative to control-fed counterparts. In addition, on day 0 OB-fed males had significantly higher concentrations of total IgG compared to OB-fed females. In contrast on day 21, FOS-fed males had higher concentrations of total IgG relative to FOS-fed females. Rats consuming DFM had significantly lower anti-KLH IgG concentrations over the primary immunization period relative to rats fed the control diet (day 5 and day 10; Fig 71 and 72). Serum concentrations of total IgG2a, but not IgG2b differed between sexes (Table 15). Anti-KLH IgG2a concentrations were higher in males compared to females at 10, but five days later, females showed a trend towards significance for anti-KLH IgG2a (Table 15). Initially on Day 10, anti-KLH IgG2b concentrations were significantly lower in RS and OB-fed rats relative to control fed rats (Fig 78; Table 15), however this effect of diet was missing five days later (Table 15). Total IgA concentrations were higher in males compared to females at all-time points examined (Fig 80; Table 15). The concentration of circulating anti-KLH IgA was significantly higher in WB-fed females relative to controlfed females 15 days post immunization (Fig 81). On day 15 females (on all diets) had higher circulating amounts of anti-KLH IgG, IgG2a and IgG2b than males. In contrast, males had higher concentrations of anti-KLH IgM on day 15. The secondary anti-KLH IgG response was approximately 10-fold higher in control-fed males compared to control fedfemales. Significance for an interaction (diet x sex; p=0.01) was also noted for the secondary anti-KLH IgG response. DFM-intake significantly (p=0.01) reduced the secondary anti-KLH IgG response in males relative to control-fed males, while no difference diet-based differences were noted between females fed the different DFM diets. 5.4 Effects of butyric acid on chemokine production by TLR agonist-challenged IEC

In vitro cell culture models were utilized to assess the time of exposure and dose effect of the bacterial fermentation metabolite butyric acid (BA) (10mM and 25mM) on proinflammatory chemokine production by TLR 4 and TLR 3 agonist challenged IECs. CINC-1 production was evaluated from IECs challenged with TLR agonists in the presence of BA. CINC-1 production was also evaluated from IEC-6 cells pretreated with BA prior to challenge with TLR agonists. The effects of butyric acid on CINC-1 secretion by β estradiol treated IEC-6 challenged with LPS were examined to explore the influence of this sex hormone on the effects of BA. The effects of β -estradiol on IL-8 production by HT-29 IEC challenged with TNF- α were also determined. Time and dose-dependent effects of BA on chemokine production by HT-29 IEC-stimulated by TLR2 agonist lipoprotein Pam₃Cys in combination with TLR3 agonist poly (I:C) were also evaluated. The effect of BA on the combination of TLR agonist-induced IL-8 production via the p38 pathway was tested using the p38 blocker (SB203580). The effects of ATP on IL-8 production by TLR challenged HT-29 cells were also assessed.



Fig 88. CINC-1 production by IEC-6 cells challenged with LPS (20 ng/ml) in the presence of 10 mM or 25 mM BA. Bars indicate mean (pg/ml) \pm SEM (n= 4 independent experiments). Significant effects of BA presence on CINC-1 (pg/ml) levels were detected by Kruskal-Wallis (p<0.001). Dunn's test detected no differences between treatment groups.



Fig 89. CINC-1 production by IEC-6 cells pretreated with 10 mM or 25 mM BA prior to challenge with LPS (20 ng/ml). Bars indicate mean (pg/ml) \pm SEM (n= 4 independent experiments). Significant effects of BA pre-treatment on CINC-1 (pg/ml) levels were detected by one-way ANOVA (p<0.0008). Differing letters indicate significance between treatment groups as determined by Tukey's multiple range test.



Fig 90. CINC-1 production by IEC-6 cells challenged with poly (I:C) (40 μ g/ml) in the presence of 10 mM or 25 mM BA. Bars indicate mean (pg/ml) ± SEM (n= 4 independent experiments). A trend towards significance for the effects of BA on CINC-1 (pg/ml) levels were detected by Kruskal-Wallis test (p<0.07), although Dunn's test did not distinguish differences between treatment groups.



Fig 91. CINC-1 production by IEC-6 cells pretreated with 10 mM or 25 mM BA prior to challenge with poly (I:C) (40 μ g/ml). Bars indicate mean (pg/ml) ± SEM (n=4 independent experiments). No significance for the effects of BA on CINC-1 (pg/ml) levels were detected by Kruskal-Wallis test.



Fig 92. Viability of IEC-6 cells treated with 10 mM or 25 mM BA for 18 hrs. Bars indicate % mean viability \pm SEM (n= 4 independent experiments). Significance for the effect of pre-treatment with BA on IEC-6 cells was detected by the Kruskal-Wallis test. Differing letters indicate significance between treatment groups as determined by Dunn's test.

Effects of dose and time of exposure to BA on chemokine production by TLR-agonist challenged IEC-6 cells

CINC-1 levels were unaffected when IEC were challenged with LPS in the presence of BA. However, levels decreased when IEC were pretreated with BA prior to LPS challenge (Fig 88 and 89). In contrast to effects on LPS-induced CINC-1 production, pre-treatment of IEC-6 cells with BA did not have an effect on CINC-1 (pg/ml) levels produced by poly (I:C)-challenged cells. CINC-1 levels produced by poly (I:C)-challenged leC in the presence of butyric acid showed a trend toward a significant increase (p = 0.07) (Figs 91 and 90). Pre-treatment of IEC-6 cells with 25 mM BA resulted in a 30% reduction in cell viability (Fig 92).



B)





A)



Fig 94. IL-8 production by HT-29 IEC treated with varying concentrations of β-estradiol (0-10 ng/ml) prior to treatment with TNF- α (50 or 100 ng/ml). Bars indicate mean (pg/ml) \pm SEM (n= 3 independent experiments). No significance for the effects of β-estradiol on TNF- α -induced IL-8 production by HT-29 IEC was detected by one-way ANOVA.

Effect of BA on chemokine production by β -estradiol treated IEC

BA had no impact on CINC-1(pg/ml) production by β -estradiol treated IEC-6 cells challenged with LPS (Fig 93). β -estradiol pre-treatment of HT-29 IEC challenged with TNF- α also had no effect on IL-8 (pg/ml) production at any concentration tested (1-10 ng/ml) (Fig 94).



B)



Fig 95. Effect of 10 mM BA on Pam3Cys (10 μ g/ml) and/or poly (I:C) (40 μ g/ml) -induced A) IL-8 and B) IP-10 production by HT-29 IEC after 6 hr. Data are shown as the mean (pg/ml) \pm SEM (n=5 independent experiments). Significance for the effect of BA on TLR-induced IL-8 and IP-10 (pg/ml) was detected by one-way ANOVA. Differing letters indicate significance between treatment groups as determined by Tukey Kramer multiple range test.



Fig 96. Viability of HT-29 IEC concurrently treated with 10 mM BA and/or TLR-agonists following a 6 hr. Data are shown as the % mean \pm SEM (n=5 independent experiments). No significant differences in % cell viability between different treatment groups were detected by one-way ANOVA.



Fig 97. Effect of a p38 inhibitor on IL-8 production by HT-29 IEC concurrently treated with 10 mM BA and/or TLR-agonists following a 4 hr. Data are shown as the mean $(pg/ml) \pm SEM$ (n=4 independent experiments). A significant effect of treatment on IL-8 (pg/ml) levels was detected by one-way ANOVA. Differing letters indicate significance between treatment groups as determined by the Tukey Kramer multiple range test.



Fig 98. IL-8 production by HT-29 IEC pre-treated with 10 mM BA and/or Pam3Cys (10 μ g/ml) followed by challenge with poly (I:C) (40 μ g/ml). Data are shown as the mean (pg/ml) \pm SEM (n=4 independent experiments). Significance for the effect of pre-incubaction on poly (I:C)-induced IL-8 (pg/ml) by HT-29 IEC was detected by one-way ANOVA. Differing letters indicate significant differences between treatment groups as determined by the Tukey Kramer multiple range test.



Fig 99. Viability of HT-29 IEC pre-treated with 10 mM BA and/or Pam3Cys followed by a 4 hr challenge with poly (I:C). Data are shown as the % mean viability \pm SEM (n=4 independent experiments). No significant differences in % cell viability between different treatment groups was detected by one-way ANOVA.

Pam3Cys+25mM+Poly (I:C) а H а 25mM+Poly (I:C) H Pam3Cys+Poly (I:C) b Poly (I:C) b Pam3Cys+25mM H а H 25mM а Pam3Cys H а Media H а 0 500 1000 1500 2000 2500 IL-8 (pg/ml)

B)



Fig 100. Effect of 25 mM BA on Pam3Cys (10 μ g/ml) and/or poly (I:C) (40 μ g/ml) -induced A) IL-8 and B) IP-10 production by HT-29 cells after a 6 hr. Data are shown as the mean (pg/ml) \pm SEM (n=5 independent experiments). Significance for the effect of BA on TLR-induced IL-8 and IP-10 (pg/ml) was detected by one-way ANOVA. Differing letters indicate significant differences between treatment groups as determined by the Tukey Kramer multiple range test.

A)



Fig 101. Vability of HT-29 cells concurrently treated with 25 mM BA and/or TLR-agonists following a 6 hr. Data are shown as the % mean viability ± SEM (n=5 independent experiments). Significant differences in % cell viability between different treatment groups were detected by Kruskal-Wallis test. Differing letters indicate significance between treatment groups as determined by Dunn's test.



Fig 102. IL-8 production by HT-29 IEC pre-treated with 25 mM BA and/or Pam3Cys (10 μ g/ml) followed by challenge with poly (I:C) (40 μ g/ml). Data are shown as the mean (pg/ml) \pm SEM (n=4 independent experiments). Significance for the effects of pre-treatment on poly (I:C)-induced IL-8 (pg/ml) by HT-29 IEC was detected by one-way ANOVA. Differing letters indicate significant differences between treatment groups as determined by the Tukey Kramer multiple range test.



Fig 103. Viability of HT-29 IEC pretreated with 25 mM BA and/or Pam3Cys followed by a 4 hr challenge with poly (I:C). Data are shown as the % mean viability \pm SEM (n=4 independent experiments). No significant differences in % cell viability between different treatment groups were detected by one-way ANOVA.

Effect of BA on TLR2 and/or TLR3 agonist-induced IL-8 (pg/ml) by HT-29 IEC

Pre-treatment of HT-29 IEC with Pam3Cys enhanced poly (I:C)-induced IL-8 production (Fig 102). However, pre-treatment of HT-29 IEC with 25 mM BA inhibited the combination of Pam3Cys and poly(I:C)-induced IL-8 production (Fig 102), suggesting BA blocked the enhancing effect of this TLR2 agonist on TLR3-induced chemokine production. This inhibitory effect was not observed when IEC were pre-treated with a lower concentration of BA (10 mM) (Fig 98). Co-incubation with 10 mM or 25 mM BA acid significantly reduced poly(I:C)-induced IL-8 and IP-10 production by HT-29 IEC (Fig 95B and 100B). Co-incubation of IEC with Pam3Cys and poly(I:C) did not enhance TLR3 agonist-induced IL-8 production, indicating the enhancing effect on IL-8 production required contact with the TLR2 agonist prior to the TLR3 agonist. The p38 blocker SB203580, significantly inhibited poly (I:C)-induced IL-8 production by HT-29 IEC, while co-incubation with SB203580 and 10 mm BA showed no additive inhibition (Fig 97).



Fig 104. IL-8 production by HT-29 IEC stimulated with LPS and/or Flagellin for 6 hr in the presence of ATP (50 or 75 nM). Data are shown as the mean (pg/ml) \pm SEM (n=4 independent experiments). Data were analyzed by Kruskal-Wallis (p<0.001). Differing letters indicate significance between treatment groups as determined by Dunn's test.



Fig 105. IL-8 production by HT-29 IEC stimulated with Pam3Cys (10 μ g/ml) in combination with LPS (20ng/ml) or Flagellin (100 ng/ml) for 6 hr in the presence of ATP (50 nM). Data are shown as the mean (pg/ml) \pm SEM (n=5 independent experiments). Data were analyzed by Kruskal-Wallis (p<0.001). Differing letters indicate significance between treatment groups as determined by Dunn's test.

Effect of ATP on chemokine production by TLR-challenged HT-29 cells

ATP had no effect on TLR-stimulated production of IL-8 (pg/ml) by HT-29 IEC (Fig 104 and 105). Significance was only observed with flagellin, or the combination of flagellin and ATP (Fig 104) and/or Pam3Cys (Fig 105) relative to media control.

6. Discussion

DFM ingestion has been shown to play an important role in host health via improvements to bowel function, serum cholesterol levels and diabetes (Slavin 2004). However, complex carbohydrates including WB, OB, RS and FOS are regularly consumed by healthy individuals. A comparative analysis of the immune changes mediated by these fermentable substrates, which have different physico-chemical and fermentative properties, would provide better understanding of how each type of substrate affects the local gut and systemic immune environments under resting conditions, potentially impacting overall host health. Considering that sex-associated differences in the faecal communities of rodents have previously been observed (Bernbom et al., 2006; Hoentjen et al., 2005), it is also necessary to understand the influence of fermentable substrate consumption between male and female rats. The current study demonstrated shifts in serum biochemical measures, SCFA profiles, cytokine concentrations and in certain cases changes to immune cell populations in non-immune challenged male and female rats and in immunized rats, following the feeding of different fermentable substrates contained within a common basal diet. These effects differed with the fermentable substrate, between sexes, and with respect to tissue location, illustrating the effects of DFM are complex and dependent upon multiple factors including diet type and sex.

6.1a) RS-ingestion differentially impacted mucosal and systemic immunological parameters than did WB or OB-ingestion under resting conditions

Differences in weight gain, serum biochemical measures and SCFA profiles were observed in DFM-fed rats. However, significant differences in certain measures were only observed between WB and RS-fed rats. For example, the amount of weight gain and BUN levels were lower, while fecal acetic acid percentages rose in WB-fed rats relative to RSfed rats. The difference in the directionality of these fermentation and biochemical measures in WB-fed rats relative to RS-fed rats suggests that differences in the gut microbiota community or their products created conditions leading to these markedly different profiles. These observations are supported by the gut microbial community analysis by our collaborators (Abnous et al., 2009; Kalmokoff et al., 2013). WB increased richness in fecal bacterial communities of rats relative to those fed an AIN-93G control diet, with the majority of the changes occurring within the C. cocciodes and C. leptum groups (Abnous et al., 2009). In contrast, the microbial community profile in rats fed the RS-supplemented diet was limited in diversity, and was dominated by the phylum Bacteroidetes in the cecum, while Ruminococcus bromii predominated in the feces (Kalmokoff et al., 2013). Microbial composition and metabolism in the large intestine affects the manner in which nitrogenous waste such as urea is excreted. The carbohydrate to nitrogen ratio decreases along the length of the large intestine and protein metabolism by *Clostridia* and other facultative bacteria leads to accumulation of nitrogenous byproducts that are excreted mainly by the kidneys. In contrast, fermentable carbohydrates have been shown to exert a urea-lowering effect and shift nitrogen excretion from the

urinary route to the fecal route (Meijers *et al.*, 2009; Evenpoel *et al.*, 2009). Kalmokoff *et al.* (2013) also reasoned the nitrogen cycling in RS-fed males was such that the cecal microbiota broke down endogenous proteins, leading to an increase in fecal urea concentration in the gut. This abundance in urea in turn led to the overgrowth of the nitrogenous utilizers within the *Bacteroidetes* in the distal colon, possibly explaining the lower BUN levels observed in RS-fed rats (Kalmokoff *et al.*, 2013). Whether DFM-induced alterations in gut microbiota nitrogen cycling may be connected to weight gain requires further examination.

Downstream gut mucosal and systemic immune parameters were mainly impacted in RS-fed rats. RS consumption induced the most changes to MLN cytokine levels. MLN CINC-1, IL-4 and TGF-β1 levels increased in RS-fed rats relative to control fed-rats, but RS consumption had no impact on MLN lymphocyte percentages. The MLN is strategically placed outside of the GALT and is important in maintaining gut homeostasis by blocking translocated commensal bacteria from leaving the MLN and entering into the circulation (Macpherson and Uhr, 2004). An unusual feature of the RS-fed cecal and fecal community is that they are both dominated by species within *Bacteroidetes* (Kalmokoff *et al.* 2013). On this basis, the increase in MLN CINC-1 might actually reflect a higher concentration of LPS in the MLN resulting from the increased proportion of these Gramnegative bacteria in the gut community, as demonstrated by others (Alonso, 1996). Rats are cecal fermenters, and RS has been noted to ferment to completion in the cecum. Cecal cytokines IL-6 and IL-4 were observed to be highest in RS-fed rats but were not significantly different from control-fed rats. The cecum can rapidly absorb SCFA via passive diffusion (Flemming *et al.*, 1991), which may have blunted the immune response in this tissue. IL-6 is secreted during epithelial repair (Reed, 2005; Rollwagen *et al.*, 2006) and its increase in relation to fermentable fibre intake suggests alterations may occur at the level of the gut epithelium due to an associated increase in SCFA production, possibly explaining the immune changes observed in the MLN of RS-fed rats.

Rats fed the WB supplement showed effects on lymphocyte population profiles. WB-fed rats showed the highest percentage of MLN B cells (CD3⁻⁴5RA⁺), but lowest total T cell (CD3⁺) percentages, reflecting a decrease in the T helper (CD4⁺) subset. This difference in lymphocyte population patterns suggests that MLN B cell activation may have increased in rats fed the WB diet, potentially reflecting the WB-associated increase in community richness. However, we observed no concurrent alterations in cecal, serum or liver IgA levels in WB-fed rats. While it cannot be ruled out that changes to IgA levels occurred at time points prior to the sampling period, it is also possible that changes were too low to be evident in the total IgA pool. WB ingestion also induced a reduction in splenic B cell (CD45RA⁺) percentages compared to control-fed rats; however no immune changes were noted in the liver with WB-fed rats. These results demonstrate systemic immune measures were also impacted differently in WB-fed rats compared to OB and RS-fed rats.

Ingestion of RS and OB-supplemented diets led to changes in TGF- β 1 concentrations at the liver. The gut normally has a TGF β -rich environment which helps maintain mucosal homeostasis by mediating the balance between regulatory and inflammatory responses within the GI tract (Konkel and Chen, 2011) and TGF- β is also essential in regulating immune activity in the liver (Crispe, 2009). Rats consuming the RS-

supplemented diet showed an increase in TGF- β 1 levels both at the MLN and liver, possibly reflecting heightened immune regulation along the gut-liver axis. The liver is pivotal in trapping gut-derived commensal microbes that reach the systemic circulation (Balmer *et al*, 2014) and TGF- β helps tolerize the liver environment against gut-derived components, including LPS (Woof and Kerr; Thomas and Knolle, 2011). SCFA produced in the GI tract can disseminate through the circulation and this has been shown following consumption of fermentable fibres (Jakobsdottir *et al.*, 2013; Arjmandi *et al.*, 1991).

Although SCFA have been reported to influence systemic immune activity (Maslowski *et al.*, 2009), few studies have addressed effects of fermentable fibres on systemic immune parameters. Since increased liver TGF- β 1 levels were observed in rats consuming either OB or RS-supplemented diets, attributing the systemic effects solely to SCFA production may be an oversimplification. Concurrent with liver TGF- β 1 levels, serum ALT levels also increased in OB-fed rats. ALT is not only abundant in the liver, but can increase in rats even in the absence of liver damage (Ozer *et al.*, 2007; Yang *et al.*, 2009), thus making it difficult to ascertain whether the increase in ALT levels observed in the liver of OB-fed rats.

 β -glucan is a component of oat bran that could be potentially be involved in the effects on ALT. Yeast-derived β -glucan has been shown to inhibit elevation of ALT levels following LPS-induced liver injury in male Wistar rats (Sandvik, *et al.*, 2007). In addition, marine β -glucan induces a decrease in ALT levels and an increase in liver macrophages following 24 hr LPS challenge in male Wistar rats (Neyrinck *et al.*, 2007). Collectively,

these studies illustrate β -glucan from varied sources can exert effects at the level of the liver. Given the very different compositions of RS and OB and the evidence for systemic effects of β -glucan, it is possible that the increase in liver TGF- β 1 levels may occur via different mechanisms in OB as opposed to RS-fed rats, with RS acting indirectly via SCFA production, in contrast to direct effects of OB mediated via fibre components.

The findings in this study demonstrate DFM ingestion can affect immune parameters differentially under steady state conditions and that differences in these effects are reflective of the physico-chemical properties and fermentation characteristics of the fermentable substrate. Based on the shifts in immune parameters in RS-fed rats, it would appear that consumption of resistant starches may heighten immune regulation under resting state conditions more so than do dietary fibres such as OB and WB.

6.1b) Physiological and immunological differences between male and female rats ingesting the WB supplement

Levels of ALP were significantly lower in male rats relative to female rats fed the control diet, indicating a sex-based difference in basal levels of ALP. However, ALP levels were significantly lower in both sexes following WB feeding. The origin of serum ALP is usually intestinal and is involved in transport of metabolites across cell membranes and of triglycerides across the trans-epithelial barrier (DeSchryver-Kecskemeti *et al.*, 1991; Dufour *et al.*, 2000). Inhibiting the intestinal transport of lipids using inhibitors was shown to block intestinal ALP secretion and lower serum ALP levels (Kaur and Gupta, 2007). Others have demonstrated lower serum ALP levels in rats fed a high fat diet supplemented with inulin, and have hypothesised the effect was due to inhibition of fat absorption by

inulin (Azorin-Ortuno *et al.*, 2009). Although serum triglyceride levels were not evaluated in the present study, a significant interaction (diet*sex) was detected for serum ALP levels, suggesting WB intake may alter lipid-mediated activity differentially between male and female rats.

Mucosal and systemic immune parameters also differed between male and female rats consuming the WB-supplemented diet. WB ingestion induced the most changes in cytokine levels at the ileum. WB feeding led to increased ileal IL-4 levels in both male and female rats. Ileal IL-10 levels decreased in females but increased in males following WB intake. WB has a short transit time in proximal regions of the colon and can induce higher enterocyte turnover and faster transit of IEC up the villous column in the small intestinal segments of rats (Roberfroid, 2000; Vahouny *et al.*, 1985). This suggests the changes in ileal cytokines levels after WB consumption may have been independent of the effects of fermentation, and may instead indicate a direct effect of this fibre. WB particles have been observed to abrade the mucosa leading to the release of mucin and exfoliated IEC (Perrin et al., 2001).

IEC are known to participate in innate immunity through PRR (Shultz and Pabst 2013; Knoop et al., 2013). The ileum contains the inductive sites of the Peyer's patches, where professional APC including macrophages and dendritic cells help facilitate the sampling of luminal contents (Coombes, 2007; Schulz and Pabst, 2013). Various components of the fibre in the diet could have also induced changes in cytokine levels at the ileum. Previous studies have shown arabinoxylan, β -glucan and gliadin can induce

innate immune activation (van Creyveld et al., 2008; Wismar et al., 2011; Thomas, 2006). Besides diet-based effects, differences in ileal cytokine levels were also observed between male and female rats. Ileal IL-6 levels were higher in females compared to males. IL-6 is involved in protecting the intestinal epithelium from injury by regulating trefoil factor, a meditator of gut epithelial repair (Rakoff-Nahoum *et al.*, 2004). These findings suggest the higher IL-6 levels in the ileum of females may reflect differences in the sensitivity of the ileal mucosa between the sexes.

Cecal and colonic tissue IL-6 levels were also higher in females than in males. The higher IL-6 levels in locations distal to the ileum may be driven by changes to the mucus lining due to the accumulation of SCFA. IL-6 helps in the production and maintenance of the intestinal mucus gel and intestinal mucin production has been shown to be sex-specific (Groux-Degroote *et al.*, 2008; Sheth *et al.*, 2010). In addition, mucin can be secreted in response to increases in acetate and butyrate levels arising from fermentation in the gut (Barcelo *et al.*, 2000). The ratios of fecal acetate and propionate were higher in females compared to males when fed the control diet, suggesting environmental pressures in the gut shaped differential basal mucosal immune responses between male and female rats.

The differences in basal mucosal cytokine levels in male and female rats may also reflect sex-based differences in cytokine-secreting immune cell populations within the GALT. Although analysis of immune cell populations between the sexes could not be thoroughly completed (due to loss of some of the samples from females), the percentages of $CD3^+4^+Foxp3^+T_{reg}$ cells were higher in males relative to females in both the MLN and

spleen. In contrast, the percentages of CD68⁺ macrophages were higher in the MLN and spleen of females than in males. The MLN and spleen are strategically positioned for circulating B and T cells to gain access to their cognate antigen, and to facilitate APC-T cell interactions and help shape immune homeostasis within these tissue environments (Macpherson and Uhr, 2004; Bronte and Pittet, 2013). However, the elevated percentages of MLN and splenic T_{regs} in males and macrophages in females suggests immune homeostasis may occur in a differential manner between males and females.

In addition to the higher CD68⁺ macrophage percentages, IL-10 levels were consistently higher in females compared to males in mucosal tissues. IL-10 is a regulatory cytokine that is secreted by M2 macrophages, in addition to other cell types (Mantovani *et al.*, 2004). Besides the heightened basal levels of IL-10 in females, WB intake led to an increase in splenic IL-10 levels in females relative to control-fed counterparts. Concurrently, levels of the pro-inflammatory cytokine CINC-1 decreased in the spleen of WB-fed females, indicating WB intake in females induced an anti-inflammatory cytokine profile at the systemic level.

In keeping with these observations it has been shown that splenocytes from rats weaned on fermentable fibre showed higher levels of mitogen-induced IL-10 production than control-fed rats (Stillie *et al.*, 2005). Insoluble fibres and glucose-based fibres have been demonstrated to increase numbers of splenic T_{reg} cells in IL-10^{-/-} mice (Bassaganya-Riera *et al.*, 2011). T_{reg} cells secrete both TGF β and IL-10 in the gut upon stimulation with SCFA (Smith *et al.*, 2013), however the possibility that SCFA production from DFM-

induced immune-modulatory effects at the spleen have only been a point of speculation to date (Stillie *et al.* 2009).

These findings illustrate DFM-mediated effects differ between sexes, at both physiological and immunological levels. Location-specific effects were not only a reflection of the physico-chemical characteristics of WB, but were possibly influenced by the differences in background immune cell populations and in the cytokine milieu in mucosal and systemic sites between male and female rats. In addition, WB intake mediated immune changes at the systemic level in female rats, while no systemic immune changes were noted in males fed WB.

6.1c) Differential metabolism of FOS resulted in altered systemic immune measures between male and female rats

In the faecal community of females, FOS supplementation altered community structure by increasing abundance within the Phylum *Bacteroidetes* and reducing abundance within the Family *Lachnospiraceae*. Differences in the metabolism of the FOS-supplemented diet appeared to reflect sex-associated differences in gut nitrogen availability. Since male and female rats consumed a proportionally equivalent amount of protein types in both diets, this indicates that either male rats have a reduced ability to digest dietary protein in the small intestine, allowing more protein to pass into the lower gut, or that additional endogenous protein sources must be present in the male gut lumen, but absent in the females. The results from the immunological analysis of these rats support the latter.

In Sprague-Dawley rats, the male colon is more permeable than that of females (Homma et al., 2005), and our results also support possible differences in gut barrier function independent of diet in male and female BioBreeding control rats. Firstly, serum LPS concentrations in male rats were higher than in females, independent of diet; this is consistent with an increased translocation of gut bacteria in males demonstrated by others (Bruggencate et al., 2005; Fontaine et al., 1996). In addition, liver tissues from males showed significantly higher levels of the pro-inflammatory cytokines IL-6 and CINC-1. Both cytokines are involved in the immune response to inflammation and tissue injury (Rakoff-Nahoum et al., 2004; Reed et al., 2005). Finally, basal liver IgA levels were significantly higher in males than females and further increased when fed the FOScontaining diet. IgA is the major immunoglobulin found in the mucosal secretions of mammals and is important in protecting the host from invasion by pathogenic bacteria (Woof and Kerr, 2006). In both rats and humans, liver macrophages (Kupffer cells) are involved in the removal of IgA-opsonized bacteria and antigens via FCR α 1 receptors, an important role when gut barrier function is reduced (Woof and Kerr, 2006; Brenchley et al., 2012). Although we did not directly analyze gut barrier function by methods such as FITC-dextran administration, these findings suggest a potential decrease in gut barrier function leading to heightened cytokine and IgA production at the systemic level in male rats compared to females.

Feeding FOS to rats is known to impact gut barrier function, inducing the shedding of mucin and increasing the translocation of gut pathogens (Fontaine *et al.*, 1996; Ten Bruggencate *et al.*, 2003; Ten Bruggencate *et al.*, 2004; Ten Bruggencate *et al.*, 2005).

Increased gut permeability in males fed FOS might also result from a higher turnover of gut mucosal cells. Colonocyte maturation and differentiation appears to be dependent on butyrate (Mariadason *et al.*, 2000; Tappenden *et al.*, 1997), which increased in fecal contents of males fed the FOS-supplemented diet. A higher rate of colonic epithelial turnover would also increase protein availability in the male lumen and may further contribute to differences in gut permeability between males and females.

In contrast to males, females appear to possess a more efficient gut barrier function. For example, intestinal permeability in Sprague Dawley rats has been linked to mucin secretion and this has been shown to be sex-specific (Sheth *et al.*, 2010). Although we did not directly measure mucin secretion, levels of ileal IL-6, a cytokine involved in inducing expression of several mucin genes (Enss *et al.*, 2000; Groux-Degroote *et al.*, 2008) were higher in females than males.

In addition, the female gastrointestinal tract tissue had high basal levels of IL-10, a cytokine known for its role in mucosal homeostasis (Malloy *et al.*, 2011). Female rats consuming the FOS-supplemented chow had the highest levels of IL-4 in caecum and colon, tissues in which IL-10 levels were high. This is a cytokine profile indicative of T_{H2} activity, a T cell phenotype that supports antibody production and mediates immune homeostasis at the gut level (Romagani, 1999; Bulek *et al.*, 2010). Additionally females had a higher percentage of MLN CD68⁺ cells, indicating an increased presence of macrophages in the MLN along with higher levels of gut mucosal IL-10 compared to males. IL-4 and IL-10 are inducers of M2 phenotype macrophages that help maintain a tolerogenic gut environment and in turn differentiate T helper cells into T_{H2} (Mantovani *et* *al.*, 2002). These cell phenotypes and cytokine profiles would support greater gut barrier integrity (Bulek *et al.*, 2010; Hardy *et al.*, 2013) potentially contributing to differences between sexes in preventing gut-derived stimuli from entering into systemic compartments. Diet and sex also had differential effects at the systemic immune level. Splenic CD3⁺ (total T cell) percentages were affected by diet, with the lowest percentages observed in female rats consuming the FOS-supplemented diet, while splenic macrophage (CD68⁺) populations were higher in females compared to males. Together, this suggests that immune population distributions can be selectively affected by diet and sex, at the systemic and mucosal level. Taken in context with data from our collaborators regarding community structure, sex-specific effects were evident at the systemic level and may be result of a naturally leakier gut in males than females.

6.2 The immune modulatory effects of butyric acid on IEC are dependent upon timing of exposure.

IEC defenses are maintained through the sensing of microbiota-derived products including SCFA, which are mediators in this host-microbiota dialogue (Alenghat *et al.*, 2014). Butyrate is anti-inflammatory due to its ability to down-regulate NF- κ B mediated pro-inflammatory cytokine production by IEC (Kumar *et al.*, 2009; Cox et al., 2009). Other SCFA, including acetate, have also been shown to halt neutrophil migration to areas of inflammation by acting through GPR43 receptors on neutrophils (Maslowski *et al.*, 2009). In contrast, Vinolo *et al.* (2009) showed butyrate and propionate, but not acetate, induced neutrophil migration and increased pro-inflammatory chemokine CINC-2αβ levels in rats
(Vinolo *et al.*, 2009). Earlier studies by Ohno *et al.* (1997) also showed butyrate upregulated macrophage inflammatory protein-2 (MIP-2) expression by IEC challenged with either LPS or IL-1 β (Ohno et al., 1997). Similarly, Fusunyan *et al.* (1999) showed butyrate increased IL-8 secretion but decreased monocyte chemokine protein-1 (MCP-1) secretion by LPS-challenged Caco-2 IEC (Fusunyan et al., 1999). These findings illustrate that the effects of butyrate on pro-inflammatory cytokine production differ depending on the type of immune cell it is acting upon and the presence of specific PRR agonists or inflammatory inducers. Within the gut environment it would be anticipated that IEC contact with butyrate depends upon fermentation substrate type and availability, and that the timing of contact of butyrate with IEC may also play an important role in the immune-modulatory outcome. Furthermore, multiple factors converge in the gut environment including TLR agonists and hormones, in addition to butyrate. All of these would be anticipated to contribute to or influence IEC signaling in response to innate immune stimuli, and so their potential interactions should be taken into consideration when examining the effects of butyrate.

The butyric acid concentrations utilized in the present study were selected based on the SCFA levels observed in DFM-fed rats, in addition to those reported in the literature (Mirmonsef et al., 2012). The effect of timing of contact with butyric acid on chemokine production by IEC-6 differed based on the type of TLR agonist these non-transformed cells were stimulated with. For example, levels of CINC-1 produced by poly (I:C)-challenged IEC-6 cells trended upward in the presence of butyric acid in a concentration-dependent manner, while the concurrent presence of butyric acid had no effect on LPS-induced CINC-1 production by IEC-6. In contrast, butyric acid pre-treatment significantly decreased LPS-

induced CINC-1 production by IEC-6. Although this decrease was observed following pretreatment with either 10mM and 25mM butyric acid, effects on IEC viability were only observed at the higher butyric acid concentration, while 10mM butyric acid pre-treatment of IEC-6 cells did not result in decreased cell viability. Butyric acid pre-treatment of IEC-6 prior to poly (I:C) challenge had no effect on CINC-1 levels induced by this TLR3 agonist. These results suggest the effects of timing of contact with butyric acid on IEC responses is TLR agonist-dependent. Butyric acid is known to promote cellular differentiation, inhibit cellular proliferation and to induce apoptosis in mammalian cell lines due to its ability to hyperacetylate histones (Davie, 2003). As cellular differentiation of IEC-6 cells has been shown to be induced by 2mM sodium butyrate after 12 hr (Patel et al., 2009), the differences observed here in the effects on TLR3 versus TLR4-induced CINC-1 production suggest that butyrate-induced differentiation has a differential impact on responses to these innate PRR ligands. In addition, Bailon et al. (2010) noted that butyrate's anti-inflammatory effects were more evident in transformed cells including HT-29 IEC, rather than non-transformed IEC, indicating this variable must be taken into context when evaluating the effects of butyric acid.

TLR2 in intestinal epithelial cells is hyporesponsive to ligand-mediated activation via the MYD88 signalling pathways and yet has been implicated as being important to intestinal barrier function and modulation of innate immune responses (Mazmanian, 2005; Hayashi *et al.*, 2010; van Bergenhenegouwen *et al.*, 2013). The bacterial cell wall component lipoprotein, ubiquitous in the gut, is the natural ligand for TLR2 and was a good candidate to examine the effect of combining TLR agonists. Effects on chemokine production by HT-29 IEC challenged with a combination of TLR2 and TLR3 agonists were dependent upon butyric acid concentrations and time of contact. Although IL-8 levels from Pam3Cys and poly (I:C) challenged HT-29 IEC decreased in the presence of 25mM butyric acid, this decrease probably reflects a reduction in the percentage of viable cells observed with butyric acid at this higher concentration. In contrast, exposure of HT-29 IEC to 25mM butyric acid for a short period of time (2 hr) significantly decreased IL-8 production induced by the combination of Pam3Cys and poly (I:C) without changes to IEC viability. However, 10mM butyric acid pre-treatment did not have this down regulatory effect. These results suggest that preconditioning of transformed IEC with high concentrations of butyric acid for short periods of time can down-regulate a dual TLR signal-induced innate response. In contrast to effects on IL-8 levels, the presence of butyric acid (at either concentration) significantly reduced poly (I:C) and the combination of poly (I:C) and Pam3Cys-induced IP-10 production by HT-29 IEC. IP-10 transcription is controlled by a MYD88-independent signalling pathway, activated following TLR3 engagement (Takeda and Akira, 2004) and IP-10 levels have been shown to be affected by butyrate, but not by acetate or propionate (Inatomi *et al.*, 2005). Taken together, these findings illustrate the presence of Pam3Cys did not impact poly (I:C)mediated IP-10 production and that butyric acid modulates IP-10 production by HT-29 IEC stimulated by dual TLR ligands differently from that of IL-8.

Collectively these results suggest that the effects of butyric acid on IEC responses are complex and are dependent upon exposure time, concentration and type of innate immune stimulant encountered. These factors would be expected to contribute to effects of DFM mediated through SCFA production at the mucosal epithelial level, and reflect the need to examine multiple products of microbial structure and metabolism in order to gain insight into the effects of DFM on the gut mucosal immune system.

6.3 The kinetics of anti-KLH antibody production are more suppressed in males than in females following DFM consumption

Microbial fermentation of DFM leads to large amounts of SCFA production, and these gut-derived metabolites have been demonstrated, in the current study and previously by others, to modulate immune responses at the gut epithelial level (Kumar et al., 2009; Smith *et al.*, 2013). The net portal flux of SCFA has been found to vary in the blood of pigs fed fermentable substrates including resistant starch and arabinoxylan (Ingerslev, et al., 2014). SCFA can also reach the circulation and levels in the portal serum of rats fed different DFM have been reported to correlate with cecal SCFA levels, providing a route for effects on immunity at the systemic level, including the liver (Jakobsdottir et al., 2013). SCFA can modulate systemic immune cell activity via G protein coupled receptors on immune cells (Maslowski et al., 2009; Vinolo et al., 2009). Other gut-derived metabolites have also been demonstrated to circulate and affect systemic immunity. Taking a metabolomics approach, Wikoff et al. (2009) was able to determine that the metabolic profile between the serum of germ free and conventionalized mice differed greatly in compounds arising exclusively from gut microbiota-driven metabolism including tryptophan (Wikoff et al., 2009). Tryptophan catabolism in the gut by indoleamine 2,3 dioxygenase enzyme leads to the release of kynurenine and picolinic acid, which have been

shown to suppress LPS-challenged IgM production by splenocytes in vitro (Harrington et al., 2008). Dietary supplements have also demonstrated an impact on systemic adaptive responses to immune challenge. Mitogen-stimulated splenocytes from lambs fed a diet supplemented with 0.05% of leucine or aketoisocaproate or isovalerate were shown to differ in their antibody production to sequential immunization with T cell-dependent and T cell-independent antigens (Khulman et al., 1988). Collectively, these results demonstrate a link between gut-derived metabolites and immune function at the systemic level. However the scope of studies researching this link are limited, and have been restricted to understanding the effect of particular metabolites in connection to systemic immune challenge (Tuyishime et al., 2014), while combinatorial effects on immune measures from a large range of gut-derived metabolites and microbial components would be anticipated to occur in vivo. Furthermore, gut-derived metabolites or microbial components could either induce systemic immune tolerance or prime the immune system which would in turn, dictate host responses during immune challenge. These effects on host systemic immune status have been shown to be partly dependent upon microbial component-PRR interactions. For example, bacterial peptidoglycan can prime the systemic immune system under resting conditions, via NOD1 receptors, to enhance neutrophil activity (Clarke et al., 2010). Recent findings by Oh et al. (2015) demonstrate the influence of bacterial flagellin-TLR5 ligation on the longevity of humoral responses to seasonal vaccination with influenza (Oh et al., 2015). It would be anticipated that diet and sex would further influence microbiota-derived metabolite profiles and influence the impact of microbiota-derived components, leading to differential effects on systemic immune parameters. To the best of our knowledge, we demonstrate for the first time that the kinetics of antibody production in response to immunization with a T cell-dependent antigen (KLH) differ between male and female rats following DFM consumption.

Effects were also observed on immune cell population percentages and cytokine levels in the PP, MLN and spleen. These changes were evaluated a week after the KLH booster was administered, after which time the cytokines involved in regulating antibody production and class switching would be returning to baseline levels. The PP are the inductive sites of the GALT where APCs process antigens to present to naïve T cells and B cells are activated upon encountering antigens. In the current study, T cell population percentages in the PP were higher in males than in females, reflecting higher percentages of CD3⁺ (total) T cells, CD3⁺4⁺ T helper cells, CD3⁺8⁺ cytotoxic T cells and CD62L⁺ (Lselectin⁺) T lymphocytes compared to females. CD62L/L-selectin is a homing receptor that recruits naïve T cells to peripheral lymph nodes and its expression on neutrophils has been shown to decrease with butyrate pre-treatment in vitro (Vinolo et al., 2009). However, in vivo effects of gut-derived metabolites would not be restricted to those of butyric acid alone, and in addition, effects of this SCFA on neutrophils and T cells may differ. Sexbased differences have also been noted in CD62L/L-selectin expression. CD62L expression on memory T cells from the spleen was observed to be lower in male mice relative to females (Barrat et al., 1997), in contrast to the pattern observed in this study. In addition, CD3⁺4⁺ T helper cell population percentages increased in control-fed rats relative to rats fed all other DFM-supplemented diets, concurrent with an increase in TGF-\beta1 in control-fed female rats compared to RS-fed female rats. Fermentable fibre feeding has been

noted to lower T cell responses to mitogen stimulation in lymphocytes from tissues such as the PP, where B cell functions predominate (Schley and Field, 2002). The parallel rise in all T cell phenotypes in the PP of males is thus interesting. In contrast to males, B cell population percentages were higher in the PP of females. CD103⁺ DC also showed significance for a diet*sex interaction in the PP, along with a trend towards higher percentages of CD103⁺DC in females relative to males. These findings suggest that B cellmediated activity was prevalent in the female rats, while T cell-driven functions may predominate in males at the PP.

These patterns in lymphocyte population profiles switched between the PP and the MLN in male and female rats. The percentages of CD3⁺ T cells was higher in females relative to males, a pattern opposite to that observed in the PP. MLN IFN- γ levels were also higher in females compared to males, although there were no changes in the percentages of CD3⁺4⁺ T helper cells in the PP. These differences were also accompanied by higher B cell percentages in control and FOS-fed males compared to females fed the same diets. Moreover, HIS36⁺ macrophage percentages were also higher in the MLN of males than in females and this was paralleled by higher IL-10 levels in the MLN of males relative to females. Although difficult to ascertain, as we did not evaluate lymphocyte mucosal homing receptors, it is nevertheless possible that B cells migrated out of the PP and into the MLN of male rats relative to female rats. Since we observed no changes in the CD3⁺4⁺ T helper cell percentages, it is reasonable to assume the high IL-10 levels were a reflection of the elevated macrophage population in the MLN, as IL-10 is secreted by

macrophages, in addition to other cell types (Mantovani *et al.*, 2004). Overall, the immune milieu in the MLN of the male rats was indicative of immune regulation.

Examination of the PP and MLN immune cell populations and cytokine levels helped illustrate the immune environment and potential mechanisms of action within these locations, possibly in response to gut-derived signals. The immune parameters in these locations were mainly affected by RS intake in either sex, or by FOS feeding in males, highlighting the observation that ingestion of fermentable substrates impacted immune measures within peripheral lymphoid tissues. In addition there were sex-based differences in immune parameters within these tissues, which possibly points to a difference in the manner by which gut mucosal homeostasis is achieved between male and female rats. Collectively the interplay between diet and sex on immune parameters within these locations would dictate the immune outcome at distal systemic sites.

DFM intake clearly affected antibody responses in male and female rats 10 days after immunization with KLH. Rats fed the DFM-supplemented diets demonstrated a decrease in the primary anti-KLH IgG response relative to control fed rats. It should be noted that anti-KLH IgG concentrations in FOS-fed rats were lower than control-fed rats on day 5, but by 10 day concentrations were on par with that of control-fed rats. In contrast, in WB-fed rats the anti-KLH IgG concentrations were not different from control-fed rats on day 5, but concentrations were lower on day 10 in WB-fed rats relative to control-fed rats. These differences may reflect differences in the impact of the metabolism of these two fermentable substrates by the gut microbiota community on the response to immunization. Given that host-microbe interactions are often considered to be bi-directional, it is also possible that immunization could influence microbial metabolism of these fermentable substrates. No evidence currently shows whether parenteral immunization could affect the gut microbiota community and this would be an interesting avenue to explore in the future.

These results indicate that DFM intake in rats, regardless of sex, delayed antibody class switching from IgM to IgG. This conclusion is supported by the observation that DFM intake by rats had no impact on anti-KLH IgM concentrations on day 5 or day 10. Anti-KLH IgG2a concentrations were unaffected by diet, while anti-KLH IgG2b concentrations were lower in rats fed RS or OB relative to control-fed rats. IgG2a production by B cells requires IL-4 (usually provided by T_H2 cells) and can also occur independently of T cell help at certain stages of the antibody response, making this subclass of antibody slower to peak (Sakai et al. 1995; Medgeysi et al., 1981). This may explain why the effects of DFM intake were not observed with anti-KLH IgG2a, but were evident with anti-KLH IgG2b. Besides the effects of diet, the primary antibody response was also observed to differ between sexes, with males demonstrating lower anti-KLH IgG antibody concentrations relative to females. On day 10, concentrations of anti-KLH IgM and IgG were higher in females compared to males, while anti-KLH IgG2a concentrations were lower in females relative to males. At day 10 concentrations of anti-KLH IgG2b were similar between the sexes. The KLH booster dose was administered on day 15. At this time point, high serum concentrations of anti-KLH IgG, IgG2a, IgG2b and IgA were observed in females compared to males. Interestingly males had higher levels of anti-KLH IgM compared to females on the day the booster was administered, yet the anti-KLH IgG secondary response was 10-fold higher in males than in females, demonstrating a diet*sex interaction. Females had higher anti-KLH IgG levels during the primary response so would have been expected to demonstrate a greater secondary response to the booster, while in males the higher concentrations of anti-KLH IgM suggested slower B cell class switching, which should have hampered the kinetics of the secondary response to the booster. In addition, splenic B cell percentages were lower in control-fed males relative to control-fed females, further confounding the observed elevation in the secondary response in males. Others have also observed this phenomenon, and have shown this to be singular to outbred rat strains including Wistar rats (Kawai *et al.*, 2013). This particular strain of rats also tend to exhibit larger inter-individual variability compared to inbred rat strains (Kawai *et al.*, 2013).

The secondary anti-KLH IgG responses were significantly suppressed in male rats fed the DFM diets, especially following FOS consumption. IL-10 levels in whole blood cultures stimulated with KLH and LPS from FOS-fed male rats demonstrated a trend towards significance relative to unstimulated control cultures. LPS has been shown to enhance the suppressive functions of T_{reg} cells, which secrete IL-10, via TLR4 activation Caramalho *et al.*, 2003). We previously noted serum LPS levels were higher in males relative to females under resting conditions, and may explain the slight elevation in IL-10 levels observed in whole blood cultures from FOS-fed males following stimulation with LPS and KLH. However, it is interesting that IL-10 rose only in FOS-fed males and was not observed in males fed the other DFM diets. We have previously also demonstrated in rats ingesting either RS or FOS an increase in immune regulatory markers such as TGF- β 1 and IgA, respectively, at the liver. Collectively, these results suggest consumption of highly fermentable substrates in males induced a greater degree of systemic tolerance, leading to a less robust host response under immune challenged conditions.

7. Conclusion

The current study has attempted to highlight differences in DFM-mediated mucosal and systemic immune measures between male and female weanling rats. The findings of our collaborators demonstrated that gut microbiota communities metabolized DFM differently between sexes. These DFM-induced alterations in community structure resulted in differential SCFA profiles, potentially contributing to differential effects on mucosal and systemic immune parameters between male and female rats. Differences in the effect of these DFM on immune measures between male and female rats may also reflect inherent differences in the immunological architecture of the GALT between sexes. Although sex hormone-mediated effects on immune cell activity are well recognized, the immune cell types and cytokine milieu observed within the gut mucosal compartments in the current study suggest gut immune homeostasis is achieved via different routes between male and female rats. This is intriguing because the gut microbiota is necessary for early GALT development and occurs postnatal, a stage at which the influence of sex hormones would be minimal. Additionally, the microbiota diversifies at the weanling stage when solid food is introduced into the diet. Since both male and female weanling rats in the current study were fed the same control diet, it would be reasonable to expect gut-microbiota diversification following consumption of control diet may alter GALT immune cell populations and activity in the same manner between the sexes, however this was not the

case. Peripheral lymphoid tissue in males demonstrated a T cell bias versus that of macrophage and B cell dominant populations in females. Steegenga et al. (2014) noted sexual dimorphism in the expression of histone-modifying genes in the small intestine and colon of pre-pubescent mice, along with sex-specific taxa present in the colonic microbiota. Their results illustrate epigenetic factors influence the bi-directional relationship between the host and the microbiota early on in GALT development, possibly explaining the differences we noted in the peripheral lymphoid immune cell populations between sexes. What is less understood is how, if at all, epigenetic factors may affect systemic immune activities via the gut-liver axis. In light of the current findings it would appear that under homeostatic conditions immune regulation occurs constantly along the gut-liver axis in males. Levels of regulatory cytokine TGF-\beta1 were especially heightened in DFMconsuming rats in mucosal and systemic tissues, possibly reflecting the higher percentages of FoxP3⁺ T_{reg} cells in these tissue compartments. Recent evidence shows SCFA exert their regulatory effects in the colon partly by stimulating histone acetylation of the FoxP3⁺ gene locus in CD4⁺ T cells, inducing their differentiation into T_{reg} (Alenghat and Artis, 2015). This may explain why highly fermentable substrates increased tissue TGF-B1 levels in male rats, and also suggests the male gut lumen may be predisposed to a microbiota that produces high amounts of SCFA. In contrast females had high background levels of IL-10 in mucosal and systemic tissues, and levels of this cytokine were affected by diet only in the female ileum. Of the two regulatory cytokines, TGF- β is well recognized to be an immune suppressant as evidenced by its prevalence in tumor micro-environments (Whiteside, 2012). The increase in MLN and liver TGF- β 1 levels in DFM-fed male rats

may suggest a mechanism through which fermentable substrates may hinder systemic immune responses in males under challenged conditions. We observed that DFM-fed males had lower anti-KLH antibody responses relative to control fed males, and this effect was less marked in females.

Host immune status is shaped by the complex interplay between diet and sex. Investigating the dynamics of these factors together, rather than separately, will provide better understanding of the potential impact on host health.

8. Future Directions

It would be interesting to examine whether the choice of basal diet may alter the outcome of DFM-mediated effects in rats. For example in the current study, male rats fed the FOS-supplemented Purina chow diet, under resting conditions, did not show alterations to TGF-B1 levels in the gut mucosal or liver tissue, unlike males fed the DFMsupplemented AIN-93G diets; nevertheless, male rats fed the FOS-supplemented AIN-93G diet still demonstrated the lowest levels of anti-KLH IgG to secondary immunization. Could this outcome have been different if the AIN-93G diet was substituted with Purina chow? Purina chow, which contains soy protein, has been criticized as a diet choice as it has the potential to affect gene expression via estrogenic effects due to the presence of high levels of circulating isoflavones after its consumption (Brown and Satchell, 2001). For this reason, it has been replaced with the soy-free AIN-93G formulated diet in the majority of rodent studies. However, Purina chow may be a better representation of a human diet than the highly-defined AIN-93G diet, and understanding DFM-mediated effects within the background of this diet and/or diets with varying sources of protein may help provide detailed insight into effects of DFM intake on host health.

9. References

- Abnous K, Brooks SP, Kwan J, Matias F, Green-Johnson J, Selinger LB, Thomas M, Kalmokoff M. Diets enriched in oat bran or wheat bran temporally and differentially alter the composition of the fecal community of rats. J Nutr. 2009; 139:2024-31.
- Abreu MT. Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. Nat Rev Immunol. 2010; 10:131-44.
- Ahluwalia A, Clodfelter KH, Waxman DJ. Sexual dimorphism of rat liver gene expression: regulatory role of growth hormone revealed by deoxyribonucleic acid microarray analysis. Mol Endocrinol. 2004; 18:747-60.
- Ahmed SA, Penhale WJ, Talal N. Sex hormones, immune responses, and autoimmune diseases. Mechanisms of sex hormone action. Am J Pathol. 1985; 121:531-51.
- Ahmed SA, Talal N. Sex hormones and the immune system-Part 2. Animal data. Baillieres Clin Rheumatol. 1990; 4:13-31
- Alenghat T, Artis D. Epigenomic regulation of host-microbiota interactions. Trends Immunol. 2014; 35:518-25.
- Alonso A, Bayón Y, Crespo MS. The expression of cytokine-induced neutrophil chemoattractants (CINC-1 and CINC-2) in rat peritoneal macrophages is triggered by Fc gamma receptor activation: study of the signaling mechanism. Eur J Immunol. 1996; 26:2165-71.

- Arjmandi BH, Ahn J, Nathani S, Reeves RD. Dietary soluble fiber and cholesterol affect serum cholesterol concentration, hepatic portal venous shortchain fatty acid concentrations and fecal sterol excretion in rats. J Nutr. 1992; 122:246-53.
- Artis D. Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nat Rev Immunol. 2008; 8:411-20.
- Atarashi K, Nishimura J, Shima T, Umesaki Y, Yamamoto M, Onoue M, Yagita H, Ishii N, Evans R, Honda K, Takeda K. ATP drives lamina propria T(H)17 cell differentiation. Nature. 2008; 455:808-12
- Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K, Kim S, Fritz JV, Wilmes P, Ueha S, Matsushima K, Ohno H, Olle B, Sakaguchi S, Taniguchi T, Morita H, Hattori M, Honda K. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature. 2013; 500:232-6.
- 12. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, Taniguchi T, Takeda K, Hori S, Ivanov II, Umesaki Y, Itoh K, Honda K. Induction of colonic regulatory T cells by indigenous *Clostridium* species. Science. 2011; 331:337-41.
- 13. Azorin-Ortuno M, Urban C, Ceron JJ, Tecles F, Allende A, Tomas-Barberan FA, Espin JC. Effect of low inulin doses with different polymerisation degree on lipid metabolism, mineral absorption, and intestinal microbiota in rats with fat supplemented diet. Food Chemistry 2009; 113:1058-1065.

- Backhed F, Ley R,Sonnenburg J, Peterson D, Gordon J. Host-Bacterial Mutualism. Science 2005; 307:1915-1920.
- Barcelo A, Claustre J, Moro F, Chayvialle JA, Cuber JC, Plaisancié P. Mucin secretion is modulated by luminal factors in the isolated vascularly perfused rat colon. Gut. 2000; 46:218-24
- 16. Barnard JA, Warwick G. Butyrate rapidly induces growth inhibition and differentiation in HT-29 cells. Cell Growth Differ. 1993; 4:495-501.
- Barrat F, Lesourd BM, Louise A, Boulouis HJ, Vincent-Naulleau S, Thibault D, Sanaa M, Neway T, Pilet CH. Surface antigen expression in spleen cells of C57B1/6 mice during ageing: influence of sex and parity. Clin Exp Immunol. 1997; 107:593-600.
- Bassaganya-Riera J, DiGuardo M, Viladomiu M, de Horna A, Sanchez S, Einerhand AW, Sanders L, Hontecillas R. Soluble fibers and resistant starch ameliorate disease activity in interleukin-10-deficient mice with inflammatory bowel disease. J Nutr. 2011; 141:1318-25.
- Bernbom N, Norrung B, Saadbye P, Lolbak L, Vogensen FK, Licht TR: Comparison of methods and animal models commonly used for investigation of caecal microbiota: effect of time, host and Sex. J Microbiol Methods. 2006; 66:87-95.
- Bird AR, Conlon MA, Christophersen CT, Topping DL. Resistant starch, large bowel fermentation and a broader perspective of prebiotics and probiotics.
 Benef Microbes. 2010; 1:423-31

- Blais M, Seidman EG, Asselin C. Dual effect of butyrate on IL-1β-mediated intestinal epithelial cell inflammatory response. DNA Cell Biol. 2007; 26:133-47.
- Bolnick DI, Snowberg LK, Caporaso JG, Lauber C, Knight R, Stutz WE. Major Histocompatibility Complex class IIb polymorphism influences gut microbiota composition and diversity. Mol Ecol. 2014; 4831-45
- 23. Bolnick DI, Snowberg LK, Hirsch PE, Lauber CL, Org E, Parks B, Lusis AJ, Knight R, Caporaso JG, Svanbäck R. Individual diet has sex-dependent effects on vertebrate gut microbiota. Nat Commun. 2014; 5:4500
- Brandtzaeg P. Homeostatic impact of indigenous microbiota and secretory immunity. Benef Microbes. 2010; 1:211-27.
- Brandtzaeg P. Mucosal immunity: induction, dissemination, and effector functions. Scand J Immunol. 2009; 70:505-15.
- Brenchley JM, Douek DC: Microbial translocation across the GI tract. Annu Rev Immunol 2012; 30:149-173.
- Bronte V, Pittet MJ. The spleen in local and systemic regulation of immunity. Immunity. 2013; 39: 806-18.
- Brooks SP, McAllister M, Sandoz M, Kalmokoff ML. Culture-independent phylogenetic analysis of the faecal flora of the rat. Can J Microbiol. 2003; 49:589-601.

- Brooks SPJ, Kalmokoff ML: Prebiotics and probiotics: Some thoughts on demonstration of efficacy within the regulatory sphere. J AOAC. Internat 2012; 95: 2-4.
- Brown NM, Setchell KD. Animal models impacted by phytoestrogens in commercial chow: implications for pathways influenced by hormones. Lab Invest. 2001; 81(5):735-47.
- Buddington KK, Donahoo JB, Buddington RK. Dietary oligofructose and inulin protect mice from enteric and systemic pathogens and tumor inducers. J Nutr. 2002; 132:472-7.
- 32. Bulek K, Swaidani S, Aronica M, Li X. Epithelium: the interplay between innate and Th2 immunity. Immunol Cell Biol. 2010; 88:257-68.
- Burkitt D. Diverticular disease of the colon epidemiological evidence relating it to fibre-depleted diets. Trans Med Soc Lond. 1973; 89:81-4.
- Butt SM, Tahir-Nadeem M, Khan MK, Shabir R, Butt MS. Oat: unique among the cereals. Eur J Nutr. 2008; 47:68-79.
- 35. Cao Y, Gao X, Zhang W, Zhang G, Nguyen AK, Liu X, Jimenez F, Cox CS Jr, Townsend CM Jr, Ko TC. Dietary fiber enhances TGF-β signaling and growth inhibition in the gut. Am J Physiol Gastrointest Liver Physiol. 2011; 301:G156-64.
- Capitán-Cañadas F, Ortega-González M, Guadix E, Zarzuelo A, Suárez MD, de Medina FS, Martínez-Augustin O. Prebiotic oligosaccharides directly modulate

proinflammatory cytokine production in monocytes via activation of TLR4. Mol Nutr Food Res. 2014; 58:1098-110.

- 37. Caramalho I, Lopes-Carvalho T, Ostler D, Zelenay S, Haury M, Demengeot J. Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. J Exp Med. 2003; 197:403-11.
- 38. Carmody RN, Gerber GK, Luevano JM Jr, Gatti DM, Somes L, Svenson KL, Turnbaugh PJ. Diet dominates host genotype in shaping the murine gut microbiota. Cell Host Microbe. 2015; 17:72-84
- Cavaglieri CR, Martins EF, Colleone VV, Rodrigues C, Vecchia MG, Curi R.
 Fiber-rich diets alter rat intestinal leukocytes metabolism. J Nutr Biochem. 2000; 11:555-561.
- Cavaglieri CR, Nishiyama A, Fernandes LC, Curi R, Miles EA, Calder PC.
 Differential effects of short-chain fatty acids on proliferation and production of pro- and anti-inflammatory cytokines by cultured lymphocytes. Life Sci. 2003; 73:1683-90.
- Cerutti A.The regulation of IgA class switching. Nature Reviews Immunol. 2008;
 8:421-434.
- 42. Chakir H, Lefebvre DE, Wang H, Caraher E, Scott FW. Wheat protein-induced proinflammatory T helper 1 bias in mesenteric lymph nodes of young diabetes-prone rats. Diabetologia. 2005; 48:1576-84.

- Chanput W, Reitsma M, Kleinjans L, Mes JJ, Savelkoul HF, Wichers HJ. β Glucans are involved in immune-modulation of THP-1 macrophages. Mol Nutr
 Food Res. 2012; 56:822-33.
- 44. Chen H, Wang W, Degroote J, Possemiers S, Chen D, De Smet S, Michiels J.Arabinoxylan in wheat is more responsible than cellulose for promoting intestinal barrier function in weaned male piglets. J Nutr. 2015; 145:51-8.
- 45. Chen KT, Malo MS, Beasley-Topliffe LK, Poelstra K, Millan JL, Mostafa G, Alam SN, Ramasamy S, Warren HS, Hohmann EL, Hodin RA. A role for intestinal alkaline phosphatase in the maintenance of local gut immunity. Dig Dis Sci. 2011; 56:1020-7.
- 46. Chen Q, Swist E, Beckstead J, Green J, Matias F, Roberts J, Qiao C, Raju J, Brooks SP, Scoggan KA. Dietary fructooligosaccharides and wheat bran elicit specific and dose-dependent gene expression profiles in the proximal colon epithelia of healthy Fischer 344 rats. J Nutr. 2011; 141:790-7
- Christensen HR, Frøkiær H, and Pestka JJ. Lactobacilli Differentially Modulate Expression of Cytokines and Maturation Surface Markers in Murine Dendritic Cells. J Immunol 2002; 168: 171-178.
- 48. Cinova J, De Palma G, Stepankova R, Kofronova O, Kverka M, Sanz Y, Tuckova L. Role of intestinal bacteria in gliadin-induced changes in intestinal mucosa: study in germ-free rats. PLoS One. 2011 Jan 13; 6:e16169.

- 49. Clarke JM, Topping DL, Bird AR, Young GP, Cobiac L. Effects of high-amylose maize starch and butyrylated high-amylose maize starch on azoxymethaneinduced intestinal cancer in rats. Carcinogenesis. 2008; 29: 2190-4.
- 50. Clarke TB, Davis KM, Lysenko ES, Zhou AY, Yu Y, Weiser JN. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat Med. 2010; 16:228-31.
- Collins D, Gibson G. Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. Am J Clin Nutr 1999; 69: 1052S– 1057S.
- 52. Conlon MA, Kerr CA, McSweeney CS, Dunne RA, Shaw JM, Kang S, Bird AR, Morell MK, Lockett TJ, Molloy PL, Regina A, Toden S, Clarke JM, Topping DL. Resistant starches protect against colonic DNA damage and alter microbiota and gene expression in rats fed a Western diet. J Nutr. 2012; 142:832-40.
- Coombes, J.L, Powrie, F. Dendritic cells in intestinal immune regulation. Nat Rev Immunol, 2008. 8(6): p. 435-46.
- Corthesy B, Gaskins H, Mercenier A. Cross-Talk between Probiotic Bacteria and the Host Immune System. J Nutr 2007; 781S-790S.
- 55. Cox MA, Jackson J, Stanton M, Rojas-Triana A, Bober L, Laverty M, Yang X, Zhu F, Liu J, Wang S, Monsma F, Vassileva G, Maguire M, Gustafson E, Bayne M, Chou CC, Lundell D, Jenh CH. Short-chain fatty acids act as antiinflammatory mediators by regulating prostaglandin E2 and cytokines. World J Gastroenterol 2009; 15: 5549-5557.

- 56. Crispe IN. The liver as a lymphoid organ. Annu Rev Immunol. 2009; 27:147-63.
- Cummings JH, Englyst HN. Gastrointestinal effects of food carbohydrate. Am J Clin Nutr. 1995; 61:938S-945S.
- 58. Cummings JH, Macfarlane GT: The control and consequences of bacterial fermentation in the human colon. J Appl Microbiol 1991, 70:443-459
- Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut. 1987; 28:1221-7.
- 60. Dahlgren UI, Hanson LA. Effect of oestradiol on the secretory immune system in the rat: an increase in biliary IgM antibodies against a T-cell independent antigen. Immunology. 1991; 74:74-7.
- De Palma G, Nadal I, Collado MC, Sanz S. Effects of a gluten-free diet on gut microbiota and immune function in healthy adult human subjects. Br J Nutr 2009; 102: 1154–1160.
- 62. Denning TL, Norris BA, Medina-Contreras O, Manicassamy S, Geem D, Madan R, Karp CL, Pulendran B. Functional specializations of intestinal dendritic cell and macrophage subsets that control Th17 and regulatory T cell responses are dependent on the T cell/APC ratio, source of mouse strain, and regional localization. J Immunol. 2011 Jul 15;187:733-47.
- Denning TL, Wang YC, Patel SR, Williams IR, Pulendran B. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. Nat Immunol. 2007; 8:1086-94.

- Dennison U, McKernan DP, Scully P, Clarke G, Cryan J, Dinan T. Menstrual cycle influences Toll-like receptor responses. Neuroimmunomodulation. 2012; 19:171-9.
- 65. Deplancke B, Gaskins, H.R. Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. Am J Clin Nutrition. 2001; 73:31S–41S.
- 66. DeSchryver-Kecskemeti K, Eliakim K, Green K, Alpers DH. A novel intracellular pathway for rat intestinal digestive enzymes (alkaline phosphatase and sucrase) via a lamellar particle. Lab Invest J Tech Meth Pathol. 1991; 65: 365-373.
- Dinarello CA. Historical insights into cytokines. Eur J Immunol. 2007;37 Suppl 1:S34-45.
- 68. Dorrestein PC, Mazmanian SK, Knight R. Finding the missing links among metabolites, microbes, and the host. Immunity. 2014 Jun 19; 40:824-32
- Dufour DR, Lott JA, Nolte FS, Gretch DR, Koff RS, Seeff LB. Diagnosis and monitoring of hepatic injury. I. Performance characteristics of laboratory tests. Clin Chem. 2000; 46:2027-49
- 70. Eaton SB. The ancestral human diet: what was it and should it be a paradigm for contemporary nutrition? Proc Nutr Soc. 2006; 65:1-6.
- 71. Enss ML, Cornberg M, Wagner S, Gebert A, Henrichs M, Eisenblätter R, Beil W, Kownatzki R, and Hedrich HR. Proinflammatory cytokines trigger *MUC* gene expression and mucin release in the intestinal cancer cell line LS180. Inflamm. res. 49 (2000) 162–169.

- 72. Evenpoel, P. M. Uremic toxins originating from colonic microbial metabolism.Kidney Inter. 2009; 75: S12-S19.
- 73. Fan MZ, Archbold T, Lackeyram D, Liu Q, Mine Y, Paliyath G. Consumption of guar gum and retrograded high-amylose corn resistant starch increases IL-10 abundance without affecting pro-inflammatory cytokines in the colon of pigs fed a high-fat diet. J Anim Sci. 2012; 90 Suppl 4:278-80.
- 74. Fish EN. The X-files in immunity: sex-based differences predispose immune responses. Nat Rev Immunol. 2008; 8: 737-744
- 75. Fleming SE, Choi SY, Fitch MD. Absorption of short-chain fatty acids from the rat cecum *in vivo*. J Nutr. 1991; 121:1787-97.
- 76. Flint H, Duncan S, Scott K, Louis P. Interactions and competition within the microbial community of the human colon: links between diet and health. Environmental Microbiol. 2007; 9: 1101–1111.
- 77. Fontaine N, Meslin JC, Lory S, Andrieux C: Intestinal mucin distribution in the germ-free rat and in the heteroxenic rat harbouring a human bacterial flora: effect of inulin in the diet. Br J Nutr. 1996; 75(6):881-892.
- 78. Fujihara M, Takahashi D, Abe H, Sakai H, Horinouchi H, Kobayashi K, Ikeda H, Azuma H. Primary and secondary immune responses to keyhole limpet hemocyanin in rats after infusion of hemoglobin vesicle, an artificial oxygen carrier. Artif Organs. 2014; 38: 234-8
- Fujihashi K, Taguchi T, Aicher WK, McGhee JR, Bluestone JA, Eldridge JH,Kiyono H. Immunoregulatory functions for murine intraepithelial lymphocytes:

gamma/delta T cell receptor-positive (TCR+) T cells abrogate oral tolerance, while alpha/beta TCR+ T cells provide B cell help. J Exp Med. 1992; 175:695-707.

- Fusunyan RD, Quinn JJ, Fujimoto M, MacDermott RP, Sanderson IR. Butyrate switches the pattern of chemokine secretion by intestinal epithelial cells through histone acetylation. Mol Med. 1999; 5:631-40.
- 81. Gannon M, Brooks S, Kalmokoff M, Raju, J, Selinger B, Inglis D, Green-Johnson J. Immunomodulatory effects of dietary fibre supplementation on lymphocyte populations, cytokine and immunoglobulin A production. Abstract # 490 96th Annual Meeting of the American Association of Immunologists, Seattle, May 9-12th, 2009.
- Gaskins HR, Croix JA, Nakamura N, Nava GM. Impact of the intestinal microbiota on the development of mucosal defence. Clin Inf Dis 2008; 46:S81-S86.
- 83. Groux-Degroote S, Krzewinski-Recchi MA, Cazet A, Vincent A: IL-6 and IL-8 increase the expression of glycosyltransferases and sulfotransferases involved in the biosynthesis of sialylated and/or sulfated Lewis x epitopes in the human bronchial mucosa. *Biochem J* 2008, 410:213-223.
- 84. Hague A, Manning AM, van der Stappen JW, Paraskeva C. Escape from negative regulation of growth by transforming growth factor beta and from the induction of apoptosis by the dietary agent sodium butyrate may be important in colorectal carcinogenesis. Cancer Metastasis Rev. 1993; 12:227-37.

- Hamaker BR, Tuncil YE. A perspective on the complexity of dietary fiber structures and their potential effect on the gut microbiota. J Mol Biol. 2014; 426:3838-50.
- 86. Hapfelmeier S, Lawson MA, Slack E, Kirundi JK, Stoel M, Heikenwalder M, Cahenzli J, Velykoredko Y, Balmer ML, Endt K, Geuking MB, Curtiss R 3rd, McCoy KD, Macpherson AJ. Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. Science. 2010; 328:1705-9.
- 87. Hardy H, Harris J, Lyon E, Beal J, Foey AD: Probiotics, prebiotics and immunomodulation of gut mucosal defences: homeostasis and immunopathology. Nutrients. 2013; 5:1869-1912.
- Harrington L, Srikanth CV, Antony R, Rhee SJ, Mellor AL, Shi HN, Cherayil BJ. Deficiency of indoleamine 2,3-dioxygenase enhances commensal-induced antibody responses and protects against *Citrobacter rodentium*-induced colitis. Infect Immun. 2008; 76:3045-53
- Harris JR, Markl J. Keyhole limpet hemocyanin: molecular structure of a potent marine immunoactivator. A review. Eur Urol. 2000; 37 Suppl 3:24-33.
- 90. Hayashi A, Sato T, Kamada N, Mikami Y, Matsuoka K, Hisamatsu T, Hibi T, Roers A, Yagita H, Ohteki T, Yoshimura A, Kanai T. A single strain of *Clostridium butyricum* induces intestinal IL-10-producing macrophages to suppress acute experimental colitis in mice. Cell Host Microbe. 2013; 13:711-22.

- 91. He B, Xu W, Santini P, Polydorides A, Chiu A, Estrella J, et al. Intestinal Bacteria Trigger T Cell-Independent Immunoglobulin A2 Class Switching by Inducing Epithelial-Cell Secretion of the Cytokine APRIL. Immunity. 2006; 26: 812-826.
- 92. Health Canada. Proposed Policy: Definition and Energy Value for Dietary Fibre. Bureau of Nutritional Sciences. Food directorate, Health Products and Food. (2010). http://www.hc-sc.gc.ca/fn-an/consult/fibre-fibres/consul-fibre-fibreseng.php#a3
- 93. Hino S, Takemura N, Sonoyama K, Morita A, Kawagishi H, Aoe S, Morita T. Small intestinal goblet cell proliferation induced by ingestion of soluble and insoluble dietary fiber is characterized by an increase in sialylated mucins in rats. J Nutr. 2012; 142:1429-36.
- 94. Hoentjen F, Welling GW, Harmsen HJ, Zhang X, Snart J, Tannock GW, Lien K, Churchill TA, Lupicki M, Dieleman LA. Reduction of colitis by prebiotics in HLA-B27 transgenic rats is associated with microflora changes and immunomodulation. Inflamm Bowel Dis. 2005; 11:977-85
- 95. Homma H, Hoy E, Xu DZ, Lu Q, Feinman R, Deitch EA: The female intestine is more resistant than the male intestine to gut injury and inflammation when subjected to conditions associated with shock states. Am J Physiol Gastrointest Liver Physiol. 2005; 288:G466–G472.
- 96. Hooper LV, Macpherson AJ. Immune adaptations that maintain homeostasis with the intestinal microbiota. Nat Rev Immunol. 2010; 10:159-169.

- 97. Hosono A, Ozawa A, Kato R, Ohnishi Y, Nakanishi Y, Kimura T, Nakamura R. Dietary fructooligosaccharides induce immunoregulation of intestinal IgA secretion by murine Peyer's patch cells. Biosci Biotechnol Biochem. 2003; 67:758-64.
- 98. Howard MD, Gordon DT, Garleb KA, Kerley MS: Dietary fructooligosaccharide, xylooligosaccharide and gum arabic have variable effects on caecal and colonic microbiota and epithelial cell proliferation in mice and rats. *J Nutr.* 1995; 125: 2604-2609.
- 99. Hu, W., T.D. Troutman, R. Edukulla, and C. Pasare, Priming microenvironments dictate cytokine requirements for T helper 17 cell lineage commitment. Immunity. 2011; 35:1010-22.
- 100. Ida JA, Shrestha N, Desai S, Pahwa S, Hanekom WA, Haslett PA. A whole blood assay to assess peripheral blood dendritic cell function in response to Toll-like receptor stimulation. J Immunol Methods. 2006; 310:86-99.
- 101. Inatomi O, Andoh A, Kitamura K, Yasui H, Zhang Z, Fujiyama Y. Butyrate blocks interferon-gamma-inducible protein-10 release in human intestinal subepithelial myofibroblasts. J Gastroenterol. 2005; 40:483-9.
- 102. Ingerslev AK, Theil PK, Hedemann MS, Lærke HN, Bach Knudsen KE. Resistant starch and arabinoxylan augment SCFA absorption, but affect postprandial glucose and insulin responses differently. Br J Nutr. 2014; 111:1564-76.

- 103. Ismail A, Hooper L. Epithelial Cells and Their Neighbors. IV. Bacterial contributions to intestinal epithelial barrier integrity. Am J Physiol Gastrointest Liver Physiol. 2005; 289: G779–G784.
- 104. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, Tanoue T, Imaoka A, Itoh K, Takeda K, Umesaki Y, Honda K, Littman DR. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell. 2009; 139:485-98.
- 105. Izcue A, Coombes JL, Powrie F. Regulatory lymphocytes and intestinal inflammation. Annu Rev Immunol. 2006; 27: 313-38.
- 106. James SL, Muir JG, Curtis SL, Gibson PR. Dietary fibre: a roughage guide.Intern Med J. 2003; 33:291-6.
- 107. Janeway CA Jr. How the immune system protects the host from infection. Microbes Infect. 2001; 3:1167-71.
- 108. Jakobsdottir G, Jadert C, Holm L, Nyman ME. Propionic and butyric acids, formed in the caecum of rats fed highly fermentable dietary fibre, are reflected in portal and aortic serum. Br J Nutr. 2013; 26:1-8.
- 109. Kalmokoff M, Zwicker B, O'Hara M, Matias F, Green J, Shastri P, Green-Johnson J, Brooks SP. Temporal change in the gut community of rats fed high amylose cornstarch is driven by endogenous urea rather than strictly on carbohydrate availability. J Appl Microbiol. 2013; 114:1516-28.

- 110. Kanga S, Tangb Q, Bluestoneb J. CD4+CD25+ Regulatory T Cells in Transplantation:Progress, Challenges and Prospects. Am J Transpl. 2007; 7: 1457–1463.
- 111. Kapp JA, Kapp LM, McKenna KC, Lake JP. γδdelta T-cell clones from intestinal intraepithelial lymphocytes inhibit development of CTL responses *ex vivo*. Immunology. 2004; 111:155-64.
- Kaur N, Gupta AK. Applications of inulin and oligofructose in health and nutrition. J Biosci. 2002; 27:703-14.
- 113. Kawai R, Ito S, Aida T, Hattori H, Kimura T, Furukawa T, Mori K, Sanbuissho A, Kawada T. Evaluation of primary and secondary responses to a T-cell-dependent antigen, keyhole limpet hemocyanin, in rats. J Immunotoxicol. 2013; 10:40-8.
- 114. Klein S. Hormonal and immunological mechanisms mediating sex differences in parasite infection. Parasite Immunology 2004; 26:247-264.
- 115. Knolle PA, Gerken G. Local control of the immune response in the liver. Immunol Rev. 2000; 174:21-34.
- 116. Knoop KA, Miller MJ, Newberry RD. Transepithelial antigen delivery in the small intestine: different paths, different outcomes. Curr Opin Gastroenterol. 2011; 29:112-8
- Konkel JE, Chen W. Balancing acts: the role of TGF-β in the mucosal immune system. Trends Mol Med. 2011;17:668-76.

- 118. Kovacs-Nolan J, Kanatani H, Nakamura A, Ibuki M, Mine Y. β-1,4-mannobiose stimulates innate immune responses and induces TLR4-dependent activation of mouse macrophages but reduces severity of inflammation during endotoxemia in mice. J Nutr. 2013; 143:384-91.
- 119. Kuhlman G, Roth JA, Flakoll PJ, Vandehaar MJ, Nissen S. Effects of dietary leucine, alpha-ketoisocaproate and isovalerate on antibody production and lymphocyte blastogenesis in growing lambs. J Nutr. 1988; 118:1564-9.
- 120. Kumar A, Wu H, Collier-Hyams LS, Kwon YM, Hanson JM, Neish AS. The bacterial fermentation product butyrate influences epithelial signaling via reactive oxygen species-mediated changes in cullin-1 neddylation. J Immunol. 2009; 182:538-46.
- 121. Kunisawa J, Kiyono H. A marvel of mucosal T cells and secretory antibodies for the creation of first lines of defense. Cell. Mol. Life Sci. 2005; 62:1308-1321.
- Lattimer JM, Haub MD. Effects of dietary fiber and its components on metabolic health. Nutrients. 2010; 2:1266-89.
- 123. Le Blay G, Michel C, Blottière HM, Cherbut C. Prolonged intake of fructooligosaccharides induces a short-term elevation of lactic acid-producing bacteria and a persistent increase in cecal butyrate in rats. J Nutr. 1999; 129:2231-5.
- 124. Le Blay GM, Michel CD, Blottière HM, Cherbut CJ. Raw potato starch and shortchain fructo-oligosaccharides affect the composition and metabolic activity of rat intestinal microbiota differently depending on the caecocolonic segment involved. J Applied Microbiol. 2003; 94:312–320.

- 125. Lebeer S, Vanderleyden D, De Keermaecker SC. Host interactions of probiotic bacterial surface molecules: comparison with commensals and pathogens. Nat Rev Microb 2010; 8: 171-184.
- 126. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD, Knight R, Gordon JI. Evolution of mammals and their gut microbes. Science. 2008; 320:1647-51.
- 127. Ley RE, Peterson DA, Gordon JI. Ecological and Evolutionary Forces Shaping Microbial Diversity in the Human Intestine. Cell. 2006; 124: 837–848.
- 128. Li M, Wang B, Zhang M, Rantalainen M, Wang S, Zhou H, Zhang Y, Shen J, Pang X, Zhang M, Wei H, Chen Y, Lu H, Zuo J, Su M, Qiu Y, Jia W, Xiao C, Smith LM, Yang S, Holmes E, Tang H, Zhao G, Nicholson JK, Li L, Zhao L: Symbiotic gut microbes modulate human metabolic phenotypes. Proc Natl Acad Sci. 2008; 105:2117-22.
- 129. Littman DR, Pamer EG. Role of the commensal microbiota in normal and pathogenic host immune responses. Cell Host Microbe. 2011; 10:311-23.
- 130. Looijer-van Langen M, Hotte N, Dieleman LA, Albert E, Mulder C, Madsen KL. Estrogen receptor-β signaling modulates epithelial barrier function. Am J Physiol Gastrointest Liver Physiol. 2011; 300(4):G621-6.
- 131. Macpherson AJ, Gatto D, Sainsbury E, Harriman GR, Hengartner H, Zinkernagel RM. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science. 2000; 288:2222-6.

- Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. Nat Rev Immunol. 2004; 4(6):478-85
- 133. Macpherson AJ, Slack E. The functional interactions of commensal bacteria with intestinal secretory IgA. Curr Opin Gastroenterolgy 2007; 23:673-8.
- Macpherson AJ, Uhr T. Compartmentalization of the mucosal immune responses to commensal intestinal bacteria. Ann N Y Acad Sci. 2004; 1029:36-43
- Macpherson AJ, Uhr T. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science. 2004; 303:1662-5.
- Malloy KJ, Powrie F: Intestinal homeostasis and its breakdown in inflammatory bowel disease. Nature. 2011, 474:298-306.
- 137. Manhart N, Spittler A, Bergmeister H, Mittlböck M, Roth E. Influence of fructooligosaccharides on Peyer's patch lymphocyte numbers in healthy and endotoxemic mice. Nutrition. 2003; 657-60.
- 138. Mann ER, Landy JD, Bernardo D, Peake ST, Hart AL, Al-Hassi HO, Knight SC. Intestinal dendritic cells: their role in intestinal inflammation, manipulation by the gut microbiota and differences between mice and men. Immunol Lett. 2013; 150:30-40.
- 139. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol. 2002; 23:549-55.
- 140. Mariadason JM, Corner GA, Augenlicht LH: Genetic reprogramming in pathways of colonic cell maturation induced by short chain fatty acids:

comparison with trichostatin A, sulindac and curcumin and implications for chemoprevention of colon cancer. Cancer Res 2000; 60(16):4561-72.

- 141. Markle JG, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, von Bergen M, McCoy KD, Macpherson AJ, Danska JS. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. Science. 201; 339:1084-8.
- Marteau P, Seksik P, Lepage P, Dore J. Cellular and Physiological Effects of Probiotics and Prebiotics. Mini-Rev Med Chem. 2004; 4: 889-896.
- 143. Martínez-Beamonte R, Navarro MA, Larraga A, Strunk M, Barranquero C, Acín S, Guzman MA, Iñigo P, Osada J. Selection of reference genes for gene expression studies in rats. J Biotechnol. 2011; 151:325-34.
- 144. Maslowski K, Vieira A, Ng A, Kranich J, Sierro F, Yu D, *et al.* Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature 2009; 461:1282-1286.
- Mazmanian S, Liu C, Tzianabos A, Kasper D. An Immunomodulatory Molecule of Symbiotic Bacteria Directs Maturation of Host Immune System. Cell 2005; 122: 107–118.
- 146. McCullough LD, de Vries GJ, Miller VM, Becker JB, Sandberg K, McCarthy MM. NIH initiative to balance sex of animals in preclinical studies: generative questions to guide policy, implementation, and metrics. Biol Sex Diff. 2014; 5:15.

- 147. McOrist AL, Miller RB, Bird AR, Keogh JB, Noakes M, Topping DL, Conlon MA. Fecal butyrate levels vary widely among individuals but are usually increased by a diet high in resistant starch. J Nutr. 2011 May; 141(5):883-9.
- 148. Medgyesi GA, Miklós K, Kulics J, Füst G, Gergely J, Bazin H. Classes and subclasses of rat antibodies: reaction with the antigen and interaction of the complex with the complement system. Immunology. 1981 May; 43(1):171-6
- 149. Medzhitov R. TLR-mediated innate immune recognition. Semin Immunol.2007 Feb; 19(1):1-2
- 150. Meijers B, De Preter V, Verbeke K, Vanrenterghem Y, Evenepoel P. p-Cresyl sulphate serum concentrations in haemodialysis patients are reduced by the prebiotic oligofructose-enriched inulin. Neph Dial Transp. 2010; 25: 219-224
- 151. Metzler-Zebeli BU, Gänzle MG, Mosenthin R, Zijlstra RT. Oat β-glucan and dietary calcium and phosphorus differentially modify intestinal expression of proinflammatory cytokines and monocarboxylate transporter 1 and cecal morphology in weaned pigs. J Nutr. 2012; 142:668-74.
- Miller S. Cellular and Physiological Effects of Short Chain Fatty Acids. Mini-Rev Med Chem. 2004; 4: 839-845.
- 153. Mirmonsef P, Zariffard MR, Gilbert D, Makinde H, Landay AL, Spear GT. Short-chain fatty acids induce pro-inflammatory cytokine production alone and in combination with toll-like receptor ligands. Am J Reprod Immunol. 2012; 67:391-400.
- 154. Moal V, Servin A. The Front Line of Enteric Host Defense against Unwelcome Intrusion of Harmful Microorganisms: Mucins, Antimicrobial Peptides and Microbiota. Clin Microb Rev. 2006; 19: 315–337.
- Mora JR. Generation of gut homing IgA secreting B cells by intestinal dendritic cells. Science. 2006; 314: 1157-1160.
- 156. Morita T, Tanabe H, Ito H, Sugiyama K, Kiriyama S. Long-term ingestion of insoluble dietary fiber increases luminal mucin content, but has no effect on nutrient absorption in rats. Biosci Biotechnol Biochem. 2008; 72:767-72.
- 157. Mosmann T. Rapid colorimetric assay for cellular growth and survival:
 application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;
 65:55-63.
- Mowat A. Anatomical basis of tolerance and immunity to intestinal antigens. Nat Immunology Reviews 2003; 3: 331-341.
- 159. Mowat AM, Agace WW. Regional specialization within the intestinal immune system. Nat Rev Immunol. 2014. 14; 667-85
- 160. Mudgil D, Barak S. Composition, properties and health benefits of indigestible carbohydrate polymers as dietary fiber: a review. Int J Biol Macromol. 2013; 61:1-6.
- 161. Nakamura Y, Nosaka S, Suzuki M, Nagafuchi S, Takahashi T, Yajima T, Takenouchi-Ohkubo N, Iwase T, Moro I. Dietary fructooligosaccharides upregulate immunoglobulin A response and polymeric immunoglobulin receptor expression in intestines of infant mice. Clin Exp Immunol. 2004;137:52-8.

- 162. Neish AS. Microbes in Gastrointestinal Health and Disease. Gastroenterology. 2009; 136: 65-80.
- 163. Neish AS. The gut microflora and intestinal epithelial cells: a continuing dialogue. Microbes Inf. 2002; 4: 309–317
- 164. Nenci A, Becker C, Wullaert A, Gareus R, van Loo G, Danese S, Huth M, Nikolaev A, Neufert C, Madison B, Gumucio D, Neurath MF, Pasparakis M. Epithelial NEMO links innate immunity to chronic intestinal inflammation. Nature. 2007; 446:557-61.
- 165. Neyrinck AM, Mouson A, Delzenne NM. Dietary supplementation with laminarin, a fermentable marine beta (1-3) glucan, protects against hepatotoxicity induced by LPS in rat by modulating immune response in the hepatic tissue. Int Immunopharmacol. 2007; 7:1497-506.
- 166. Nofrarías M, Martínez-Puig D, Pujols J, Majó N, Pérez JF. Long-term intake of resistant starch improves colonic mucosal integrity and reduces gut apoptosis and blood immune cells. Nutrition. 2007; 23:861-70.
- 167. Oh JZ, Ravindran R, Chassaing B, Carvalho FA, Maddur MS, Bower M, Hakimpour P, Gill KP, Nakaya HI, Yarovinsky F, Sartor RB, Gewirtz AT, Pulendran B. TLR5-mediated sensing of gut microbiota is necessary for antibody responses to seasonal influenza vaccination. Immunity. 2014; 41:478-92

- 168. Ohno Y, Lee J, Fusunyan RD, MacDermott RP, Sanderson IR. Macrophage inflammatory protein-2: chromosomal regulation in rat small intestinal epithelial cells. Proc Natl Acad Sci. 1997; 94:10279-84
- 169. Onodera S, Suzuki K, Matsuno T, Kaneda K, Takagi M, Nishihira J. Macrophage migration inhibitory factor induces phagocytosis of foreign particles by macrophages in autocrine and paracrine fashion. Immunology. 1997; 92:131-7.
- 170. Ortega-González M, Ocón B, Romero-Calvo I, Anzola A, Guadix E, Zarzuelo A, Suárez MD, Sánchez de Medina F, Martínez-Augustin O. Nondigestible oligosaccharides exert nonprebiotic effects on intestinal epithelial cells enhancing the immune response via activation of TLR4-NFκB. Mol Nutr Food Res. 2014; 58:384-93.
- Ozer J, Ratner M, Shaw M, Bailey W, Schomaker S. The current state of serum biomarkers of hepatotoxicity. Toxicology. 2008; 245(3):194-205.
- 172. Parhar K, Ray A, Steinbrecher U, Nelson C, Salh B. The p38 mitogen-activated protein kinase regulates interleukin-1β-induced IL-8 expression via an effect on the IL-8 promoter in intestinal epithelial cells. Immunology. 2003; 108:502-12.
- 173. Parnell JA, Reimer RA. Effect of prebiotic fibre supplementation on hepatic gene expression and serum lipids: a dose–response study in JCR:LA-cp rats. Br J Nutr. 2010; 103:1577-1584.
- 174. Perez de la Cruz A, Abiles J, Perez Abud R. Perspectives in the design and development of new products for enteral nutrition. Nutr. Hosp. 2006; 21:98-108.

- 175. Perrin P, Pierre F, Patry Y, Champ M, Berreur M, Pradal G, Bornet F, Meflah K, Menanteau J. Only fibres promoting a stable butyrate producing colonic ecosystem decrease the rate of aberrant crypt foci in rats. Gut. 2001; 48:53-61.
- 176. Peterson DA, McNulty NP, Guruge JL, Gordon JI. IgA response to symbiotic bacteria as a mediator of gut homeostasis. Cell Host Microbe. 2007; 2:328-39.
- 177. Qu H, Madl RL, Takemoto DJ, Baybutt RC, Wang W. Lignans are involved in the antitumor activity of wheat bran in colon cancer SW480 cells. J Nutr. 2005; 135:598-602
- 178. Quigley E. Prebiotics and probiotics, modyfying and mining the microbiota.Pharmacol Res. 2010; 61: 213-218.
- 179. Racanelli V, Rehermann B. The liver as an immunological organ. Hepatology. 2006; 43 Suppl:S54-62.
- 180. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R: Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell. 2004; 118:229–241.
- 181. Ramakers JD, Volman JJ, Biörklund M, Onning G, Mensink RP, Plat J. Fecal water from ileostomic patients consuming oat beta-glucan enhances immune responses in enterocytes. Mol Nutr Food Res. 2007; 51:211-20.
- 182. Reed KL, Fruin AB, Gower AC, Gonzales KD, Stucchi AF, Andry CD, O'Brien M, Becker JM. NF-kappaB activation precedes increases in mRNA encoding neurokinin-1 receptor, proinflammatory cytokines, and adhesion molecules in

dextran sulfate cccccfsodium-induced colitis in rats. Dig Dis Sci. 2005; 50:2366-78.

- 183. Reed KL, Fruin AB, Gower AC, Gonzales KD, Stucchi AF, Andry CD, O'Brien M, Becker JM: NF-kappaB activation precedes increases in mRNA encoding neurokinin-1 receptor, proinflammatory cytokines, and adhesion molecules in dextran sulfate sodium-induced colitis in rats. Dig Dis Sci 2005; 50:2366-2378.
- Roberfroid M. Prebiotics and probiotics: are they functional foods? Am J Clin Nutr. 2000; 71:1682S-1687S
- 185. Roberfroid M. Prebiotics: The Concept Revisited. J Nutr. 2007; 830S-837S.
- 186. Roberts BJ, Dragon JA, Moussawi M, Huber SA. Sex-specific signaling through Toll-Like Receptors 2 and 4 contributes to survival outcome of Coxsackievirus B3 infection in C57Bl/6 mice. Biol Sex Differ. 2012; 3:25.
- 187. Rocha B, von Boehmer H, Guy-Grand D. Selection of intraepithelial lymphocytes with CD8 alpha/alpha co-receptors by self-antigen in the murine gut. Proc Natl Acad Sci. 1992; 89:5336-40.
- 188. Rollwagen FM, Madhavan S, Singh A, Li YY, Wolcott K, Maheshwari R. IL-6 protects enterocytes from hypoxia-induced apoptosis by induction of bcl-2 mRNA and reduction of fas mRNA. Biochem Biophys Res Commun. 2006; 347:1094-8
- 189. Romagnani S: Th1/Th2 cells. Inflamm Bowel Dis. 1999; 5:285-94.
- 190. Rook GAW, Burnett LR. Microbes, immunoregulation, and the gut. Gut Inter.2004; 317-320.

- 191. Round J, Mazmanian S. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol. 2009; 9:313-323.
- 192. Ryz NR, Meddings JB, Taylor CG. Long-chain inulin increases dendritic cells in the Peyer's patches and increases ex vivo cytokine secretion in the spleen and mesenteric lymph nodes of growing female rats, independent of zinc status. Br J Nutr. 2009; 101:1653-63.
- Sabater-Molina ML. Dietary fructooligosaccharides and potential benefits on health. J. Physiol. Biochem. 2009; 65: 315-328.
- 194. Sakai T, Agui T, Muramatsu Y, Nagasawa H, Himeno K, Matsumoto K. Low level of immunoglobulin G2a subclass correlates with a deficiency in T helper cell function in LEC mutant rats. J Vet Med Sci. 1995; 57:527-9.
- 195. Sanderson IR. Dietary modulation of GALT. J Nutr. 2007;137(11 Suppl):2557S-2562S.
- 196. Sandvik A, Wang YY, Morton HC, Aasen AO, Wang JE, Johansen FE. Oral and systemic administration of β-glucan protects against lipopolysaccharideinduced shock and organ injury in rats. Clin Exp Immunol. 2007; 148:168-77.
- 197. Satchithanandam S, Vargofcak-Apker M, Calvert RJ, Leeds AR, Cassidy MM. Alteration of gastrointestinal mucin by fiber feeding in rats. J Nutr. 1990; 120:1179-84.
- 198. Schiffrin E, Blum S. Interactions between the microbiota and the intestinal mucosa. Eur J Clin Nutr. 2002; 56: S60–S64.

- Schilderink R, Verseijden C, de Jonge WJ. Dietary inhibitors of histone deacetylases in intestinal immunity and homeostasis. Front Immunol. 2013; 4:226.
- Schley PD, Field CJ. The immune enhancing effects of dietary fibres and prebiotics. Br J Nutr 2002; 87:S221-S230.
- 201. Schulz O, Pabst O. Antigen sampling in the small intestine. Trends Immunol.2013; 34:155-61.
- 202. Sghir A, Chow JM, Mackie RI: Continuous culture selection of bifidobacteria and lactobacilli from human faecal samples using fructooligosaccharide as selective substrate. J Appl Microbiol. 1998, 85:769–777.
- 203. Shan M, Gentile M, Yeiser JR, Walland AC, Bornstein VU, Chen K, He B, Cassis L, Bigas A, Cols M, Comerma L, Huang B, Blander JM, Xiong H, Mayer L, Berin C, Augenlicht LH, Velcich A, Cerutti A. Mucus enhances gut homeostasis and oral tolerance by delivering immunoregulatory signals. Science. 2013; 342:447-53.
- 204. Sheth SU, Lu Q, Twelker K, Sharpe SM, Qin X, Reino DC, Lee MA, Xu DZ, Deitch EA: Intestinal mucus layer preservation in female rats attenuates gut injury after trauma-hemorrhagic shock. J Trauma. 2010; 68:279-288.
- 205. Shingadia D, O'Gorman M, Rowley AH, Shulman ST. Surface and cytoplasmic immunoglobulin expression in circulating B-lymphocytes in acute Kawasaki disease. Pediatr Res. 2001; 50:538-43.

- Singh RS, Singh RP: Fructooligosaccharides from inulin as prebiotics. Food Technol Biotechnol. 2010; 48:435–450.
- 207. Slack E, Hapfelmeier S, Stecher B, Velykoredko Y, Stoel M, Lawson MA, Geuking MB, Beutler B, Tedder TF, Hardt WD, Bercik P, Verdu EF, McCoy KD, Macpherson AJ. Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. Science. 2009; 325:617-20.
- Slavin J. Fiber and prebiotics: mechanisms and health benefits. Nutrients. 2013;
 5:1417-35.
- 209. Slavin J. Whole grains and human health. Nutr Res Rev. 2004; 17:99-110.
- 210. Smith DW, Nagler-Anderson C. Preventing Intolerance: The induction of nonresponsiveness to dietary and microbial antigens in the intestinal mucosa. J Immunol. 2005; 3851-3857.
- 211. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN, Garrett WS. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science. 2013; 341:569-73.
- 212. Sokol H, Pignuer B, Waterlott L, *et al. Faecalibacterium prausnitzii* is an antiinflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. PNAS USA 2008; 105: 16731-16736.
- 213. Steegenga WT, Mischke M, Lute C, Boekschoten MV, Pruis MG, Lendvai A, Verkade HJ, Boekhorst J, Timmerman HM, Plösch T, Müller M. Sexually dimorphic characteristics of the small intestine and colon of prepubescent C57BL/6 mice. Biol Sex Differ. 2014; 29:5-11.

- 214. Stevenson L, Phillips F, O'Sullivan K, Walton J. Wheat bran: its composition and benefits to health, a European perspective. Int J Food Sci Nutr. 2012; 63:1001-13.
- 215. Stillie R, Bell RC, Field CJ. Diabetes-prone BioBreeding rats do not have a normal immune response when weaned to a diet containing fermentable fibre. Br J Nutr. 2005; 93:645-53
- 216. Strober W, Murray P, Kitani A, Watanabe T. Signalling pathways and molecular interactions of NOD1 and NOD2. Nat Rev Immunol. 2006; 6: 9-20.
- 217. Takaishi K, Ohtsuka T, Tsuneyoshi S, Maehara N, Harada M, Yoshida H, Watanabe K, Tsurufuji S. Inhibition of the production of rat cytokine-induced neutrophil chemoattractant (CINC)-1, a member of the interleukin-8 family, by adenovirus-mediated overexpression of IκBα. J Biochem. 2000; 127:511-6.
- 218. Takeda K, Akira S. TLR signaling pathways. Semin Immunol. 2004; 16:3-9.
- 219. Takeuchi O, Sato S, Horiuchi T, Hoshino K, Takeda K, Dong Z, Modlin RL, Akira S. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J Immunol. 2002; 169:10-4.
- 220. Talke H, Schubert GE. Enzymatic urea determination in the blood and serum in the Warburg optical tests. Klin Wochenschr. 1965; 43:174-5.
- 221. Tappenden KA, Thomson AB, Wild GA, McBurney MI. Short chain fatty acidsupplemented total parenteral nutrition enhances functional adaptation to intestinal resection in rats. Gastroenterol. 1997; 112:792-802.

- 222. Taylor ME, Drickamer K. Convergent and divergent mechanisms of sugar recognition across kingdoms. Curr Opin Struct Biol. 2014; 28:14-22.
- 223. Ten Bruggencate SJ, Bovee-Oudenhoven IM, Lettink-Wissink ML, Katan MB, van Der Meer R: Dietary fructo-oligosaccharides and inulin decrease resistance of rats to Salmonella: protective role of calcium. Gut. 2004; 53:530–5.
- 224. Ten Bruggencate SJ, Bovee-Oudenhoven IM, Lettink-Wissink ML, Katan MB, van der Meer R: Dietary fructooligosaccharides affect intestinal barrier function in healthy men. J Nutr. 2006; 136:70-74.
- 225. Ten Bruggencate SJ, Bovee-Oudenhoven IM, Lettink-Wissink ML, van der Meer R: Dietary fructo-oligosaccharides dose-dependently increase translocation of Salmonella in rats. J Nutr. 2003; 133:2313-2318.
- 226. Ten Bruggencate SJ, Bovee-Oudenhoven MJ, Lettink-Wissink ML, van der Meer
 R: Dietary fructooligosaccharides increase intestinal permeability in rats. J Nutr.
 2005; 135:837-842.
- 227. Thangaraju M, Cresci GA, Liu K, Ananth S, Gnanaprakasam JP, Browning DD, Mellinger JD, Smith SB, Digby GJ, Lambert NA, Prasad PD, Ganapathy V. GPR109A is a G-protein-coupled receptor for the bacterial fermentation product butyrate and functions as a tumor suppressor in colon. Cancer Res. 2009; 69:2826-32.
- 228. Thomas, K.E., A. Sapone, A. Fasano, and S.N. Vogel, Gliadin stimulation of murine macrophage inflammatory gene expression and intestinal permeability

are MyD88-dependent: role of the innate immune response in Celiac disease. J Immunol. 2006; 176:2512-21.

- Thomson AW, Knolle PA. Antigen-presenting cell function in the tolerogenic liver environment. Nat Rev Immunol. 2010; 10:753-66.
- 230. Tlaskalova-Hogenova H, Tuckova L, Mestecky J, Kolinska J, Rossmann P, Stepankova R, Kozakova H, Hudcovic T, Hrncir T, Frolova L, Kverka M. Interaction of mucosal microbiota with the innate immune system. Scand J Immunol. 2005; 62 S1:106-13.
- Topping D, Clifton P. Short-Chain Fatty Acids and Human Colonic Function:Roles of Resistant Starch and Nonstarch Polysaccharides. Physiol Rev. 2001; 81:1031-1064.
- Tsuji M, Suzuki K, Kinoshita K, Fagarasan S. Dynamic interactions between bacteria and immune cells leading to intestinal IgA synthesis. Semin Immunol. 2008; 20: 59-66.
- 233. Tuyishime S, Haut LH, Zhu C, Ertl HC. Enhancement of recombinant adenovirus vaccine-induced primary but not secondary systemic and mucosal immune responses by all-trans retinoic acid. Vaccine. 2014; 32(27):3386-92.
- Vahouny GV, Le T, Ifrim I, Satchithanandam S, Cassidy MM. Stimulation of intestinal cytokinetics and mucin turnover in rats fed wheat bran or cellulose.
 Am J Clin Nutr. 1985; 41:895-900
- 235. Van Craeyveld V, Swennen K, Dornez E, Van de Wiele T, Marzorati M,Verstraete W, Delaedt Y, Onagbesan O, Decuypere E, Buyse J, De Ketelaere B,

Broekaert WF, Delcour JA, Courtin CM. Structurally different wheat-derived arabinoxylooligosaccharides have different prebiotic and fermentation properties in rats. J Nutr. 2008; 138:2348-55

- 236. Van den Abbeele P, Gérard P, Rabot S, Bruneau A, El Aidy S, Derrien M, Kleerebezem M, Zoetendal EG, Smidt H, Verstraete W, Van de Wiele T, Possemiers S. Arabinoxylans and inulin differentially modulate the mucosal and luminal gut microbiota and mucin-degradation in humanized rats. Environ Microbiol. 2011; 13:2667-80.
- 237. Van Tassell ML, Miller MJ. Lactobacillus adhesion to mucus. Nutrients. 2011;3:613-36
- 238. Veldhoen, M., R.J. Hocking, C.J. Atkins, R.M. Locksley, and B. Stockinger, TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity, 2006. 24:179-89.
- Verthelyi D, Zuener RA. Differential signaling by CpG DNA in DCs and B cells: not just TLR9. Trends Immunol. 2003; 24: 519-522.
- 240. Verthelyi D. Female's heightened immune status: estrogen, T cells, and inducible nitric oxide synthase in the balance. Endocrinol. 2006; 147:659-61.
- 241. Vinolo MA, Rodrigues HG, Hatanaka E, Hebeda CB, Farsky SH, Curi R. Shortchain fatty acids stimulate the migration of neutrophils to inflammatory sites. Clin Sci (Lond). 2009; 117:331-8.
- 242. Vinolo MA, Rodrigues HG, Nachbar RT, Curi R. Regulation of inflammation by short chain fatty acids. Nutrients. 2011; 3:858-76.

- 243. Vogt LM, Meyer D, Pullens G, Faas MM, Venema K, Ramasamy U, Schols HA, de Vos P. Toll-like receptor 2 activation by β2→1-fructans protects barrier function of T84 human intestinal epithelial cells in a chain length-dependent manner. J Nutr. 2014; 144:1002-8.
- 244. Volman JJ, Mensink RP, Buurman WA, Onning G, Plat J. The absence of functional dectin-1 on enterocytes may serve to prevent intestinal damage. Eur J Gastroenterol Hepatol. 2010; 22:88-94.
- 245. Volman JJ, Ramakers JD, Plat J. Dietary modulation of immune function by βglucans. Physiol Behav. 2008; 94:276-84.
- Watzl B, Girrbach S, Roller, M. Inulin, oligofructose and immunomodulation. Br J Nutr. 2005; 93: S49-S55
- 247. Weaver GA, Tangel CT, Krause JA, Parfitt MM, Jenkins PL, Rader JM, Lewis BA, Miller TL, Wolin MJ. Acarbose enhances human colonic butyrate production. J Nutr. 1997; 127:717-23.
- 248. Weiner HL. Oral tolerance: immune mechanisms and the generation of Th3-type TGF-β-secreting regulatory cells. Microb Infec 2001; 3: 947-954.
- 249. Whiteside TL. What are regulatory T cells (Treg) regulating in cancer and why?Semin Cancer Biol. 2012; 22:327-34.
- 250. Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC, Siuzdak G. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc Natl Acad Sci USA. 2009; 106:3698-703

- 251. Wismar R, Brix S, Laerke HN, Frøkiaer H. Comparative analysis of a large panel of non-starch polysaccharides reveals structures with selective regulatory properties in dendritic cells. Mol Nutr Food Res. 2011; 55:443-54
- 252. Wolford ST, Schroer RA, Gohs FX, Gallo PP, Brodeck M, Falk HB, Ruhren R. Reference range data base for serum chemistry and hematology values in laboratory animals. J Toxicol Environ Health. 1986; 18:161-88.
- 253. Woof JM, Kerr MA: The function of immunoglobulin A in immunity. J Pathol.2006; 208:270-282.
- 254. Wu J, Zhou Z, Hu Y, Dong S. Butyrate-induced GPR41 activation inhibits histone acetylation and cell growth. J Genet Genomics. 2012; 39:375-84.
- 255. Wu Y, Kenneth K, DeWitt R, Tolley E, Li J. Route and Type of Nutrition Influence IgA-Mediating Intestinal Cytokines. Annals Surg. 1999; 229: 662– 668.
- 256. Yamanaka T, Helgeland L, Farstad IN, Fukushima H, Midtvedt T, Brandtzaeg P. Microbial colonization drives lymphocyte accumulation and differentiation in the follicle-associated epithelium of Peyer's patches. J Immunol. 2003; 170:816-22
- 257. Yang X, Schadt EE, Wang S, Wang H, Arnold AP, Ingram-Drake L, Drake TA, Lusis AJ. Tissue-specific expression and regulation of sexually dimorphic genes in mice. Genome Res. 2006; 16:995-1004.
- 258. Yang RZ, Park S, Reagan WJ, Goldstein R, Zhong S, Lawton M, Rajamohan F, Qian K, Liu L, Gong DW. Alanine aminotransferase isoenzymes: molecular

cloning and quantitative analysis of tissue expression in rats and serum elevation in liver toxicity. Hepatology. 2009 Feb;49(2):598-607.

- 259. Yoshida H, Miura S, Kishikawa H, Hirokawa M, Nakamizo H, Nakatsumi RC, Suzuki H, Saito H, Ishii H. Fatty acids enhance GRO/CINC-1 and interleukin-6 production in rat intestinal epithelial cells. J Nutr. 2001; 131:2943-50.
- 260. Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D. Cutting edge: recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. J Immunol. 1999; 163:1-5
- 261. Zenhom M, Hyder A, de Vrese M, Heller KJ, Roeder T, Schrezenmeir J. Prebiotic oligosaccharides reduce proinflammatory cytokines in intestinal Caco-2 cells via activation of PPARγ and peptidoglycan recognition protein 3. J Nutr. 2011; 141:971-7
- 262. Zoetendal EG, Collier CT, Koike S, Mackie RI, Gaskins HR. Molecular ecological analysis of the gastrointestinal microbiota: a review. J Nutr. 2004; 134:465-72.
- 263. Zoran DL, Turner ND, Taddeo SS, Chapkin RS, Lupton JR. Wheat bran diet reduces tumor incidence in a rat model of colon cancer independent of effects on distal luminal butyrate concentrations. J Nutr. 1997; 127:2217-25.

10. Appendix



Figure AI. Change in the male and female faecal community in response to the FOSsupplemented diet. Bar graphs indicate the distribution of phylotypes (%) at the Family level. Rats fed rodent chow (**•**) or rodent chow supplemented with OF (**□**). **Left panel**: females. **Right panel**: males. Arrows indicate major taxa changing in response to OF A) Family *Porphyromonadaceae* (Genus *Barnesiella*). B) Family *Prevotellaceae* (Genus *Prevotella*) C and D) Family *Lachnospiraceae*. **Inset**: Pie charts show the distribution of phylotypes at the level of Phylum in rats fed rodent chow (upper pie charts) or rodent chow supplemented with OF (lower pie charts).