MODULATION OF THE IMMUNE SYSTEM IN THE MAMMALIAN INTESTINE AS AN ALTERNATE EXPLANATION FOR THE ACTION OF ANTIMICROBIAL GROWTH PROMOTERS

ESTELA COSTA Doctor of Veterinary Medicine, Santa Catarina State University, 2007

> A Thesis Submitted to the School of Graduate Studies of the University of Lethbridge in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

Department of Biological Sciences University of Lethbridge LETHBRIDGE, ALBERTA, CANADA

© Estela Costa, 2010

Dedication

To my beloved husband,

Anderson Macedo da Silva

ABSTRACT

Modulation of the immune system in the mammalian intestine as an alternate explanation for the action of antimicrobial growth promoters

The novel hypothesis that antimicrobial growth promoters (AGP) function by modulating the mammalian immune system was tested. Sampling methods to characterize the mucosa-associated microbiota of the murine intestine by terminal restriction fragment length polymorphism (T-RFLP) analysis indicated that direct plug extraction was superior to wash methods. Using T-RFLP analysis, non-therapeutic administration of chlortetracycline (CTC) and sulfamethazine to beef cattle did not affect the composition of bacterial communities associated with intestinal mucosa and in digesta, with exception of those associated with mucosa of the proximal jejunum. Similarly, oral administration of non-therapeutic concentrations of CTC did not affect the mucosa-associated microbiota of the murine intestine. Oral administration of nontherapeutic concentrations of CTC prevented weight loss, reduced pathologic changes, modulated transcription levels of inflammatory cytokines in *C. rodentium*-infected mice, and did not consistently affect the colonic microbiota. These findings support the hypothesis that AGP primarily function by modulating the intestinal immune system.

ACKNOWLEDGEMENTS

I sincerely thank my supervisors, Dr. Doug Inglis and Dr. Brent Selinger, for giving me the opportunity of expanding my knowledge in science, and of working in excellent laboratories, as well as for their support and guidance over the years. I also express my gratitude to Dr. John Kastelic, for his support, friendship, and immeasurable generosity towards me and my husband when we first arrived in Canada. I also thank members of my Committee, Dr. Richard Uwiera, for his guidance in histology, immunology, and molecular biology, and Dr. Ute Kothe, for her advice in microbiology and molecular biology. I also express sincere gratitude to my work colleagues: Jenny, Tara, Kathaleen, Lisa, Randy, Matt, Kristen, Nathan, Rachel, Larissa, and Amy for their substantial help with laboratory, computer, and animal techniques, as well as for their friendship. I am also grateful to Helena Danyk, Dr. Roy Golsteyn, and Dr. Sophie Kerneis-Golsteyn for giving me the opportunity of expanding my teaching skills.

I am deeply thankful for the unconditional love and support from my family. I feel blessed to have my parents Joao and Beatriz, my beautiful sister Maria Eduarda, my dearest sister Gisele, my brother-in-law José Roberto, my gorgeous niece Beatriz, my mother-in-law Zenaide, my brother-in-law Alysson, and my sister-in-law Andressa as part of my loving family. Their encouragement, love, and prayers greatly help me through difficult times. Most importantly, I would like to deeply thank my husband and best friend Anderson, for his enduring love, patience, support, and for accompanying me through this and all journeys of life.

v

TABLE OF CONTENTS

Title Page	i
Signature Page	ii
Dedication Page	iii
Abstract	iv
Acknowledgements	V
Table of Contents	vi
List of Tables	ix
List of Figures	X
List of Abbreviations	xii
Introduction	1
Chapter 1. Literature review	3
1.1 Non-human use of antimicrobial agents	3
1.1.1 Use of antimicrobial agents as growth promoters	4
1.1.1.1 Use of antimicrobial growth promoters in beef	5
1.1.1.2 Use of antimicrobial growth promoters in poultry	7
1.1.1.3 Use of antimicrobial growth promoters in swine	7
1.1.2 Antimicrobial resistance	8
1.1.2.1 Mechanisms of antimicrobial resistance	9
1.1.3 Restrictions on the use of antimicrobial growth promoters	9
1.1.3.1 Consequences of the restricted use of antimicrobial growth	
promoters	11
1.2 Morphophysiology of the mammalian gastrointestinal tract	12
1.2.1 Gastrointestinal microbiota	15
1.2.1.1 Methods to characterize the gastrointestinal microbiota	16
1.2.2 Mucosal immunity	19
1.2.2.1 Innate and adaptive immunity	19
1.2.2.2 Host-pathogen interaction	22
1.2.2.3 The acute-phase response	22

1.2.2.4 Regulation of the T-cell response	24
1.2.2.5 Commensal microbiota and immunity	27
1.3 Animal models	
1.3.1 Enteric inflammation models	29
1.3.1.1 Gnotobiotic animals	29
1.3.1.2 Citrobacter rodentium model	
Chapter 2. Hypotheses and objectives	
2.1 Microbiota modulation hypothesis	
2.2 Alternative hypotheses	
2.3 Research hypotheses and objectives	
Chapter 3. Characterization of mucosa-associated bacterial communities of the	murine
intestine by terminal restriction fragment length polymorphism	39
3.1 Introduction	40
3.2 Materials and methods	42
3.3 Results	49
3.4 Discussion	52
Chapter 4. Effect of long-term oral administration of non-therapeutic doses of a	a model
antimicrobial growth promoter on the intestinal microbiota compos	ition of
cattle and mice	58
4.1 Introduction	59
4.2 Materials and methods	61
4.3 Results	68
4.4 Discussion	79
Chapter 5. Non-therapeutic concentrations of orally administered chlortetracyc	line
modulate immune responses to Citrobacter rodentium in mice	86
5.1 Introduction	86
5.2 Materials and methods	89
5.3 Results	98
5.4 Discussion	112
Chapter 6. General discussion and future directions	124
6.1 Characterization of intestinal microbial communities	124

6.2 Variation of intestinal microbiota composition among individuals	125
6.3 General conclusions	126
6.4 Restricted use of antimicrobial growth promoters	127
6.5 Alternatives to antimicrobial growth promoters	127
6.6 Future research	130
6.7 Final conclusions	135
References	136
Appendices	154

LIST OF TABLES

Chapter 1.	
Table 1.1.	Antimicrobial agents registered in Canada for use in animals for growth
	promotion, weight gain and/or feed efficiency, and disease prevention,
	prophylaxis and/or control, along with those registered for human
	therapy
Chapter 3.	
Table 3.1.	Group significance analysis of T-RFLP community profiles of murine
	intestinal samples submitted to different treatments before DNA
	extraction
Chapter 4.	
Table 4.1.	Group significance analysis of T-RFLP community profiles
	(presence/absence data) by pairwise comparisons within cattle intestinal
	communities associated with digesta and mucosal samples72
Table 4.2.	Group significance analysis of T-RFLP community profiles
	(presence/absence data) by global comparisons within murine intestinal
	communities associated with the mucosa77
Chapter 5.	
Table 5.1.	Description of the histological inflammation scoring criteria applied to
	cross-sections of the murine distal colon for this study96
Table 5.2.	Non-parametric statistical analysis of histological inflammation scoring
	performed in cross-sections of the murine distal colon104
Table 5.3.	Group significance analysis of T-RFLP community profiles by global
	and pairwise comparisons

LIST OF FIGURES

Chapter 1.
Figure 1.1. Total administration of AMA in Denmark from 1996 to 200113
Figure 1.2. Tissue organization and structure of the small intestine14
Figure 1.3. A schematic showing the thickness of the firmly adherent and loosely
adherent mucus layer throughout the GI tract of rats
Figure 1.4. Schematic representation of an acute-phase response following
stimulation of the immune system
Chapter 2.
Figure 2.1. Schematic representation of the interactions involved in this project37
Chapter 3.
Figure 3.1. Schematic diagram of the sampling process applied to each
intestinal location (i.e. terminal ileum, largest portion of cecum and
descending colon) of mice in this study, with the purpose of negating
spatial bias44
Figure 3.2. T-RFLP profiles (16S rRNA gene) of mucosa-associated bacterial
communities from the murine intestine before statistical detection of
T-RFs (i.e. no threshold applied)
Figure 3.3. T-RFs of mucosa-associated bacteria from the murine cecum (i.e. no
threshold applied)
Chapter 4.
Figure 4.1. Schematic representation of the experimental units and the treatments
applied in this study
Figure 4.2. Non-metric multi-dimensional scaling applied to presence/absence
T-RFLP data from mucosa and digesta of small and large intestine
cattle
Figure 4.3. Non-metric multi-dimensional scaling applied to presence/absence
T-RFLP profiles from digesta and mucosa of the cattle intestine to
compare the composition of bacterial communities between
treatments71

Figure 4.4	. Non-metric multi-dimensional scaling applied to presence/absence and
	relative abundance T-RFLP profiles to compare bacterial community
	composition between mucosa and digesta from the small and large
	intestine of cattle
Figure 4.5	. Venn diagram illustrating the distribution of unique, shared, and
	common T-RFs among intestinal mucosa and digesta of cattle from
	Control and AS700 treatment
Figure 4.6	. Non-metric multi-dimensional scaling applied to presence/absence
	T-RFLP profiles from mucosa of small and large intestine of mice76
Figure 4.7	. Non-metric multi-dimensional scaling applied to presence/absence
	T-RFLP data from small and large intestine of mice from the Control
	treatment, and of those that received various doses of CTC78
Figure 4.8	. Dendrogram of bacterial community relatedness generated from
	presence/absence T-RFLP data of the intestinal mucosa of mice not
	administered CTC
Chapter 5.	
Figure 5.1	. Quantities of C. rodentium cells in feces and associated with colonic
	mucosa
Figure 5.2	. Change in body weight101
Figure 5.3	. Gross pathology of the murine large intestine (cecum to rectum)103
Figure 5.4	. Microphotographs of the murine distal colon105
Figure 5.5	. Epithelial crypt height106
Figure 5.6	. Relative mRNA expression of cytokines in colonic tissues108
Figure 5.7	. Temporal transcript levels of cytokine mRNA in the distal colon of
	treatment mice
Figure 5.8	. Non-metric multi-dimensional scaling applied to presence/absence
	T-RFLP profiles of mucosa-associated bacterial communities in the
	murine distal colon at days 3, 8, 14, and 21 p.i111
Figure 5.9	. Non-metric multi-dimensional scaling applied to presence/absence
	and relative abundance of T-RFLP profiles of mucosa-associated
	bacterial communities in the murine distal colon

LIST OF ABBREVIATIONS

AAFC	Agriculture and Agri-Food Canada			
ACC	Animal Care Committee			
ACTH	Adrenocorticotropic hormone			
AFMNet	Advanced Foods and Materials Network			
AGP	Antimicrobial growth promoters			
AMA	Antimicrobial agents			
AMR	Antimicrobial resistance			
ANOVA	Analysis of variance			
ASF	Altered Schaedler Flora			
ATCC	American Type Culture Collection			
BMR	Basal metabolic rate			
bp	Base pairs			
CCAC	Canadian Council on Animal Care			
CD	Cluster of differentiation			
cDNA	Complimentary deoxyribonucleic acid			
CFU	Colony-forming units			
CMIB	Compendium of Medicating Ingredient Brochures			
CR	Citrobacter rodentium			
CSF	Colony-stimulating factor			
Ct	Cross-threshold			
CTC	Chlortetracycline			
CXCL	CXC chemokine ligand			
DANMAP	Danish Veterinary Institute			
DGGE	Denaturing gradient gel electrophoresis			
DNA	Deoxyribonucleic acid			
DSS	Dextran sulfate sodium			
EDTA	Ethylenediaminetetraacetic acid			
eNOS	Endothelial nitric oxide synthases			
FDA	Food and Drug Administration			

FOS	Fructo-oligosaccharide			
GALT	Gut-associated lymphoid tissue			
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase			
GI	Gastrointestinal			
НС	Health Canada			
H&E	Hematoxylin and eosin			
HPA	Hypothalamic-pituitary-adrenal			
IFN	Interferon			
Ig	Immunoglobulin			
IL	Interleukin			
iNOS	Inducible nitric oxide synthases			
LB	Luria-Bertani			
LPS	Lipopolysaccharide			
LRC	Lethbridge Research Centre			
MIC	Minimum inhibitory concentration			
MHC	Major Histocompatibility Complex			
MLS _B	Macrolide-lincosamide-streptogramin B			
mRNA	Messenger ribonucleic acid			
MRSA	Methicillin-resistant Staphylococcus aureus			
NAHMS	National Animal Health Monitoring System			
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells			
NGP	Non-antibiotic growth promoters			
NMS	Non-metric multi-dimensional scaling			
nNOS	Neuronal nitric oxide synthases			
NO	Nitric oxide			
NOD	Nucleotide-binding oligomerization domain isoform			
O.D.	Optical Density			
PAMP	Pathogen-associated molecular pattern			
PBS	Phosphate buffer saline			
PCR	Polymerase Chain Reaction			
PRB	Population Reference Bureau			

PRR	Pattern recognition receptors		
rDNA	Ribosomal deoxyribonucleic acid		
RNA	Ribonucleic acid		
rRNA	Ribosomal ribonucleic acid		
SAS	Statistical Analysis Software		
SOC	Super Optimal broth with Catabolite repression		
TBE	Tris-borate-EDTA		
TGF	Transforming growth factor		
TGGE	Thermal gradient gel electrophoresis		
Th	T-helper		
TLR	Toll-like receptor		
ТМСН	Transmissible murine colonic hyperplasia		
TNBS	2,4,6-trinitro benzene sulfonic acid		
TNF	Tumor necrosis factor		
Treg	T-regulatory		
T-REX	T-RFLP analysis Expedited		
T-RFLP	Terminal restriction fragment length polymorphism		
T-RFs	Terminal restriction fragments		
TSA	Tryptic soy agar		
UPGMA	Unweighted pair-group methods with arithmetic means		
VRE	Vancomycin-resistant enterococci		
WHO	World Health Organization		

INTRODUCTION

The global human population will reach 7 billion by 2011, and is projected to increase to 8.1 billion in 2025 (PRB, 2009). This continuous increase translates to a higher demand for high-quality animal products, which resulted in the development of techniques to optimize animal production and maintain animal health. In the last decades, animal production has been intensified by improvements in husbandry, animal reproduction, genetic traits, disease control, and nutrition. Sustainability of animal production has been greatly improved with in-feed administration of non-therapeutic concentrations of antimicrobial agents (AMA), which are known as antimicrobial growth promoters (AGP).

Unfortunately, administration of AGP has contributed to the emergence of antimicrobial resistance (AMR) in zoonotic pathogens (e.g. Inglis *et al.*, 2005), which may compromise the therapeutic use of AMA to treat bacterial infections in humans and animals. From 1986 to 2006, as a consequence of widespread public health concerns regarding AGP use, the European Union gradually banned all AGP, which resulted in production losses (Casewell *et al.*, 2003), and several adverse effects for human and animal health (Castanon, 2007). It is anticipated that the AGP ban will progressively be imposed in other countries. A recent draft guidance issued by the United States Food and Drug Administration (FDA) recommended restrictions that would limit the use of AGP to assure animal health in North America (FDA, 2010). Therefore, identification and development of alternatives to AGP is an urgent issue. However, a lack of knowledge

regarding the exact mode of action of AGP has hampered the development and application of efficacious substitutes to AGP.

The difficulty of demonstrating the mode of action of AGP is attributable to the complexity and numerous interactions within the mammalian gastrointestinal (GI) tract, which harbors more microorganisms than the number of cells in the host (Savage, 1977). That germ-free mice do not exhibit enhanced growth as a result of AGP administration (Coates *et al.*, 1963) has promulgated the belief that modulation of the intestinal microbiota is the primary mode of action of AGP (Dibner and Richards, 2005). However, scientific evidence to support the microbiota modulation hypothesis is lacking.

Many AMA, including those frequently used as AGP, have anti-inflammatory properties (Rubin and Tamaoki, 2005). To our knowledge, whether these properties relate to the growth promoting action of AGP has not been critically tested. Therefore, the overall objective of the current investigations was to further our understanding of the mechanisms of action of AGP by testing an alternate hypothesis. Obtaining this information is fundamental to the development of efficacious alternatives to AGP. Since the current project involved interactions among AGP, the intestinal microbiota, and host immune responses, the first study focused on examining the utility of various strategies to characterize the mucosa-associated microbiota of the murine intestine, using terminal restriction fragment length polymorphism (T-RFLP) analysis. Subsequent studies aimed at evaluating the effect of growth promoting concentrations of orally administered chlortetracycline (a commonly used AGP in North America) on the intestinal microbiota composition of cattle and mice. The final study examined a potential immunomodulatory effect of AGP in mice infected with *Citrobacter rodentium* (an inflammation incitant).

CHAPTER ONE

Literature review

1.1 Non-human use of antimicrobial agents

The term "antibiotic" was originally defined as: "a chemical substance, produced by microorganisms, which has the capacity to inhibit the growth of and even to destroy bacteria and other micro-organisms" (Waksman, 1947). Antimicrobial agents comprise all drugs that kill or inhibit the growth of microorganisms, such as bacteria, viruses, fungi, and protozoa. The use of AMA in food animals, companion animals, aquaculture, and horticulture is divided by the World Health Organization (WHO) into four categories: therapeutic, prophylactic, metaphylactic, and growth promoting (WHO, 2003). Therapeutic antimicrobial use is the treatment of established infections. Metaphylaxis is a term used for group-medication procedures, intended to treat sick animals, while medicating others in the group to prevent disease. Prophylaxis is defined as the preventative use of AMA in either individuals or groups to avoid the development of infections. Therapeutic, metaphylactic, and prophylactic use of AMA involve administration to the animals by various routes (e.g. orally or intramuscularly) at therapeutic concentrations for short intervals. An AMA is a growth promoter when administered in/on the feed of food animals to promote growth and enhance feed efficiency. Growth promoters are usually administered in relatively low concentrations, ranging from 2.5 to 125 mg kg⁻¹ (ppm), depending on the drug and animal species treated. In many countries, these low concentrations are used for growth promotion as well as for prophylaxis, making the distinction between prophylaxis and growth

promotion less clear than between prophylaxis and therapy. "Sub-therapeutic" doses of AMA are commonly referred to as drug concentrations that are usually lower than therapeutic concentrations. Historically, in the United States, the term "sub-therapeutic" was defined by FDA as the administration of doses less than or equal to 200 g per ton of animal feed for 2 weeks or longer (FDA, 1980). According to the WHO, this term is no longer used, since it does not consider animal species or drug potencies (WHO, 2003). The term "non-therapeutic" is used as a general term for AMA added in low doses to feed or water of healthy animals (Mellon *et al.*, 2001).

1.1.1 Use of antimicrobial agents as growth promoters

Healthy animals are administered AGP to increase weight gain per unit of feed consumed. In other words, AGP are used to improve feed efficiency, making the production system more profitable for the producer. Indications of the beneficial effect of AGP on host health and nutrition were reported as early as 1946 (Moore *et al.*, 1946). The use of AMA as feed additives for farm animals without prescription was first approved by the FDA in 1951 (Jones and Ricke, 2003), and since then, the use of AGP in livestock has become a common practice around the world. It is noteworthy that approximately 90% of the AMA used in agriculture are administered as growth promoters and as prophylactic agents, and recommended concentrations for growth promotion have increased by 10- to 20-fold since the 1950s (Khachatourians, 1998). Quantities of AMA administered annually to Canadian livestock for non-therapeutic purposes are largely uncertain, since only a few surveys of AMA treatment practices have been conducted (Dunlop *et al.*, 1998; Fraser *et al.*, 2004). However, in the United States,

where animal production and treatment practices are somewhat similar to Canada, total non-therapeutic AMA administered to cattle, poultry and swine in the late 1990s was estimated at 11.1 million kg of AMA, representing eight times more AMA used in animal production than in human medicine (Mellon *et al.*, 2001). From these estimates, the authors also stated that the use of AMA in livestock increased by approximately 50% since 1985, showing that previous assessments of the total non-therapeutic use of AMA were greatly underestimated (Mellon *et al.*, 2001). In Canada, many AMA registered as AGP are also registered for use in human medicine (Table 1.1).

1.1.1.1 Use of antimicrobial growth promoters in beef

Typically, beef cattle are raised on pasture, weaned at 7 months of age, shipped to backgrounding farms, and eventually to feedlots where they are confined in large groups and fed high-energy rations (McEwen and Fedorka-Cray, 2002). Various AMA are used in beef production to reduce pneumonia and diarrhea in calves, to control liver abscesses, to prevent or treat respiratory outbreaks, and to accelerate growth (McEwen and Fedorka-Cray, 2002). According to the United States National Animal Health Monitoring System (NAHMS), 83.2% of feedlots administered at least one type of AGP to cattle in feed or water for prophylaxis or growth promotion in 1999 (NAHMS, 1999). Chlortetracycline (CTC) was administered at 51.9%, CTC-sulfamethazine in combination at 16.8%, oxytetracycline at 19.3%, and tylosin at 20.3% of feedlots; in addition, tetracyclines were fed 4-12 days on average, whereas tylosin was fed 138-145 days, depending on the weight of the animal at the arrival at the feedlot (NAHMS, 1999).

Registered in animal species*			
Antimicrobial agent	Growth promotion,	Disease prevention,	Drugs in the same class registered for human
class and drug	weight gain and/or	prophylaxis, and/or	brugs in the same class registered for human
	feed efficiency	control	therapy
Aminoglycosides			
Neomycin	С	C, Sw, Ch, T, Sh, H, D, M	Streptomycin, Neomycin, Gentamicin, Amikacin
Gentamicin		Ch, T	
Spectinomicin		Sw, Br, Brl	
Streptomycin		Pi	
Cephalosporins			
Ceftiofur			Ceftriaxone, Cefadroxil, Cefaclor, Cefepime,
		т	Cefixime, Cefotaxime, Cefotetan, Cefoxitin,
		1	Cefprozil, Ceftazidime, Ceftriaxone,
			Cefuroxime, Cephalexin, Cephalothin
Lincosamides			
Lincomycin	Dr	Sw Pr T Du C	Clindamuain Linaamuain hudraahlarida
hydrochloride	DI	Sw, Ы, 1, Du, G	Chindamychi, Lincomychi hydrochionde
Macrolides			
Erythromycin	Br, Brl	Sw, Pi, Ch, T, Sh	En throws in Arithmania
Tylosin	Sw	C, Sw, Ch	Erythromycin, Azithromycin
Nitrofurans			
Nitrofurazone		C. Sw. Ch. Sh. Go. H	Nitrofurantoin
Penicillins		- , , - , - , ,	
Penicillin G			
potassium	Br, Brl, T	Т	Ampicillin, Sulbactam, Cloxacillin sodium,
Penicillin G			Penicillin G benzathine, Penicillin G
procaine	Sw, Ch, I	C, Sw, 1, Sh	potassium, Piperaciilin, Ticarciilin
Streptogramins			
Virginiamycin		Sw, Brl	Quinupristin, dalfopristin
Tetracyclines		,	
Chlortetracycline	C Sw Br L T Sh	C Sw Ch T Sh	
Oxvtetracvcline	C Sw Ch T Sh	C Sw T Ch Be	Tetracycline hydrochloride, Doxycycline
Pleuromutilins	0, 511, 01, 1, 51	0, 011, 1, 011, 20	
Tiamulin		Sw	
Sulfonamidas		5₩	
Sulfaquanidina		C Sw Sh U	
Sulfamethazine	C Sw	C, Sw, Sh, H	Sulfamethovazola
Jononhores	C, 5w	C, 5w, 5ll, 11	Sunamethoxazole
Ionophores	0	01	
	C	Ch	
Maduramicin	c.	Ch, T	
Monensin	C	C, Ch, T	
Narasın	Sw	Ch	
Salinomycin	C, Sw	Ch	
sodium	,		
Miscellaneous	~		
Arsanilic acid	Sw, Brl, T	~ -	
Bacıtracın	C, Sw, Ch, T	Sw, Br	Bacitracin
Bambermycins	Brl, T		
Carbadox	Sw	Sw	

Table 1.1. Antimicrobial agents registered in Canada for use in animals for growth promotion, weight gain and/or feed efficiency, and disease prevention, prophylaxis and/or control, along with those registered for human therapy

* C = cattle, Sw = swine, Pi = piglets, Ch = chicken (Br = breeder, Brl = broiler, L = layer), T = turkey, Du = duck, G = geese, Sh = sheep, Go = goat, H = horse, D = dog, M = mink, Be = bees. Source: Health Canada (HC, 2002)

1.1.1.2 Use of antimicrobial growth promoters in poultry

Broilers and turkeys are raised in barns with thousands of birds, and many rations contain AMA to prevent coccidiosis, as well as to enhance feed efficiency; nearly all medications are administered to entire poultry flocks through feed or water (HC, 2002). Bacitracin, one of the most commonly used AMA in starter and grower broiler feeds, is used for growth promotion and to control necrotic enteritis caused by *Clostridium perfringens* (McEwen and Fedorka-Cray, 2002). In 2000, AMA were used in starter, grower, and final withdrawal feeds by 64.8, 66.9, and 48.1% of broiler production units in the United States, respectively (Chapman and Johnson, 2002).

1.1.1.3 Use of antimicrobial growth promoters in swine

A substantial amount of AMA is used in swine production for prophylaxis and growth promotion in North America. In 2006, the NAHMS reported that 43.2, 31.1, and 25.9% of nursery pig sites administered CTC, carbadox, and tiamulin, respectively, via feed (NAHMS, 2006). This report further revealed that 52.6, 44.2, and 29.1% of grower/finisher sites administered CTC, tylosin, and bacitracin via feed (NAHMS, 2006). A survey made in Ontario revealed that out of 630 operations, 86% added AMA to starter (i.e. weanling pig) rations, whereas 29% added AMA to finisher pig rations; the most common AMA used were tylosin, carbadox, and furazolidone in weanling pigs, and tylosin, lincomycin and tetracycline in finishers (Dunlop *et al.*, 1998).

1.1.2 Antimicrobial resistance

Regardless of the contribution of AMA to animal performance, nutrition and health, their use as growth promoters has been increasingly scrutinized due to concerns regarding selection for AMR and potential transmission of resistant strains to humans. The misuse and overuse of AMA in human medicine, agriculture, and animal production is thought by many to be a primary reason for the development of AMR (Conly, 2002), which hampers the ability to treat diseases in humans and animals.

That many food animals are important reservoirs of several human bacterial pathogens highlights the importance of AMR. In Canada, the most important human pathogens isolated from food animals include *Campylobacter jejuni* (Rudi *et al.*, 2004), Salmonella enterica (Perron et al., 2008), and Escherichia coli (Pruimboom-Brees, 2000). Bacterial microorganisms with increasing rates of resistance to commonly used AMA include methicillin-resistant Staphylococcus aureus (MRSA; Smith et al., 2009), vancomycin-resistant enterococci (VRE; Emborg et al., 2003), Shigella spp. (Ahmed et al., 2006) and Salmonella spp. (Perron et al., 2008) resistant to multiple AMA, enteric Gram-negative bacilli (Klebisiella and Enterobacter species) resistant to extendedspectrum β -lactams (Emborg *et al.*, 2003; French *et al.*, 1996), penicillin-resistant Streptococcus pneumoniae (Goldstein, 1999), Campylobacter spp. and Escherichia coli O157:H7 resistant to multiple AMA (Meng et al., 1998; Miflin et al., 2007). The emergence of resistance in bacteria that are typically non-pathogenic (e.g. commensal bacteria within the GI tract) to humans is also a concern to public health, since these bacteria provide a pool of resistance genes that can be transferred to human pathogens (HC, 2002).

1.1.2.1 Mechanisms of antimicrobial resistance

There are four main mechanisms of resistance to AMA that evolve in a threatened microbial population: (i) acquisition of genes with resistance determinants, such as β lactamases that destroy AMA before it can impart an effect; (ii) acquisition of efflux pumps that extrude the AMA from the cell; (iii) acquisition of several genes for a metabolic pathway which ultimately produces altered bacterial cell walls that no longer contain the binding site of the AMA; and (iv) acquisition of mutations that limit access of AMA to the intracellular target site, via downregulation of porin genes (Davies, 1994; Hughes and Datta, 1983; Tenover, 2006; Wood et al., 1996). Enzymatic inactivation of AMA is one of the most common biochemical processes that generate resistance in a wide variety of bacteria; genes encoding enzymes that catalyze covalent modifications of AMA can undergo mutations that remodel the active site of the enzyme, changing the spectrum of antimicrobial substrates that may be modified (Davies, 1994). Acquisition of genetic material from other resistant bacteria (i.e. "horizontal evolution") is accomplished through conjugation, transduction, or transformation, and may occur between strains of the same species or between different bacterial species or genera (Tenover, 2006). Transposons may facilitate the incorporation of multiple resistance genes into the host's genome or plasmids (Tenover, 2006).

1.1.3 Restrictions on the use of antimicrobial growth promoters

Management options aimed at minimizing adverse human health consequences due to AMR have primarily focused on decreasing antimicrobial selection pressure in animals. The WHO provided recommendations to reduce the overuse and misuse of

AMA in food animals to protect human health, with the overall objective: "*To minimize the negative impact of the use of antimicrobial agents in food-producing animals whilst at the same time providing for their safe and effective use in veterinary medicine*" (WHO, 2000). These recommendations included prohibiting the growth-promoting administration of AMA used in human medicine. Subsequent WHO recommendations include: (i) to avoid group medication added to animal feed or water whenever possible; (ii) to restrict the use of antimicrobials for prescription only; (iii) to ban AGP (WHO, 2003); and (iv) to implement risk-assessment studies and establish surveillance programs to monitor AGP use and AMR in bacteria from food animals (WHO, 2004).

In 1986, Sweden banned the use of AGP; this was followed by the ban of avoparcin and virginiamycin in Denmark between 1995 and 1998. Subsequently, the European Union banned the use of avoparcin in 1997, and the four remaining AMA used for growth promotion (i.e. bacitracin, spiramycin and tylosin, and virginiamycin) in 1999, on the basis of the "Precautionary Principle" (Casewell *et al.*, 2003). In 2006, the European Union instituted a ban on all AMA and hormones used for animal growth promotion, and is expected to prohibit the use of anticoccidial substances as feed additives before 2013 (Castanon, 2007). This will limit the use of medical substances in animal production to veterinary prescription only. In Canada, the recommendations of restricted use of AGP from the 2002 Health Canada's *Report of the Advisory Committee on Animal Uses of Antimicrobials and Impact on Resistance and Human Health* (HC, 2002) have not been fully implemented, nearly 7 years after publication of the report (Holtz, 2009). Many AMA used in human therapy are still used in livestock for growth promotion and prophylaxis (Table 1.1), and are listed in the Compendium of Medicating Ingredient Brochures (CMIB); the CMIB lists medicated ingredients permitted in Canada to be added to livestock, and specifies directions for use and dosage (CMIB, 2008). Oxytetracycline, CTC, and the combination product of CTC/sulfamethazine/penicillin deserve special attention, due to their large number of claims at CMIB and their clinical importance in human medicine (HC, 2002).

1.1.3.1 Consequences of the restricted use of antimicrobial growth promoters

Despite the implementation of strategies designed to compensate for the lack of AMA usage, the ban of AGP in the European Union has had a profound impact on animal and human health (Casewell et al., 2003), although the economic implications of the AGP ban are still subject to debate (Graham et al., 2007). The AGP ban in the European Union resulted in reduced average weight gain, and increased prevalence of diarrhea, enteritis, and mortality in early post-weaning pigs and broilers (Casewell et al., 2003). Regarding human infections, there was some decrease in vancomycin resistance in enterococci isolated from asymptomatic human carriers, but the prevalence of enterococci infection did not decrease (Casewell et al., 2003). Despite the lower incidence of AMR in some pathogens, resistance level in *Salmonella* spp. and *Campylobacter* spp. (major zoonotic pathogens in Europe) was not reduced, likely because the AMA banned primarily have a Gram-positive spectrum of activity (Casewell et al., 2003). Furthermore, an increase in human infection by vancomycin-resistant staphylococci was reported and related to a higher usage of vancomycin to treat methicillin-resistant staphylococci (Casewell et al., 2003). The increasing rate of health problems in livestock due to the AGP ban in Denmark have led to higher therapeutic use

of AMA (Figure 1.1). Although the AGP ban in Denmark decreased the occurrence of AMR in enterococci isolated from human and animal feces (Aarestrup *et al.*, 2001; Casewell *et al.*, 2003), it is not known to what extent the increase of therapeutic use of AMA will contribute to the emergence of AMR.

The economic implications of an AGP ban remain controversial. Based on Swedish and Danish experiences, it was estimated that an AGP ban would increase the cost of production by \$700 million in the United States pork industry over a 10 year period (Jensen and Hayes, 2003). Conversely, an economic analysis of the United States poultry industry illustrated that the benefits associated with AGP use are not sufficient to compensate the costs of the AMA (Graham *et al.*, 2007). This analysis, however, did not include veterinary cost changes due to an AGP ban (Graham *et al.*, 2007).

1.2 Morphophysiology of the mammalian gastrointestinal tract

The lumen of the mammalian GI tract is lined with a thin layer of epithelial cells with a large surface area (e.g. over 400 m² in humans), forming with the complex indigenous microbiota, a barrier to protect the host against pathogens (MacDonald and Monteleone, 2005). Mucosa, which includes the epithelium and lamina propria (i.e. thin layer of connective tissue that underlies the epithelium) collectively, along with submucosa, muscularis, and serosa, comprise a series of functional layers of the GI tract (Mueller and Macpherson, 2006). As a consequence of constant contact with microorganisms, the GI mucosa has evolved multiple layers of protection (Figure 1.2). Epithelial cells are joined by tight junctions, which exclude large molecules and microorganisms from contact with immune cells in the lamina propria (Kelsall, 2008).



Figure 1.1. Total administration of AMA in Denmark from 1996 to 2001 (Metric Tons of active ingredients). Source: Iowa Ag Review Online. Adapted from Danish Veterinary Institute - DANMAP 2002; Jensen and Hayes, 2003).



Figure 1.2. Tissue organization and structure of the small intestine. Adapted from Tortora and Derrickson, (2006).

The small intestine consists of proximal, central, and distal areas, designated the duodenum, jejunum, and ileum, respectively (Kelsall, 2008). The primary function of the small intestine is nutrient absorption (Kelsall, 2008). The initial section of the large intestine is the cecum, followed by the colon and rectum. The primary function of the large intestine is absorption of water and electrolytes (Turnberg, 1970). Additionally, bacteria present in the large intestine may produce biologically active metabolites from dietary components (e.g. short-chain fatty acids) which are beneficial to the nutrition and health of the host (Davis and Milner, 2009).

1.2.1 Gastrointestinal microbiota

The human GI microbiota represents a highly diverse and complex community, composed of up to 10^{14} cells (Ley *et al.*, 2006), representing more than 500 distinct bacterial species (Eckburg *et al.*, 2005). Two controlling factors have roles in the complexity of microbial communities and associated host-functions within the GI tract: bacteria-bacteria interactions and bacteria-host interactions. The functional adaptability of the GI microbial community and host selection factors for specific bacteria are fundamental to the stability of the microbiota, which contributes to the host's health and nutrition (Backhed *et al.*, 2005). Essential attributes that the host has not evolved on its own and that are provided by the GI microbiota include: physiological transformations to enhance uptake of nutrients, establishment and maintenance of immune homeostasis, prevention of diseases, modulating the immune response, and eliminating opportunistic pathogens from the GI tract by competitive exclusion (Backhed *et al.*, 2005). The most evident physiologic and morphologic differences between germ-free (i.e. "axenic") and

conventional mice are manifested in the GI tract, and include cecal enlargement, and loss of connective tissue and small vessels in the lamina propria in germ-free mice (Coates, 1975). A decrease in blood flow to organs that would normally have a close contact to microorganisms (e.g. skin, liver, digestive tract), and reduced cardiac output have also been reported in germ-free mice (Coates, 1975; Gordon and Pesti, 1971). Reduced cardiac output was attributed to the fact that, in conventional mice, there are stimulatory effects of the microorganisms on the chemical energy and oxygen requirements of organs they are in contact with. Therefore, the weights of organs that harbour microorganisms in conventional animals, as well as heart weight and blood flow, are reduced in germ-free mice. The growth of germ-free animals is consistently enhanced, probably due to more efficient digestion and absorption of food, attributed to the lack of microorganisms that compete for nutrients (Coates, 1975). Conversely, the defense systems of germ-free animals are less developed (Coates, 1975).

1.2.1.1 Methods to characterize the gastrointestinal microbiota

The study of GI microbial ecology involves assessments of abundance and diversity of microorganisms, their activity and their relationship with each other and with the animal host (Zoetendal *et al.*, 2004). Although the application of anaerobic cultivation techniques contributed to the identification of numerous taxa, it was the development of molecular-based techniques that expressively expanded our knowledge of bacterial community structure and function, and shed light on the richness and complexity of the GI microbiota (Weng *et al.*, 2006).

The 16S rRNA gene is widely used for phylogenetic studies, since it is universally distributed, and includes both conserved regions among various species of bacteria (Weisburg *et al.*, 1991), and variable regions that can be taxon specific (Stackebrandt and Goebel, 1994). However, sequence analysis of clone libraries containing the near complete 16S rRNA gene (i.e. \approx 1500 bp; Sanger sequencing) are impractical for studies involving large number of samples containing diverse microbial communities. Pyrosequencing methods enable direct sequencing of the target gene (i.e. without cloning), and are faster than the Sanger method; however, sequence reads with pyrosequencing are currently restricted to 450 bp or less (Ronaghi, 2001). Despite the success of sequencing methods to explore microbial diversity and to identify species in microbial communities, fingerprinting methods, such as denaturing/thermal gradient gel electrophoresis (DGGE/TGGE; Muyzer and Smalla, 1998), and T-RFLP (Liu et al., 1997) enable the comparative assessment of community diversity and composition, which is an important goal in microbial ecology. The T-RFLP technique comprises the isolation of total community DNA and amplification of 16S rRNA genes with universal or domain-specific primers, at least one of which is fluorescently labelled; this is followed by a restriction enzyme digestion and the determination of terminal restriction fragments (T-RFs) with the use of automated genetic analyzers (Liu et al., 1997). Fingerprinting profiles in T-RFLP analysis largely depend on the restriction enzyme used, and HaeIII has been effective in T-RFLP analyses of fungi (Edwards and Turco, 2005), archaea (Moeseneder et al., 2001), and bacteria (Zhang et al., 2008). The use of more restriction digests in independent reactions improves species resolution. However, it has not been determined whether the use of multiple enzymes in a single reaction

improves species resolution, especially in highly complex communities. Despite the use of T-RFLP technique for species identification through the distinctive restriction site position in each taxon (i.e. T-RF length), there has been limitations in accurately predicting microbial community composition due to: (i) inconsistencies between true T-RF length and observed T-RF length when using primers labelled with different fluorescent dyes (Pandey *et al.*, 2007), (ii) shared T-RFs across species, and (iii) multiple T-RFs within the same species (Dickie and FitzJohn, 2007). Nevertheless, T-RFLP has been considered reliable for comparative microbial community analysis (Hartmann and Widmer, 2008). It has been reported that similar fingerprint profiles of bacterial diversity in soils were obtained by DGGE and T-RFLP techniques (Smalla *et al.*, 2007). Conversely, assessment of bacterial diversity in a marine environment revealed that more phylogenetic groups were observed using T-RFLP and clone library analysis than using DGGE, which usually produced fewer operational taxonomic units (Zhang *et al.*, 2008).

One of the limitations of PCR-based technologies is potential PCR bias, which may substantially affect genetic profiles and analyses. Artifacts generated in PCR reactions can originate from chimeric sequences, nucleotide misincorporation, heteroduplexes (Qiu *et al.*, 2001), partially single-stranded DNA (Egert and Friedrich, 2005), residual activity of DNA polymerases (Hartmann *et al.*, 2007), and differential copy number of the target gene among taxa (Klappenbach *et al.*, 2001). Nevertheless, advancements in limiting PCR-related bias (i.e. treatment of partially single-stranded amplicons with Klenow fragment; Egert and Friedrich, 2005), and in statistical analyses of genetic profiles (Abdo *et al.*, 2006; Culman *et al.*, 2008) have resulted in more reliable and accurate characterization of microbial communities.

1.2.2 Mucosal immunity

The mammalian GI tract is one of the largest immunological organs of the body, containing more lymphocytes and plasma cells than the spleen, bone marrow, and lymph nodes combined (Stokes and Bailey, 2000). The gut-associated lymphoid tissue (GALT) contains cells organized within the lymphoid follicles of the Peyer's patches, as well as those distributed throughout the lamina propria and intestinal epithelium (Stokes and Bailey, 2000). The mucus layers overlying the intestinal epithelium are fundamental for the structure and functional stability of the microbiota, which facilitates nutrient exchange and induces host innate immunity (Sonnenburg et al., 2004), and are variable in thickness along the GI tract (Figure 1.3). The inner mucus layer (i.e. firmly adherent layer) of the murine colon is formed by a dense concentration of mucins, which serve as a functional physical barrier to reduce the direct contact of resident bacteria with the underlining intestinal epithelium (Johansson et al., 2008). Conversely, there are substantial amounts of bacteria present in the outer loose mucus layer of the murine colon, which seems to provide an ideal environment for resident microbial communities (i.e. mucosa-associated; Johansson *et al.*, 2008). It is now recognized that mucosaassociated microbial communities differ from those present in digesta or feces (Eckburg et al., 2005; Zoetendal et al., 2002).

1.2.2.1 Innate and adaptive immunity

The mucus and epithelial barriers do not completely prevent luminal antigens from entering underlying intestinal tissues. Innate mechanisms of defense, which occur naturally as a result of the genetic constitution and microbial environment within the GI



Figure 1.3. A schematic showing the thickness of the firmly adherent and loosely adherent mucus layer throughout the GI tract of rats. In the stomach and colon, the firmly adherent layer was continuous, but in the small intestine it had a "patchy" distribution and was absent on individual villi. The loosely adherent mucus layer was removable by careful suction, whereas the firmly adherent mucus layer was not. Values on the x-axis represent mean (standard errors) thickness values at each location. Figure recreated from Atuma *et al.* (2001).

tract, regulate intestinal homeostasis and contribute to the control of inflammatory reactions in the lamina propria (Moal and Servin, 2006). As indicated above, the surface of intestinal epithelial cells is covered by a well-developed layer of membrane-bound glycoproteins and mucus that effectively prevent bacterial attachment (Kelsall, 2008). Intestinal epithelial cells modulate mucosal and adaptive immune responses by secreting products that activate or inhibit immune cells (Rumbo and Schiffrin, 2005). Inflammation is a biological response of tissues to harmful stimuli, such as pathogens, damaged cells or irritants, protecting the host by removing the injurious stimuli and initiating the healing process for the tissue (Kelsall, 2008). Both innate and adaptive (acquired) immune responses are involved in clearing bacteria from the lamina propria.

Synthesis of antimicrobial peptides, pro-inflammatory cytokines, chemokines, and secondary anti-inflammatory responses, are part of innate immune responses (Thoma-Uszynski *et al.*, 2001). Adaptive immune responses, which are long-lasting and specific cellular responses to antigens, contribute to innate responses by limiting exposure of the mucosal immune system to commensal bacteria (Kelsall, 2008). Adaptive immune responses are divided into humoral immunity, which comprise antibodies, secreted by B lymphocytes and circulating in the blood, and cell-mediated immunity, which comprise T lymphocytes that are able to either directly destroy infected cells or activate phagocytes to kill pathogens (Dempsey *et al.*, 2003). An effective overall immune response depends on the interplay and regulation between the innate and adaptive immune systems. It is accepted that innate immune signals play a critical role in initiating and instructing the development of adaptive effector mechanisms, despite the independent evolution of these two classes of immunity (Dempsey *et al.*, 2003). The intersection between innate and

adaptive responses is intricate and finely balanced, and depends on the accurate and effective translation of the innate signals of infection or damage.

1.2.2.2 Host-pathogen interaction

Microorganisms can be recognized by intestinal epithelial cells when they penetrate mucins and resist host's antimicrobial peptides. Innate immunity is initiated by germ-line encoded pattern recognition receptors (PRRs) that recognize conserved molecular patterns (i.e. pathogen-associated molecular patterns, PAMPs) of microorganisms (Uematsu and Akira, 2006). Toll-like receptors (TLR) and nucleotide-binding oligomerisation domain isoforms (NOD)-like receptors are PRRs that detect a wide range of microorganisms that may be associated with mucosal surfaces (e.g. bacteria, fungi, protozoa, and viruses; Uematsu and Akira, 2006). Recognition of PAMPs by PRRs triggers the activation of signal transduction pathways, which induce dendritic cell maturation and cytokine production, resulting in an adaptive immune response that can eliminate pathogens and shape the intestinal microbiota (Akira *et al.*, 2006; Kelly *et al.*, 2005).

1.2.2.3 Acute-phase response

When an immune response is triggered by a microorganism, several proinflammatory cytokines, such as interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)- α are released by macrophages and monocytes, mediating a systemic stress response known as the acute-phase response (Klasing and Korver, 1997). Cytokines are polypeptidic factors produced by a wide variety of cells, including leukocytes (e.g.

monocytes and lymphocytes); some can be considered mediators of immune-neuroendocrine interactions, modulating the length, intensity, and type of immune response (Besedovsky and del Rey, 1996). A reciprocal expression of receptors for products of the nervous, endocrine, and immune systems, and the extensive neural supply to immune tissues, such as the spleen and lymph nodes, constitutes the basis of immune-neuroendocrine interactions (Besedovsky and del Rey, 1996). Innate immune responses at systemic and localized levels are generally inhibited by the neuro-endocrine stress response and the sympathetic and parasympathetic nervous system, whereas local innate immune responses tend to be enhanced by the peripheral immune system (Sternberg, 2006).

The hypothalamic-pituitary-adrenal (HPA) axis provides a physiological feedback loop on inflammation (Sternberg, 2006). In response to antigenic stimuli, cells from the paraventricular nucleus of the hypothalamus produce corticotrophin-releasing hormone, which stimulates leukocytes (Weigent and Blalock, 1995) and the pituitary gland to release adrenocorticotropic hormone (ACTH) into the blood; substantial amounts of corticosterone are released from the adrenal glands, inhibiting protein synthesis of skeletal muscles. Cytokines can systemically limit growth by direct action on tissues, or indirectly through their effects on the endocrine (Klasing and Korver, 1997) and nervous systems (Sternberg, 2006). They induce several metabolic, cellular and behavioral alterations, such as anorexia, increased basal metabolic rate (BMR), and protein turnover, contributing to the development of fever and to an increase in the rate of feed conversion (i.e. feed mass necessary to be converted to 1 kg of body mass) and degradation of skeletal muscle protein (Klasing and Korver, 1997). The amino acids made available due
to decreased rates of muscle accretion are subsequently utilized by the liver for the synthesis of acute-phase proteins, which enhance innate immunity and modulate cytokine activity (Klasing and Korver, 1997). Hepatic glycogenolysis is induced by IL-1 β , and the glucose released is used by muscles, representing a shift away from fatty acids and toward carbohydrates as a primary energy substrate by some tissues (Sakurai *et al.*, 1994). The changes in metabolism following an inflammatory response by the immune system (Figure 1.4) have been referred to as "immunologic stress" (Klasing *et al.*, 1987). Immunologic stress is defined as a response that modifies the partitioning of dietary nutrients away from growth and skeletal muscle accretion, and towards metabolic processes that support the immune response and disease resistance (Roura *et al.*, 1992). Hepatic acute-phase response is an anabolic process that increases nutrient use by the immune system (e.g. up to 6-fold in case of lysine uptake; Klasing, 2007).

1.2.2.4 Regulation of the T-cell response

Dendritic cells belong to a heterogeneous population of cells present in an immature state within the GI mucosa and skin, and along with macrophages, play a critical role in communicating innate signals of infection or damage to the adaptive immune system (Dempsey *et al.*, 2003). Central to T-cell activation, is the recognition of antigens in the peptide-binding groove of major histocompatibility complex (MHC) molecules, which are expressed by antigen-presenting cells, such as macrophages and dendritic cells (Vyas *et al.*, 2008). Maturation signals obtained from the activation of the innate immune system produce varied profiles of cytokines and costimulatory receptors, and determine the type of adaptive immune response mounted by the host.



Figure 1.4. Schematic representation of an acute-phase response following stimulation of the immune system. Cytokines IL-1, IL-6, and TNF- α orchestrate this response by direct actions on tissues (bold arrows) and by indirect actions (dashed arrows) through the endocrine system, resulting in metabolic, behavioral and cellular modifications that limit growth and skeletal muscle deposition. Adapted from Klasing and Korver (1997), and Niewold (2007).

T-helper (Th)1 cells produce predominantly IFN-γ, IL-2, and TNF-α, and facilitate cell-mediated immunity, phagocyte-dependent inflammation, and favor production of immunoglobulin (Ig)G1 and IgG3 opsonizing and complement-fixing antibodies (Dempsey *et al.*, 2003). Th2 cells produce IL-4, induce IgE production by B cells, evoke strong antibody responses, induce eosinophil accumulation, and inhibit several functions of phagocytic cells (i.e. phagocyte-independent inflammation; Dempsey *et al.*, 2003; Romagnani, 2000). It has been shown that Th1 and Th2 are mutually regulated. For instance, IL-12, IFN-γ and IFN-α favor the development of Th1 cells, but inhibit development of Th2 cells, whereas IL-4 favor development of Th2 cells, but inhibit development of Th1 cells (Parronchi *et al.*, 1992). T-regulatory (Treg) cells regulate both Th1 and Th2 responses, and include Th3 cells that exert their suppressive activity by the production of TGF-β (Weiner, 2001). In addition, type 1 Treg cells (Tr1) are induced in the presence of IL-10 and exert their suppressive activity by producing IL-10 and TGF-β (Roncarolo *et al.*, 2001).

More recently, a distinct subset of effector Th cells, named Th17 cells, was described, and are thought to be involved in enhancing antimicrobial host protection against mucosal and epithelial extracellular bacteria and some fungi in support of Th1and Th2-mediated immunity (Weaver *et al.*, 2007). Furthermore, Th17 cells have been shown to be involved in the pathogenesis of some inflammatory and auto-immune disorders (van de Veerdonk *et al.*, 2009). Th17 cells are thought to originate by the combined activity of IL-6 and TGF- β , and by the induction of IL-23 produced by dendritic cells (Romagnani, 2006). The cytokines produced by Th17 cells, such as IL-17A and IL-22, evoke inflammation by stimulating fibroblasts, endothelial cells,

epithelial cells, and macrophages to produce chemokines, as well as granulocyte colonystimulating factor (CSF) and granulocyte-macrophage CSF, which induces the recruitment of polymorphonuclear leucocytes (Ye *et al.*, 2001). Whereas IL-4 and IFN- γ inhibit Th17 responses, it is not clear if Th17 cells exert inhibitory effects on development of Th1 and Th2 cells, and the precise effects of Treg cells on Th17 cells are yet unknown (Dempsey *et al.*, 2003).

1.2.2.5 Commensal microbiota and immunity

Microbial communities associated with mucosal surfaces are thought to be remarkably stable throughout the GI tract (Savage, 1987). The stability of the intestinal microbiota is probably required to maintain host homeostasis (Schiffrin and Blum, 2002). Nevertheless, the GI microbiota can be constantly shaped by both host (e.g. secretory substances and peristalsis) and environmental factors (e.g. dietary components, drugs, food structure, and ingestion of microorganisms; Egert *et al.*, 2006), which can profoundly affect host health. A disruption in the ecological balance of the GI microbiota may be detrimental to the host, since the microbiota is thought to provide protection against colonization by pathogenic microorganisms (i.e. colonization resistance; Vollaard and Clasener, 1994).

The close and intermittent contact of the GI mucosa with the microbiota results in a constant state of "controlled" physiological inflammation (Biancone *et al.*, 2002; Ma *et al.*, 2003). Despite the crucial role in maintaining a protective and readily-generated local immune response, the controlled inflammation in the mucosa of the GI tract is also associated with a catabolic cost to the host (Klasing and Korver, 1997).

Immunoregulatory molecules released by activated immunocytes in the GI tract ensure maintenance of an adequate balance between luminal antigens, including resident microorganisms, and the host immune response (Biancone *et al.*, 2002). The "normal" indigenous microbiota does not typically exert a strong intestinal epithelial response, but may contribute to host-immune modulation (Schiffrin and Blum, 2002). Pathogen recognition by immune cells is well known; however, the effect of TLR and NOD on the recognition of commensal microbiota is relatively unexplored (Kelly *et al.*, 2005). It is well established, however, that resident microorganisms do interact with antigenpresenting cells (e.g. dendritic cells) in the lamina propria and/or actually cross the intestinal epithelial barrier, without causing inflammation (Strober, 2006). Local tolerance to commensal microbiota is achieved by the active suppression of T-cell response by Treg cells (Kelly *et al.*, 2005).

1.3 Animal models

Animal models are used in experimental medicine to unravel mechanisms of pathogenesis, and to facilitate development of therapeutic and prophylactic methods to mitigate or eradicate disease in humans and animals. The complexity of mammalian biological systems, combined with the ethical limitation of conducting scientific experiments in humans, have necessitated the development of suitable animal models to study host-pathogen interactions. Rodents have many advantages as animal models, including ease of rearing and tractability, they possess a short life span, there are many defined genotypes available, and their microbiota can be readily eliminated or defined.

1.3.1 Enteric inflammation models

In the mammalian GI tract, the association among the microbiota, epithelial cells, and mucosal immune cells is essential for the health of the host; it is not possible to study such a complex and dynamic interaction *in vitro*. Many animal models have utilized induced mutations or genetic alterations that modify components of the GI tract (i.e. microbiota, mucosal immune cells, and/or epithelial cells), with the goal of elucidating factors that contribute to disease (Lorenz *et al.*, 2005).

1.3.1.1 Gnotobiotic animals

The absolute complexity, richness, and potential variation of microbial communities within the GI tract have greatly hampered our understanding of the host-microbiota interaction. To circumvent this difficulty, and to achieve more effective assessments of host and microbial relationships, gnotobiotic animals were introduced. Gnotobiotic animals are derived by aseptic caesarean section, and are continuously maintained with "germ-free" techniques. In gnotobiotic animals, the microbiota is fully defined, and can be classified as follows: (i) germ-free or axenic animals, in which the gnotobiotes are devoid of microorganisms; and (ii) defined flora animals, in which the gnotobiotes are maintained under isolator conditions in association with one or more known types of microorganisms (Gordon and Pesti, 1971). Gnotobiotic animals may be used to study external or endogenous factors (i.e. nutrition, immune reactions, responses to various forms of injury), where the unconfounded actions of such factors, affected or unaffected by associates in the host, are of interest (Gordon and Pesti, 1971). Germ-free

mice have been essential in investigating intrinsic mechanisms of disease, such as the susceptibility of mice to *C. rodentium* (Itoh *et al.*, 1988).

Due to the high susceptibility of germ-free mice to microorganisms, enteric infections were a common occurrence in early research with gnotobiotic animals (Gordon and Pesti, 1971). This susceptibility can be attributed to the lack of colonization resistance in germ-free animals (van der Waaij et al., 1971). The introduction of a defined-flora to germ-free mice was found to confer colonization resistance (Gordon and Pesti, 1971). The most complex defined-flora model to date is the Altered Schaedler Flora (ASF), which is produced by the colonization of germ-free rodents with four members of the original Schaedler Flora (i.e. the two lactobacilli, Bacteroides distasonis, and an extremely oxygen sensitive fusiform bacterium), a spiral-shaped bacterium (*Flexistipes* sp.), and three new fusiform extremely oxygen-sensitive bacteria (*Clostridium* spp., and *Eubacterium plexicaudatum*; Sarma-Rupavtarm *et al.*, 2004). The ASF model is an important tool to assess host responses to pathogens and to the intestinal microbiota, in a defined and controlled environment. For instance, the use of gnotobiotic mice colonized with ASF have revealed that microbial perturbation with *Helicobacter* billis or Brachyspira hyodysenteriae induced differential immune responses to nonpathogenic resident bacteria, which exacerbates intestinal inflammation (Jergens et al., 2006).

1.3.1.2 Citrobacter rodentium model

Citrobacter rodentium (i.e. formerly *C. freundii* biotype 4280 and *Citrobacter* genomospecies 9; Luperchio *et al.*, 2000), is a gram negative bacterial pathogen that

causes transmissible murine colonic hyperplasia (TMCH) in immunocompetent laboratory mice (Barthold *et al.*, 1978; Mundy *et al.*, 2005). *C. rodentium* is considered a rodent equivalent of *Escherichia coli* infection in humans (Schauer *et al.*, 1995), and elicits a host response similar to that found in murine models of inflammatory bowel disease (Higgins *et al.*, 1999). Age, host, genetic background, diet, and the indigenous microbiota have all been shown to influence colitis incited by *C. rodentium* (Barthold *et al.*, 1977; Luperchio and Schauer, 2001), and mice that recover from TMCH may be refractory to reinfection (Barthold *et al.*, 1978). Infection in adult mice is subclinical and self-limiting, whereas suckling mice exhibit severe colitis with necrosis and mucosal erosions, as well as visible clinical signs (Luperchio *et al.*, 2000). The degree of mucosal hyperplasia caused by *C. rodentium* is severe in NIH Swiss mice and moderate in C3H/HeJ mice, whereas C57BL/6J and DBA/2J mice exhibit the least degree of mucosal hyperplasia (Barthold *et al.*, 1977).

C. rodentium infection in laboratory mice has proven to be a robust model to assess mechanisms of pathogen-induced host responses (Borenshtein *et al.*, 2008), since it causes attaching/effacing lesions in the intestinal epithelium, which lead to strong host responses (Skinn *et al.*, 2006). *C. rodentium* colonizes the apical surface of the large intestinal mucosa, causing mucosal hyperplasia with limited inflammation and epithelial cell hyperproliferation in the descending colon, characterized by crypt elongation, increased mitotic activity, mucosal thickening, variable mucosal inflammation, crypt abscesses, occasional erosions and ulcers, and goblet cell depletion (Barthold *et al.*, 1978). Infection with *C. rodentium* generates a predominantly lymphocytic infiltrate, characterized by CD4⁺ T cells situated near the proliferative epithelial crypts, and up-

regulation of Th1-related cytokines (i.e. IL-12, IFN- γ , and TNF- α ; Higgins *et al.*, 1999; Spahn *et al.*, 2008), and Th17-related cytokines (IL-22, IL-17, and IL-6; Symonds *et al.*, 2009; Zheng *et al.*, 2008), which are critical for resolution of infection. T and/or B lymphocytes mediate most of the pathology and inflammation in the later stages of infection by *C. rodentium* and are required for clearance of the bacterium (Vallance, 2002). Bacteremia and extra-intestinal infection are not hallmarks of TMCH, though recovery of bacteria from blood, liver, and spleen has been reported (Luperchio and Schauer, 2001).

CHAPTER TWO

Hypotheses and objectives

2.1 Microbiota modulation hypothesis

Suppression of the intestinal microbiota is frequently regarded as the main mechanism of action of AGP (Dibner and Richards, 2005). In this regard, studies of mechanisms of growth promotion by AGP have focused almost exclusively on interactions between the microbiota and AMA. With respect to livestock production, AGP are frequently thought to modulate microbial communities, which provides maximum benefits with minimum costs to the host (Dibner and Richards, 2005). That AGP do not exert beneficial effects on the growth performance of germ-free mice, combined with the observation that AGP-fed animals have a thinner intestinal wall, have sustained this belief (Niewold, 2007). In this regard, several mechanisms to explain the contribution of AGP to animal health and growth are prominent in the literature: (i) AGP decreases opportunistic pathogens, therefore limiting subclinical infections and reducing the metabolic costs for the host; (ii) AGP reduce microbial production of growthdepressing metabolites (e.g. short-chain fatty acids from microbial fermentation); (iii) AGP reduce microbial use of nutrients; and (iv) AGP enhance host uptake of nutrients (i.e. digestibility), due to a thinner intestinal wall (Dibner and Richards, 2005; Niewold, 2007).

2.2 Alternative hypotheses

Scientific evidence supporting the microbiota modulation hypothesis of AGP action is lacking, and Niewold (2007) recently emphasized several reasons why the intestinal microbiota is an unlikely target for the action of AGP: (i) the AMA dose used for growth promotion is normally lower than the minimum inhibitory concentration (MIC) for enteric pathogens and other bacteria; (ii) AMA promote a similar growth effect in various production animals, despite considerable differences in their intestinal bacterial communities; (iii) major shifts occur in the composition of the intestinal microbiota as an animal develops; (iv) there are no available data on whether AGP actually enlarges the total mucosal surface by thinning the intestinal wall; (v) the use of various AMA, which have varying spectra of action, can promote similar growth promotion in animals; (vi) not all AMA have growth-promoting properties, whereas all of them should affect the microbiota according to the original hypothesis; (vii) management strategies using alternatives to AGP, including agents known to affect the intestinal microbiota, have highly variable and unexpected effects when compared to AGP (Utiyama, 2004); (viii) *Lactobacillus* spp. and *Enterococcus* spp. are probiotic bacteria used for promoting growth in livestock, but bacteria in both genera appear to produce growth-depressing metabolites; and (ix) it is unlikely that the composition of the microbiota can be manipulated towards a desired community composition, considering the complexity of the intestinal ecosystem (Savage, 1977). Furthermore, recent findings indicate the AMR to AMA is prevalent amongst the intestinal microbiota of livestock. For example, the fecal community of intensively raised swine continuously fed AGP contained a high level of erythromycin methylase genes conferring resistance to macrolide-lincosamide-

streptogramin B AMA (Kalmokoff *et al.*, 2010). This further challenges the microbiota modulation hypothesis. Hence, other mechanisms of AGP action, including an alternative target, should be considered to explain the consistent effects of AGP in such a complex environment.

It has been suggested that AGP may directly regulate tissue deposition via an endocrine involvement. Rumsey *et al.* (1999) suggested that an effect of CTC on the pituitary gland of ruminants might be the route by which AGP improve energy efficiency in tissue deposition, but not necessarily in muscle deposition.

Niewold (2007) proposed an interaction with host factors to explain the consistency of AGP activity. He suggested that AGP would decrease "physiologic" and constant inflammation of the intestinal mucosa, which would enhance nutrient utilization for growth purposes by decreasing the catabolic costs of mounting an immune response. An immunomodulatory effect of AGP would limit a cascade of immunological events (i.e. acute-phase response to antigenic stimuli) that demands great metabolic expenditure from the host. It has been reported that the growth promotion effect of AGP was enhanced in broiler chicken that were maintained in an unsanitary environment, by decreasing immunologic stress (Roura et al., 1992). These authors, however, did not attribute the decrease in circulating IL-1 (i.e. cytokine involved in the stress response) to a direct effect of AGP on the host immune system (Roura *et al.*, 1992). Some AMA (e.g. macrolides, tetracyclines and sulfonamides) have immunomodulatory properties; they can down-regulate prolonged inflammation, increase mucus clearance, stimulate or impair activation of the host immune system, and modify phagocyte activity by altering their functions, including chemotaxis, phagocytosis, oxidative burst, bacterial killing, and

cytokine production (Rubin and Tamaoki, 2005). Furthermore, therapeutic concentrations of CTC modulated acute response in *ex vivo* perfused pig livers, and decreased the secretion of TNF- α by cultured Kupffer cells (Akunda *et al.*, 2001).

2.3 Research hypotheses and objectives

I hypothesize that AGP do not exert a substantial effect on the composition of the intestinal microbiota of mammalian livestock, and that AGP function by modulating intestinal immune responses. This is referred to as "immunomodulation hypothesis" hereafter (Figure 2.1). To test the immunomodulation hypothesis, the following research objectives were formulated:

1) Test the efficiency of various sampling methods to characterize mucosaassociated bacterial communities (Chapter 3);

2) Assess the utility of Klenow fragment and mung bean nuclease, which are deemed to reduce T-RFLP bias, on the characterization of microbial communities (Chapter 3);

3) Evaluate the effect of orally administered non-therapeutic doses of CTC and sulfamethazine (i.e. AS700; model AGP for beef cattle) on the composition of bacterial communities associated with the intestinal mucosa and digesta of cattle (Chapter 4);

4) Establish a murine AGP model, and evaluate the effect of orally administered non-therapeutic doses of CTC on the composition of bacterial communities associated with the intestinal mucosa of mice (Chapter 4);

5) Using the murine AGP model, assess the modulatory effects of orally administered non-therapeutic doses of CTC on inflammatory cytokine transcription



Figure 2.1. Schematic representation of the interactions involved in this project. The dark arrow represents the immunomodulation hypothesis for the growth promoting action of AGP.

levels, pathologic changes, and weight loss associated with *C. rodentium* infection, as well as on the composition of microbiota of the murine colon (Chapter 5).

I contend that the development of alternatives to AGP will only be effectively accomplished once the appropriate target for their growth promoting action is identified.

CHAPTER THREE

Characterization of mucosa-associated bacterial communities of the murine intestine by terminal restriction fragment length polymorphism¹

ABSTRACT

Terminal restriction fragment length polymorphism is a molecular technique used for comparative analysis of microbial community composition and dynamics. Three sampling methods for recovering bacterial community DNA associated with intestinal mucosa of mice were evaluated: mechanical agitation with PBS, hand washing with PBS containing Tween 80, and direct DNA extraction from mucosal plugs. In addition, the utility of two methods (i.e. Klenow fragment and mung bean nuclease) to reduce singlestranded DNA artifacts was tested. Based on T-RFLP analysis, there were diverse communities of bacteria associated with mucosa of the murine ileum, cecum, and descending colon. Although there was no significant difference in bacterial community composition between the mechanical agitation and direct DNA extraction methods regardless of intestinal location, community diversity was reduced for the wash method in the colon. The use of Klenow fragment and mung bean nuclease has been reported to eliminate single-stranded DNA artifacts (i.e. pseudo-T-restriction fragments); however, neither method was beneficial for characterizing mucosa-associated bacterial communities of the murine cecum. In conclusion, mechanical agitation and direct plug extraction yielded equivalent bacterial community DNA from the mucosa of the small

¹ Chapter published as: Costa, E., Puhl, N. J., Selinger, L. B. and Inglis, G. D. (2009). Characterization of mucosa-associated bacterial communities of the mouse intestine by terminal restriction fragment length polymorphism: Utility of sampling strategies and methods to reduce single-stranded DNA artifacts. *Journal of Microbiological Methods*, **78**(2): 175-180.

and large intestine of mice, but the latter method was superior for logistical reasons. In addition, statistical detection of T-RFs, analysis of variance for T-RF number, and group significance test through statistical randomization were used to ensure more reliable and comprehensive analysis of T-RFLP data.

3.1 INTRODUCTION

The ultimate goal of modern microbial ecology is not only to identify particular species within bacterial communities, but also to explore community structure and dynamics in complex natural environments. Molecular approaches, such as PCR-based methods coupled with rRNA gene-based phylogeny, enable the identification of uncultured organisms, contributing to the characterization of diverse bacterial communities (Weng *et al.*, 2006). Sequence analysis of 16S rRNA gene libraries for species determination can be laborious and time consuming, becoming impractical for substantive studies of microbial diversity and function in highly complex communities. Rapid and more comprehensive quantitative assessment of bacterial communities is possible using T-RFLP analysis, a molecular technique based on restriction site polymorphisms of the 16S rRNA gene (Liu *et al.*, 1997). Despite being often reduced to the level of species (i.e. unique restriction site position to differentiate species), T-RFLP analysis is also used for temporal and spatial comparative analyses of microbial community composition (Clement *et al.*, 1998).

Assessments of intestinal microbial communities are challenging, as luminal bacteria (e.g. bacteria released from mucosal surfaces into ingesta) are transient, and may not necessarily represent microbial communities of a particular location of the intestine

(Savage, 1977). Mucosa-associated bacteria are likely to best represent intestinal communities, as they are uniformly distributed, differ from fecal communities, and are influenced by host factors (Zoetendal *et al.*, 2002). Effective recovery of DNA from samples is essential for DNA-based community analyses (Apajalahti *et al.*, 1998). The sampling process and DNA extraction may result in random bias (Frostegard *et al.*, 1999), which could increase with the extraction of DNA from the whole intestinal tissue, due to a higher chance of insufficient or preferential disruption of bacterial cells (Ingrassia *et al.*, 2001). The release of bacteria from mucosal surfaces by hand washing with buffer containing a surfactant has been used (Gong *et al.*, 2002; Li *et al.*, 2003), as has agitation of tissues in buffer using a bead-beater (Sait *et al.*, 2003). Nevertheless, the efficacy of these methods for recovering bacteria from the surface of mucosa has not been adequately investigated (Li *et al.*, 2003), and it is not known if these strategies will affect random sampling bias.

Another potential drawback of the T-RFLP technique is PCR-related bias (Egert and Friedrich, 2003; Frey *et al.*, 2006; Hartmann *et al.*, 2007; Pinard *et al.*, 2006), especially in the form of partially single-stranded amplicons (Egert and Friedrich, 2005). The occurrence of pseudo-T-RFs has been reported to be consistently decreased by postamplification treatment with Klenow fragment, which restores the partially singlestranded amplicons (Egert and Friedrich, 2005). Furthermore, single-strand-specific mung bean nuclease completely eliminated pseudo-T-RFs (Egert and Friedrich, 2003). Techniques that prevent sampling bias and artifact formation in T-RFLP analyses are particularly valuable for the characterization of highly complex bacterial communities, especially those associated with intestinal mucosa, which are poorly characterized. The

objectives of the current study were to: (i) use T-RFLP to empirically compare the efficacy of three sampling strategies on the recovery of mucosa-associated bacteria from the intestine of mice; (ii) assess the effect of mung bean nuclease and Klenow fragment on the formation of T-RFs; and (iii) apply novel statistical methods on T-RF selection, T-RF number and group similarity assessments, providing a quantitative interpretation of T-RFLP data.

3.2 MATERIALS AND METHODS

Mice

Mice were housed at the small animal facility located at the Agriculture and Agri-Food Canada (AAFC) Lethbridge Research Centre (LRC). All requirements specified by the Canadian Council on Animal Care (CCAC) were met, and the project was approved by the LRC Animal Care Committee (ACC) before commencement of the study (Animal Use Protocol Review 0703). Swiss Webster mice (Charles River, Montreal, QC) at 16 weeks of age were used.

Sample collection

Animals (n = 9) were sedated with isofluorane and euthanized by CO_2 asphyxiation. Immediately after euthanasia, a midline incision was made and the intestines were aseptically exposed. Sections approximately 2 cm in length were collected from the terminal ileum, central portion of the cecum, and distal descending colon. Intestinal sections were longitudinally opened, and the mucosal surfaces were gently rinsed with cold sterile phosphate buffer with saline (PBS; 10 mM sodium phosphate buffer with 130 mM sodium chloride; pH 7.2) to remove ingesta. Three adjacent tissue plugs were obtained by punching the antimesenteric side of each intestinal section with a 3-mm-diameter biopsy punch. All samples were kept on ice and processed within 60 min of euthanasia. To negate spatial sampling bias, samples collected from each intestinal location of three arbitrarily-selected mice were pooled to comprise each of the three replicates (Figure 3.1).

Sampling methods

Three sampling methods were evaluated. For method one (i.e. the hand wash method), bacteria were washed from mucosal surfaces using the protocol of Gong et al. (2002), with minor modifications. Briefly, 1 mL of PBS containing 0.1% (v/v) Tween 80 was added to each of three replicate samples, samples were vigorously shaken by hand for 1 min, and the resultant wash solutions were transferred to new tubes after a 5 s centrifugation pulse (13200 x g). The wash procedure was conducted three times. Tubes containing the washes were centrifuged at 27000 x g for 20 min to pellet bacterial cells, the supernatant was discarded, pellets were re-suspended in 20 µL of PBS, and the three washes per replicate were pooled. Samples were then centrifuged again at 27000 x g for 20 min and the supernatant was discarded. For method two (i.e. the mechanical agitation method), tissue samples were processed as described by Sait et al. (2003) with some modifications. Samples in 1 mL of PBS were agitated mechanically using a Mini-Beadbeater (Biospec Products, Bartlesville, OK) for 20 s. The procedure was conducted three times, and the washes were combined and centrifuged as described above. For method three (i.e. direct DNA extraction), intestinal tissue samples were left untreated. Each method consisted of three independent replicates (n = 9 mice per method).



Figure 3.1. Schematic diagram of the sampling process applied to each intestinal location (i.e. terminal ileum, largest portion of cecum and descending colon) of mice in this study, with the purpose of negating spatial bias. Each pool of three tissue plugs comprised one replicate. This process was conducted three times to comprise the three replicates used in the study (i.e. nine animals per method).

Genomic DNA extraction

Total genomic DNA was extracted from all samples using the protocol for Grampositive bacteria of the DNeasy Blood & Tissue Kit, according to the manufacturer's recommendations (Qiagen Sciences Inc., Germantown, MD). Cells recovered using the hand and mechanical washing methods were incubated in enzymatic lysis buffer at 37 °C for 30 min, whereas samples for direct DNA extraction were incubated for 90 min in the lysis buffer. All samples were eluted with 50 μ L of optima water and genomic DNA was verified by electrophoresis in a 1% Tris-borate-EDTA (TBE) agarose gel.

Bacterial community DNA amplification

DNA encoding the 16S rRNA gene was amplified by PCR. Reaction mixtures consisted of 2 μ L (10 ng) of genomic DNA, 1X PCR buffer, 0.2 mM of each deoxynucleoside triphosphate, 0.1 μ g μ L⁻¹ of acetylated bovine serum albumin (Promega, Madison, WI), 0.625 U of Taq DNA polymerase (Qiagen Sciences Inc.), 0.5 μ M of the universal eubacterial primers, 5' FAM (6-carboxyfluorescein)-labeled 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'), and optima water to a final volume of 25 μ L. PCR conditions were 95 °C for 15 min; 30 cycles consisting of 94 °C for 30 s, 50 °C for 1 min and 30 s, and 72 °C for 1 min; and a final extension period at 72 °C for 10 min. All PCR reactions were performed in triplicate, pooled, purified using QIAquick PCR-purification Kit (Qiagen Sciences Inc.), and eluted with optima water. Samples were quantified by agarose gel electrophoresis (1% TBE agarose) relative to a 100 bp DNA ladder (Promega). A gel purification kit (Qiagen Sciences, Inc.) was used to isolate the \approx 1500 bp amplicons in samples in which non-specific amplification occurred.

Restriction of bacterial community DNA

Digestions were carried out in duplicate in a mixture containing 75 ng of the purified PCR-product, 3 U of restriction endonuclease *Hae*III (Invitrogen Corp., Carlsbad, CA), 2.5 μ L of enzyme buffer, and optima water (final volume 25 μ L). Samples were incubated at 37 °C for 2 h in the dark, and the reaction was stopped by ethanol precipitation.

Capillary gel electrophoresis and fragment size determination

Samples were air-dried overnight in the dark after ethanol precipitation, resuspended in 9.25 μ L of Hi-Di formamide and 0.25 μ L of LIZ500 size standard marker (Applied Biosystems, Foster City, CA), denatured at 95 °C for 3 min, and immediately placed on ice. Fluorescently-labelled T-RFs were separated in POP7 polymer using a 3130 Genetic Analyzer (Applied Biosystems-Hitachi).

Enzymatic treatment of single-stranded DNA artifacts

DNA extracted from cecal samples (i.e. hand wash, mechanical wash, and direct extraction) was used, and each method was replicated three times. To assess the occurrence of single-stranded artifacts (i.e. pseudo-T-RFs), bacterial community DNA was treated with mung-bean nuclease and Klenow fragment before restriction digestion. The resultant T-RFLP community profiles were compared to those obtained from DNA subjected directly to restriction digestion after purification and quantification. Protocols were performed as described previously for mung-bean nuclease (Egert and Friedrich, 2003) and Klenow fragment (Egert and Friedrich, 2005). Digestion with mung-bean nuclease was stopped by ethanol precipitation, and samples treated with Klenow fragment were purified using QIAquick PCR-purification kit (Qiagen Sciences Inc.)

immediately after incubation. Amplification of community DNA, restriction digestion, capillary gel electrophoresis, and fragment size determination were performed as described above.

T-RFLP electropherograms

Two electropherograms (i.e. separate runs) represented by peaks were obtained per sample; each electropherogram was analyzed separately using GeneMapper software Version 4.0 (Applied Biosystems) with the Local Southern peak calling method. Fragment sizes were assigned to bins (i.e. operational taxonomic units) with a resolution of 1 bp each using a rounding method (Dunbar *et al.*, 2001), where peaks that differed by less than 0.5 bp were considered identical. Manual binning was also performed on bins generated by the software that were less than 1 bp in length. A baseline for peakdetection in fluorescence units by the software was assigned as zero, meaning that every peak would be detected and represented on the electropherograms.

Statistical Analyses

To prepare data for statistical analysis of T-RFs (i.e. "true peaks"), the presence or absence of peaks ranging from 50 to 500 bp, as well as peak height and peak area were determined for each replicate. Data was analyzed using SAS (SAS Institute Inc., Cary, NC). Analysis was based on a statistical method for identification of T-RFs as previously described (Abdo *et al.*, 2006). T-RFs were detected based on variance, which was calculated by assuming that the true mean of the background noise height was zero. Data points that were larger than three standard deviations were identified as T-RFs and assigned the value one; pseudo-T-RFs (i.e. "false peaks"; Egert and Friedrich, 2003), were assigned the value zero, and were detected either by variance or by replicate

comparison (i.e. if a T-RF was detected in only one of the two replicates, it was considered a pseudo-T-RF). The SAS program written is provided in Appendix one. The binary dataset (i.e. 1/0 values) obtained from the SAS analysis was imported to Bionumerics software version 5.1 (Applied Maths Inc., Austin, TX) for cluster and genetic similarity analyses. Cluster analysis was performed by using the Dice coefficient, and unweighted pair-group methods with arithmetic means (UPGMA).

To test the statistical significance of each group, multivariate analysis was performed by grouping the treatment replicates within each intestinal location, and comparing the within- and between-group similarities with randomization tests (i.e. resampling by statistical bootstrapping) within the Bionumerics software (Applied Maths Inc.). The test determined significance among treatments based on comparisons between overall and group similarity averages. The script was set for 1000 iterations (i.e. resamplings). The *P*-values for this analysis represented how often the randomized average was higher than the observed group average, based on pairwise similarity measures (Kropf *et al.*, 2004).

An analysis of variance (ANOVA) for occurrence of T-RFs and for total peak area was performed as a completely randomized design, using the MIXED procedure of SAS, with location, treatment, and their interaction included in the model as fixed effects. Least squares were generated for significant effects and Fisher's protected least significant difference test was used to compare differences among means of interest. The UNVARIATE procedure of SAS was used to produce normal probability plots to check for normality and outliers. Obvious outliers were removed before completion of the final analysis.

3.3 RESULTS

Amplification of 16S rRNA gene from tissue plugs and cell pellets

It was not possible to generate enough DNA for mung-bean nuclease and Klenow fragment reactions from all ileal and descending colon samples. Despite yielding a much lower concentration of genomic DNA, cell pellets generated from mechanical and hand washing produced similar 16S rRNA gene amplicon concentration to that produced from whole tissue samples (data not shown). Extraction of DNA from cell pellets generated from the washes did not decrease non-specific amplification relative to the direct method (data not shown).

Bacterial communities recovered from different sampling methods

Diverse bacterial communities in association with intestinal mucosa were observed in the murine terminal ileum, cecum, and descending colon of mice using T-RFLP, regardless of the sampling method employed (Figure 3.2, A; all peaks shown). For analysis purposes, pseudo-T-RFs were removed and samples from each intestinal location were grouped, generating dendrograms containing pairwise similarity measures (Figure 3.2, B). Composition of bacterial communities associated with mucosa within the colon, but not the ileum or cecum, were similar for the hand wash method among replicates (Figure 3.2, B).

The hand wash method applied to colonic mucosal samples generated distinct (P=0.03) T-RFLP community profiles (Table 3.1). However, in the ileum and cecum, there was no difference (P>0.05) in T-RFLP communities among the hand wash, mechanical wash, or direct methods.



Figure 3.2. (A) T-RFLP profiles (16S rRNA gene) of mucosa-associated bacterial communities from the murine intestine before statistical detection of T-RFs (i.e. no threshold applied). The three replicates per treatment are overlaid. (B) dendrograms of bacterial community relatedness generated from the binary data containing true bacterial T-RFs; T-RFLP patterns were analyzed by Dice's coefficient (>50% mean) and UPGMA algorithm for cluster analysis. Acronyms on (A) and (B) represent the sampling method applied to recover bacterial DNA from tissue plugs (sampling method): direct DNA extraction (DE); hand-washing with PBS containing Tween 80 (HW); mechanical agitation with PBS (MA). Numbers on (B) represent each of the replicates.

Table 3.1. G different trea	troup signi atments be	fficance and fore DNA (alysis of T-R extraction (E	FLP comm)E: direct ex	unity profil traction; H	es of murine W: hand was	intestinal sar sh; MA: mecl	nples submi nanical agita	tted to tion)
		<i>P</i> -value		Within-g	roup simil:	arity (%)	Between-	group simil	arity (%)
	DE	ΜH	MA	DE	ΜH	MA	DE	ΗW	MA
Ileum	0.489	0.810	0.735	53.00	34.17	40.66	51.97	46.78	47.11
Cecum	0.847	0.768	0.611	47.39	52.90	58.25	59.63	60.54	60.43
Colon	0.749	0.033*	0.791	35.52	80.22	22.22	46.61	50.74	38.61

 Values represent unique groups 	(P<0.05)
 Values represent unique 	groups
 Values represent 	unique
· Values	represent
× .	· Values

Based on analysis of variance, T-RF number did not differ among sampling methods as a function of the intestinal location (P=0.21), and sampling method alone did not differ (P=0.45) among the three methods. However, there was a difference in T-RF number (P<0.001) among intestinal locations; more T-RFs (P<0.05) were observed from cecal mucosa (58.4 ± 3.2) than from the other two sites, and more T-RFs (P<0.05) were observed from the ileal mucosa (27.1 ± 3.9) relative to the descending colon (17.6 ± 3.0).

Occurrence of pseudo-T-RFs

Peaks generated from samples treated with mung bean nuclease had overall weaker fluorescence when compared to those generated from Klenow-treated and control samples (Figure 3.3). The total peak area (relative fluorescent units) from the mung bean nuclease-treated samples $(1.9 \times 10^5 \pm 4.1 \times 10^4)$ was 47.1% lower (*P*<0.05) than the direct-extracted samples $(3.5 \times 10^5 \pm 6.4 \times 10^4)$, and 53.3% lower (*P*<0.05) than the Klenow fragment-treated samples $(4.2 \times 10^5 \pm 1.2 \times 10^5)$. Nevertheless, differences in community profiles among the untreated, Klenow fragment, and mung bean nuclease treatments did not differ (*P*>0.05). Furthermore, there was no difference in T-RF number among the treatments (*P*=0.13), sampling method (*P*=0.81), or treatment as a function of sampling method (*P*=0.98).

3.4 DISCUSSION

In this study, three tissue plugs from each intestinal location (i.e. ileum, cecum, and descending colon) were collected and subsequently combined to ensure spatial consistency from these locations, and thereby facilitate an accurate comparison of



Figure 3.3. T-RFs of mucosa-associated bacteria from the murine cecum (i.e. no threshold applied). Untreated controls (UC), treatment with Klenow fragment (KF) and mung bean nuclease (MB) before restriction digestion. Each electropherogram contains nine replicates of the specified enzymatic treatment, three from each sampling method applied before DNA extraction (i.e. direct extraction, hand wash and mechanical wash).

treatments on microbial communities. Nonetheless, there was considerable variability among replicates, suggesting that a significant degree of inter-animal variability in mucosa-associated communities of the ileum and cecum occurred, despite the similarity in diet, environment, and genetics of the mice used in the current study.

Samples from the descending colon subjected to hand washes generated distinct T-RFLP profiles, and fewer T-RFs than the direct and mechanical wash methods; therefore, it was inferred that the hand washing method provided an underestimation of diversity of bacteria associated with the mucosa in this location. This effect was location-specific, as there was no difference in T-RFLP profiles among the three treatments for samples obtained from the cecum or ileum. These findings contrasted with those of Li et al. (2003), who reported that > 93% of bacterial cells were released from the colonic mucosa, as well as from the ileum and cecum, by hand washing the tissues with buffer containing Tween 80. They compared the number of bacterial cells released from the mucosa by washing, relative to those scraped from the mucosa with a glass slide and enumerated by epifluorescence microscopy. However, they did not assess the efficacy of the scraping method in removing and enumerating mucosa-associated bacteria. It was concluded that DNA extracted directly from tissue provided an accurate and reliable representation of bacterial communities associated with the intestinal mucosa. Whole tissue, however, may hamper the disruption of bacterial cells in the enzymatic lysis (Ingrassia et al., 2001). Increasing the enzymatic lysis period, as in the current study, negated this possibility (More et al., 1994). In addition, DNA extracted directly from tissue may also generate pseudo-T-RFs as a result of non-specific amplification of mammalian DNA. Amplification of the 18S rRNA gene using 16S rRNA gene primers

was previously encountered in our laboratory (data not published). However, 16S rRNA gene amplicons were gel-purified to ensure that non-target amplicons were eliminated. Interestingly, dislodgement of bacterial cells from the mucosa by washing was not effective in preventing non-specific amplification, and gel purification was also required for washed samples. Given the considerable logistical advantages of the direct method (i.e. tissues can be archived for later processing), combined with amplification efficacy equivalent to the wash methods, it was concluded that the direct tissue method was preferred for T-RFLP analysis of mucosa-associated bacteria of the intestine.

DNA treated with Klenow fragment and mung bean nuclease did not generate significantly different T-RFLP profiles, indicating that single-stranded DNA artifacts were either not eliminated or their presence was not frequent enough to significantly affect the comparative analysis. Mung bean nuclease decreased the DNA concentration, and as with the Klenow fragment, required a relatively large amount of DNA for the reactions (i.e. 1 µg). Therefore, their use for routine characterizations of mucosa-associated bacteria, which are not always abundant (e.g. particularly in the small intestine), is not practical. Although Klenow fragment and mung bean nuclease were not beneficial for comparative analysis of intestinal mucosa-associated bacteria in the present study, their use should be considered when it is possible to produce enough DNA for the reactions, and for more specific applications, as the analysis of sequence diversity for cloning (Egert and Friedrich, 2005).

Despite being a very powerful and high-throughput technique for analysis of microbial communities, the T-RFLP method is still in development. Several key issues must be addressed, especially when the objective of experimentation is to widely

characterize community structure and dynamics. Appropriate T-RF selection and alignment for the analysis, as well as accurate diversity (Blackwood et al., 2007) and abundance (Frey et al., 2006) assessments of microbial profiling remain issues of concern. Determination of group significance by randomization tests using pairwise similarity measures (Kropf et al., 2004), as well as variance and ordination analysis (Culman *et al.*, 2008) have proven effective for comparative analysis of bacterial community fingerprinting data. In the current study, a statistical method based on variance for selecting T-RFs was used, combined with statistical methods of randomization and variance to determine group significance and T-RF occurrence, thereby providing a comprehensive, reproducible, and reliable analysis of T-RFLP profiles. Currently, most T-RFLP data analyses are based on qualitative assessments of the electropherograms which are commonly generated by establishing a fixed-percentage threshold (Dunbar et al., 2001) to exclude the background noise (i.e. "false peaks"). Statistical detection of T-RFs negates the possibility of excluding small reproducible peaks (Abdo et al., 2006).

The present findings emphasized the utility of the T-RFLP method for comparative analyses of complex bacterial communities. Using quantitative assessments of the electropherograms and dendrograms, T-RFLP analysis were applied to characterize complex bacterial communities associated with the intestinal mucosa of mice. In addition to considerable logistical advantages, direct extraction of DNA from intestinal tissues provided equivalent representation of bacterial communities associated with intestinal mucosa. Furthermore, it was demonstrated that neither Klenow fragment nor mung bean nuclease were crucial for T-RFLP analysis of intestinal bacterial communities.

ACKNOWLEDGEMENTS

We thank Toby Entz (AAFC) for his assistance with statistical analyses, including the design of the SAS script used in this study; Tara Shelton (University of Lethbridge) for her assistance with mouse handling and sample collection; and the AAFC LRC Vivarium staff for maintaining mice. This work was funded in part by a Discovery Grant to Dr. Doug Inglis and Dr. Brent Selinger from the Advanced Foods and Materials Network (AFMNet) and an AAFC Peer Review Project Grant to Dr. Doug Inglis.

CHAPTER FOUR

Effect of long-term oral administration of non-therapeutic doses of a model antimicrobial growth promoter on the intestinal microbiota composition of cattle and mice

ABSTRACT

Despite the multitude of studies regarding the prevalence of resistant bacteria in animals administered AGP, little attention has been directed towards determining the true mode of action of AGP, and its impact on the intestinal microbiota. In this study, the effect of orally administered non-therapeutic CTC and sulfamethazine (AS700) on the composition of the intestinal microbiota of cattle was examined by T-RFLP analysis. Bacterial communities associated with the intestinal mucosa differed from those associated with the adjacent digesta in beef cattle. However, administration of AS700 did not affect the composition of bacterial communities associated with the mucosa or digesta of cattle, with exception of the mucosa of the proximal jejunum. Similarly to cattle, nontherapeutic administration of CTC administered to mice did not substantially affect bacterial communities associated with the murine intestinal mucosa. It was also demonstrated that bacterial community composition varied along the intestinal tract of cattle and mice, with an obvious distinction between the small and large intestine. The results from this study do not support the microbiota modulation hypothesis as the primary mechanism of action of growth promotion by AGP, and indicate that AGP may function in an alternate manner.

4.1 INTRODUCTION

Antimicrobial agents from the tetracycline class are widely used because of their favorable effects, such as absence of major side effects and broad-spectrum of action, and their activity against a wide range of Gram-positive and Gram-negative bacteria, including chlamydiae, mycoplasmas, and rickettsiae (Chopra and Roberts, 2001). Chlortetracycline was the first of the tetracycline class of AMA to be discovered (Duggar, 1948), and has been widely used at therapeutic concentrations to treat infections in human and veterinary medicine, and at non-therapeutic concentrations to prevent infections and promote growth in livestock (Chopra and Roberts, 2001). Unfortunately, the extensive use of CTC in human and veterinary medicine, as well as in agriculture (Chopra and Roberts, 2001), has resulted in elevated selective pressure for AMR in enteric pathogens (Alexander et al., 2008; Emborg et al., 2003; Inglis et al., 2005), substantially decreasing the effectiveness of CTC (Chopra and Roberts, 2001). Resistance to tetracyclines in bacteria has emerged due to the acquisition of resistant determinants, such as tet genes (Burdett et al., 1982). The long-term administration of AGP, including CTC and sulfamethazine in livestock, is generally regarded as a primary reason for the high prevalence of AMR (McEwen and Fedorka-Cray, 2002; Shea *et al.*, 2004). A concern is that AGP are still widely used in North American livestock production, and many AMA used in human medicine and as AGP belong to the same class (i.e. sulfonamide, tetracycline, penicillin, macrolide, aminoglycoside, lincosamide; HC, 2002). The National Animal Health Monitoring System (NAHMS) reported that 52% of all United States feedlots in 1999 (NAHMS, 1999) and 43% of all United States swine production nursery sites in 2006 (NAHMS, 2006) administered CTC in water or feed.
Measures to restrict AGP use in North America, will soon be implemented, as has already occurred in the European Union (Castanon, 2007). The prevalence of AMR in specific zoonotic pathogens and pathogen surrogates due to AGP administration in livestock has been the subject of numerous studies (Conly, 2002; McEwen and Fedorka-Cray, 2002). However, limited research has examined the effect of AGP on the composition of livestock microbiota or on AMR in commensal intestinal bacteria.

T-RFLP is a cultivation-independent community fingerprinting technique that has been successfully used to characterize and explore complex microbial communities (Dunbar *et al.*, 2001; Weng *et al.*, 2006). The relative simplicity of T-RFLP to explore community structures is a result of 16S rRNA gene sequence polymorphisms (Liu *et al.*, 1997). Recent advancements in statistical analysis of T-RFLP data (Abdo *et al.*, 2006; Costa *et al.*, 2009; Culman *et al.*, 2009) enable a highly comprehensive and practical comparative assessment of the intestinal microbiota composition among intestinal locations and experimental treatments.

Examining potential effects of AGP on the intestinal microbiota is critical to unraveling the mechanisms of action of AGP, and will assist in the development of alternatives. Despite being the most accepted hypothesis (Dibner and Richards, 2005), modulation of the microbiota by AGP has not been thoroughly tested. Furthermore, it has been suggested that low concentrations of AGP would not be expected to exert a substantive effect on the composition of the intestinal microbiota (Niewold, 2007). The objective of the present study was to assess the effect of non-therapeutic concentrations of orally administered CTC and sulfamethazine (i.e. AS700) on the composition of the cattle intestinal microbiota using T-RFLP analysis. In addition, doses of CTC given to

cattle were extrapolated for use in a murine model, and the effect of three non-therapeutic doses of CTC on the composition of bacterial communities associated with the intestinal mucosa of mice were also assessed. The following hypothesis was tested: the administration of model AGP (i.e. AS700 for cattle, or CTC for mice) will not impart substantial modifications to the intestinal microbiota of cattle and mice.

4.2 MATERIALS AND METHODS

Cattle

Beef cattle were housed in an experimental feedlot located at the AAFC LRC. All of the animals were cared for according to the guidelines set out by the CCAC. Cattle originated from a common location and did not receive AMA before commencement of the experiment.

Treatments of cattle

Upon arrival at the feedlot, cattle were randomly assigned to one of two treatments: (1) Control, which received no AMA; and (2) AS700, which received 350 mg hd⁻¹ d⁻¹ CTC and 350 mg hd⁻¹ d⁻¹ sulfamethazine (Aureo S®-700 G, Alpharma Inc., NJ). AS700 is commonly used in the Canadian beef industry, and was fed at the concentration recommended by the manufacturer. Each experimental unit consisted of a separate pen containing ten steers, and each treatment was replicated five times. Water troughs were shared between adjacent pens, but treatments were arranged in a manner so that only cattle that received the same antimicrobial agent shared water troughs (Figure 4.1). Steers entering the feedlot were fed a forage-based diet for the first 115 d consisting of 70%



Figure 4.1. Schematic representation of the experimental units and the treatments applied in this study. Cross-hatched rectangles with a number correspond to a separate pen in the experimental feedlot, and represent one experimental unit.

barley silage, 25% barley grain and 5% (dry matter basis) supplement containing vitamins and minerals (i.e. backgrounding period). Cattle were subsequently switched from the forage-based diet to a grain-based diet (85% barley, 10% barley silage, 5% supplement) for 21 d, and then were maintained on a grain-based diet for an additional 179 d (i.e. finishing period). Cattle were fed once daily in a manner that ensured that all feed that was allotted to each pen was consumed. AS700 was introduced into the diets 5 d after cattle arrived at the feedlot, and they were included in the forage-based diet for 56 d and reintroduced for additional 42 d when the grain-based diet was fed. AS700 was administered in the diet until 28 days before slaughter. To avoid cross contamination, AMA were mixed with 5 kg of a supplement containing minerals and vitamins, and the mixture was manually spread over the surface of feed within each of the appropriate pens during the morning feeding. All animals in the pen were capable of feeding at the feed trough at the same time.

Collection of digesta and intestinal tissues from cattle

One animal per replicate from each of the Control and AS700 treatments was selected for sampling in the abattoir (n = five animals per treatment); the abattoir was a provincially inspected, medium-capacity plant (Ben's Quality Meats, Picture Butte, AB). Animals were transported to the abattoir on the evening prior to slaughter, and those from the AS700 treatment were transported 1 d after animals from the Control treatment. At the abattoir, animals were maintained on a barley silage diet, and were humanely euthanized. The intestinal tract of each animal was removed approximately 10 min after euthanasia, and placed on a clean sheet of plastic on a cool cement floor in the abattoir.

Nine intestinal sections, each approximately 20 cm long, were obtained from each animal at the following locations: (i) descending portion of the duodenum (i.e. following the cranial flexure); (ii) proximal jejunum; (iii) central jejunum; (iv) distal jejunum; (v) ileum (\approx 10 cm before the ileal-cecal junction); (vi) terminus of the cecum; (vii) central flexure of the ascending colon; (viii) descending colon (≈ 20 cm before the sigmoid colon), and (ix) rectum. Before excision of the intestinal sections, bilateral ligatures were applied adjacent to the excision site to minimize external contamination of the tissues with digesta. Tissues samples were placed in individual bags on ice and processed within 6 h of collection. Intestinal sections were aseptically excised longitudinally and digesta was aseptically removed. Digesta from each sample was weighed (200 mg \pm 20 mg), and placed in DNA-free tubes. Following removal of the majority of digesta, the mucosal surface within each intestinal section was gently washed with chilled sterile PBS, taking care to remove residual digesta while minimizing disruption of mucus on the mucosal surface. Mucosal sections were removed with a sterile 3-mm-diameter Biopsy Acu-Punch (CDMV) and individual plugs were placed in DNA-free tubes. Digesta and mucosal plugs were stored at -20 °C until processed.

Mice

Twelve C57BL/6J 4 week-old female mice were purchased from Charles River Laboratories International, Inc. (Montreal, QC). Mice were housed at the small animal facility located at the AAFC LRC. All requirements specified by the CCAC were met, and the project was approved by the LRC ACC before commencement of the study (Animal Use Protocol Review 0703). Mice were maintained individually in filter-topped cages under specific pathogen free (SPF) conditions, and transferred to sterile cages daily

during the experiment. All mice were maintained on a 12 h light/dark cycle and had free access to sterile autoclaved food (Prolab RMH 3500 Autoclavable; PMI Nutrition International, Brentwood, MO) and water.

Treatments of mice

Mice were randomly assigned to each treatment (n = three mice per treatment). Mice from CTC treatments received 2, 8, or 32 mg L⁻¹ of CTC (Sigma, St. Louis, MO) added to autoclaved water, whereas mice from the Control treatment received only autoclaved water. CTC was administered for 3 weeks; fresh CTC solutions were provided twice a week. Sucrose (5% w/v; Sigma) was added to the water for all treatments, and all water bottles were covered with aluminum foil.

The three doses of CTC used in mice were extrapolated from non-therapeutic doses administered in cattle using three approaches. The first approach was based on body weight ratio, which is approximately 20 g in mice versus 400 kg in beef cattle (ratio of 1/8000). Based on this ratio and a non-therapeutic dose of 75 mg d⁻¹ in cattle, the average daily intake in mice would be approximately 9 μ g (achieved with a water concentration of 2 mg L⁻¹). The second approach was based on the ratio of non-therapeutic to therapeutic doses of CTC in cattle (approximately 75 versus 6000 mg d⁻¹, ratio of 1/80). Using this ratio and an average therapeutic dose of CTC in mice of 640 mg L⁻¹, a dose of 8 mg L⁻¹ was obtained. Finally, since average therapeutic doses of CTC in mice are approximately four-fold higher than those used in cattle (approximately 60 versus 15 mg kg⁻¹ d⁻¹), a third dose, four-fold higher (32 mg L⁻¹), was selected.

Collection of feces and intestinal tissues from mice

Fecal samples of mice from Control and CTC treatments were collected at 1, 8, 12, and 20 d after commencement of CTC administration. On day 21, mice were humanely euthanized by CO₂ asphyxiation following sedation by isofluorane (Halocarbon Products Corporation, River Edge, NJ). The GI tract was removed, and 2 cm sections of the proximal duodenum, central jejunum, ileum (immediately before the ileal-cecal junction), terminus of the cecum, ascending and descending colon were aseptically collected. Each intestinal section was longitudinally excised, rinsed with chilled sterile PBS, and mucosal samples were collected with a sterile 3-mm-diameter Biopsy Acu-Punch (CDMV, St. Hyacinthe, QC).

Estimation of residual CTC in murine feces

To estimate the concentration of residual CTC in fecal samples, an agar diffusion method was performed as described previously (Brooks *et al.*, 2009), with the following modifications. Residual CTC was extracted by suspending known amounts (10-50 mg) of murine feces in 0.01 N HCl, which was followed by homogenization and centrifugation (12,000 x g, 20 min). The supernatant (i.e. acidic extract) was recovered, sterilized by filtration through a 0.2 μ m filter (Nalgene, Rochester, NY), and added to Triptic Soy Agar (TSA; BD, Franklin Lakes, NJ) in Petri dishes to which cells of *Staphylococcus aureus* strain ATCC 29213 were distributed over the agar surface. *S. aureus* cells were obtained from an overnight culture on TSA at 37 °C. Cells were suspended in Columbia broth (BD) and adjusted to an optical density (O.D.) equivalent to a 0.5 McFarland standard (O.D. 0.1 at λ 600 nm). A 100 μ L aliquot of the suspension of *S. aureus* cells were then spread on TSA. Regular spaced wells were perforated in the agar with a sterile

cork borer (4-mm diameter), and 50 µL of the acidic extract from feces was added to each well. Assays were carried out in triplicate for each sample, and the zones of inhibition in the *S. aureus* lawn were measured following 18 h of incubation at 37 °C (Appendix two). The diameters of the inhibition zones were measured in two directions at 90° to each other and an average value was calculated. Estimation of CTC concentrations in fecal samples was interpolated from standard curves of inhibitions zones obtained from wells to which known concentrations of CTC were added.

Characterization of bacterial communities by T-RFLP analysis²

Extraction of community DNA from intestinal samples, amplification of bacterial community 16S rDNA, restriction digestion of amplified rDNA, capillary gel electrophoresis, and analysis of T-RFLP electropherograms were conducted as described in Chapter 3. Extraction of community DNA from cattle digesta samples was performed with the QIAamp DNA Stool Kit according to the manufacturer's recommendations (Qiagen Sciences Inc., Germantown, MD).

Statistical analyses

Statistical analysis for selection of T-RFs ("true peaks") was performed with T-REX software for the analysis of T-RFLP data (Culman *et al.*, 2009), which provided highly automated processing of the T-RFLP data. The T-REX software was used for analyses of presence/absence of T-RFs and relative abundance; the latter used peak height data (relative fluorescence). Matrices produced by the T-REX software were imported into Bionumerics for cluster analyses and group significance tests, as described in Chapter 3. Cluster analysis for the relative abundance data (i.e. numerical data), was

² Kristen Reti (University of Lethbridge) conducted the T-RFLP analysis for the beef cattle component of this study.

performed with the Bray-Curtis coefficient, which corresponds to the Dice coefficient that is applied to binary data (i.e. presence/absence of T-RFs). Non-metric multidimensional scaling analysis (NMS) of the similarity matrices obtained from Bionumerics analysis was performed in the SAS software (SAS Institute Inc., Cary, NC). Group significance tests were applied to compare the composition of the microbiota among intestinal locations, and to compare communities associated with the digesta and mucosa of the cattle intestine. Samples were combined across locations that were not significantly different for subsequent comparisons of microbiota composition between treatments with NMS plots and group significance test. Venn diagrams with unique, common, and shared T-RFs were constructed with a macro in Excel software (Microsoft Corp., Redmond, Washington) to assess commonality of bacterial communities among treatments and between mucosa and digesta of cattle with information obtained from T-REX.

4.3 RESULTS

Distinctive microbiota composition along the intestinal tract of cattle

Analysis of T-RFLP data from digesta and mucosa of cattle revealed a distinctive microbiota composition between the small (P<0.001) and large intestine (P<0.001) for Control and AS700 treatments (Figure 4.2). For the mucosa, differences in microbiota composition were not observed among individual locations of the small intestine (P>0.05), and neither between the cecum and descending colon (P>0.05), regardless of treatment. However, the composition of the microbiota of the cecum (P=0.041) and rectum (P<0.001) were different than those of the descending colon. Thus, the locations



Figure 4.2. Non-metric multi-dimensional scaling applied to presence/absence T-RFLP data from mucosa and digesta of small and large intestine cattle. (A) mucosa, Control treatment; (B) mucosa, AS700 treatment; (C) digesta, Control treatment; and (D) digesta, AS700 treatment. (SI) small intestine; (LI) large intestine; (PJ) proximal jejunum; (CE) cecum; (AC) ascending colon; (DC) descending colon; (RE) rectum. Dashed-line ellipsoids in A and B contain mainly samples from the small intestine, with a few outliers from the large intestine. Closed-line ellipsoids in A and B comprise exclusively samples of the large intestine, with exception of one outlier in A. The dashed-line and closed-line ellipsoids in C and D represent the formation of unique groups (P<0.05) for the small and large intestine, respectively.

defined for comparison of treatments for cattle mucosa were: (i) small intestine, (ii) cecum, (iii) ascending colon, (iv) descending colon, and (iv) rectum. For digesta, there was no significant difference in microbiota composition between individual intestinal locations. Thus, the locations defined for comparison of treatments for cattle digesta were: i) small intestine and ii) large intestine.

AS700 treatment did not consistently affect intestinal microbiota of cattle

The composition of the microbiota varied amongst individual animals (Figure 4.3). There was no difference (P>0.05) between Control and AS700 treatments in the composition of the microbiota in the digesta of either the small or large intestine (Table 4.1, Figure 4.3). Similarly, there was no difference (P>0.05) between treatments in the composition of the microbiota associated with mucosa of the duodenum, central jejunum, distal jejunum, ileum, cecum, ascending colon, descending colon, and rectum. However, the composition of the microbiota associated with mucosa of the proximal jejunum differed (P<0.05) between the two treatments (Table 4.1, Figure 4.3).

Microbiota composition differed between mucosa and digesta of the cattle intestine

Based on analysis of presence/absence T-RFLP data, the composition of the microbiota in digesta differed (P<0.05) from that in association with adjacent mucosa in the small (i.e. distal jejunum and ileum) and large (i.e. cecum, descending colon) intestines (Figure 4.4, A, B), regardless of treatment. Venn diagram analysis further illustrated this difference (Figure 4.5). Similarly to the presence/absence T-RFLP data, analysis of the relative abundance T-RFLP data revealed differences (P<0.05) between bacterial communities associated with mucosa and in digesta (Figure 4.4, C, D).



Figure 4.3. Non-metric multi-dimensional scaling applied to presence/absence T-RFLP profiles from digesta and mucosa of the cattle intestine to compare the composition of bacterial communities between treatments. (A) mucosa, small intestine; (B) mucosa, large intestine; (C) digesta, small intestine; and (D) digesta, large intestine. (SI) small intestine; (PJ) proximal jejunum; (CE) cecum; (AC) ascending colon; (DC) descending colon; and (RE) rectum. No difference in microbiota composition was observed between treatments with the group significance test, with the exception of the mucosa of the proximal jejunum. Each data point corresponds to one individual sample. Inter-animal variability is observed by the dispersion of data points from the same treatment group.

	P values	
Digesta	Control	AS700
Small intestine	0.860	0.308
Large intestine	0.607	0.060
Mucosa		
Duodenum	0.300	0.834
Proximal jejunum	0.033*	0.066
Central jejunum	0.098	0.267
Distal jejunum	0.831	0.653
Ileum	0.834	0.171
Cecum	0.587	0.512
Ascending colon	0.074	0.751
Descending colon	0.343	0.955
Rectum	0.672	0.215

Table 4.1. Group significance analysis of T-RFLP community profiles (presence/absence data) by pairwise comparisons within cattle intestinal communities associated with digesta and mucosal samples

*Values represent unique groups



Figure 4.4. Non-metric multi-dimensional scaling applied to presence/absence and relative abundance T-RFLP profiles to compare bacterial community composition between mucosa (\bullet) and digesta (\bigcirc) from the small and large intestine of cattle. (A) presence/absence data, small intestine; (B) presence/absence data, large intestine; (C) relative abundance data, small intestine; and (D) relative abundance data, large intestine. Closed-line ellipsoids represent unique groups (P<0.05).



Figure 4.5. Venn diagram illustrating the distribution of unique, shared, and common T-RFs among intestinal mucosa and digesta of cattle from the Control and AS700 treatment. T-RFs were combined across animals from the same treatment (total = 178 T-RFs). (A) digesta, Control treatment; (B) digesta, AS700 treatment; (C) mucosa, Control treatment; (D) mucosa, AS700 treatment.

Distinctive microbiota composition along the intestinal tract of mice

Group significance test and ordination of presence/absence T-RFLP data by NMS revealed the formation of three distinct communities along the mouse intestinal tract: the upper small intestine (i.e. duodenum and jejunum), ileum, and large intestine (i.e. cecum, proximal colon, and distal colon; Figure 4.6). Bacterial communities associated with the mucosa of the ileum did not possess a unique microbiota composition for either treatment (P>0.05, Figure 4.6). Additionally, communities associated with mucosa in the upper small intestine and large intestine for the Control (Figure 4.6, A) and 2 mg L⁻¹ (Figure 4.6, B) treatments possessed a unique microbiota composition (P<0.05), whereas communities associated with the mucosa in the large intestine, but not the upper small intestine, possessed a unique microbiota composition within the 8 mg L⁻¹ (Figure 4.6, C) and 32 mg L⁻¹ (Figure 4.6, D) treatments (P<0.05). No significant difference (P>0.05) in microbiota composition was observed between the duodenum and jejunum, and neither among the cecum, proximal and distal colon.

CTC treatment did not consistently affect intestinal microbiota of mice

To assess the effect of CTC treatment on the composition of the intestinal microbiota, global (i.e. comparing treatments among all the defined locations; Table 4.2) and pairwise comparisons of T-RFLP data for the upper small intestine, ileum, and large intestine were performed. For the upper small intestine, the 2 mg L⁻¹ treatment mice possessed a unique microbiota composition relative to the other treatments (Table 4.2, Figure 4.7). Pairwise comparisons revealed no difference (P>0.05) among treatments in the upper small intestine. There was no significant difference between treatments in the ileum (Table 4.2, Figure 4.7). In the large intestine, both the 2 mg L⁻¹ and 8 mg L⁻¹



Figure 4.6. Non-metric multi-dimensional scaling applied to presence/absence T-RFLP profiles from mucosa of small and large intestine of mice. (A) Control treatment; (B) 2 mg L⁻¹ treatment; (C) 8 mg L⁻¹ treatment; (D) 32 mg L⁻¹ treatment; (\bullet) upper small intestine (i.e. duodenum and jejunum); (O) ileum; and (\blacksquare) large intestine (i.e. cecum, proximal and distal colon). Closed-line ellipsoids represent unique groups (*P*<0.05).

Comparisons	P value	
Upper small intestine		
Control versus (2 mg L^{-1} + 8 mg L^{-1} + 32 mg L^{-1})	0.086	
$2 \text{ mg } \text{L}^{-1} \text{ versus (Control} + 8 \text{ mg } \text{L}^{-1} + 32 \text{ mg } \text{L}^{-1})$	0.001*	
8 mg L^{-1} versus (Control + 2 mg L^{-1} + 32 mg L^{-1})	0.885	
$32 \text{ mg } \text{L}^{-1} \text{ versus (Control} + 2 \text{ mg } \text{L}^{-1} + 32 \text{ mg } \text{L}^{-1})$	0.137	
Ileum		
Control versus (2 mg L^{-1} + 8 mg L^{-1} + 32 mg L^{-1})	0.510	
$2 \text{ mg } \text{L}^{-1} \text{ versus (Control} + 8 \text{ mg } \text{L}^{-1} + 32 \text{ mg } \text{L}^{-1})$	0.073	
8 mg L^{-1} versus (Control + 2 mg L^{-1} + 32 mg L^{-1})	0.237	
$32 \text{ mg } \text{L}^{-1} \text{ versus (Control} + 2 \text{ mg } \text{L}^{-1} + 32 \text{ mg } \text{L}^{-1})$	0.449	
Large intestine		
Control versus (2 mg L^{-1} + 8 mg L^{-1} + 32 mg L^{-1})	0.100	
2 mg L^{-1} versus (Control + 8 mg L^{-1} + 32 mg L^{-1})	<0.001*	
8 mg L^{-1} versus (Control + 2 mg L^{-1} + 32 mg L^{-1})	0.047*	
$32 \text{ mg } \text{L}^{-1} \text{ versus (Control} + 2 \text{ mg } \text{L}^{-1} + 32 \text{ mg } \text{L}^{-1})$	0.306	

Table 4.2. Group significance analysis of T-RFLP community profiles (presence/absence data)

 by global comparisons within murine intestinal communities associated with the mucosa

* Values represent unique groups



Figure 4.7. Non-metric multi-dimensional scaling applied to presence/absence T-RFLP data from small (A; USI=upper small intestine; IL=ileum) and large intestine (B) of mice from the Control treatment, and of those that received various doses of CTC. Closed-line ellipsoid in (A) is composed mainly of samples from the ileum. Closed-line ellipsoid in (B) is composed of samples from mice that received 8 mg L⁻¹ CTC, which formed a unique group when globally to the other treatments, but not when compared to the 32 mg L⁻¹ treatment. Dashed-line ellipsoid in (B) is composed of samples from mice that received 2 mg L⁻¹ CTC, which formed a unique group when global and pairwise comparisons.

treatment mice possessed a unique microbiota composition relative to the other treatments (Table 4.2, Figure 4.7). Pairwise comparisons in the large intestine revealed that the 2 mg L⁻¹ treatment mice possessed a unique (P<0.05) microbiota composition relative to the Control, 8 mg L⁻¹, and 32 mg L⁻¹ treatment mice, whereas the 8 mg L⁻¹ treatment mice possessed a unique (P<0.05) microbiota composition relative to the Control and 2 mg L⁻¹ treatment mice (Figure 4.7). A considerable degree of inter-animal variability was observed. For instance, there was a higher similarity among samples from different locations within the same mouse than among samples of the same location from different mice (Figure 4.8, Control treatment).

Residual CTC in murine feces

Residual CTC was detected in acidic extracts of feces using the bacterial sensitivity bioassay. Mean zones of inhibition from mice administered CTC were $3.2 \pm 2.0 \ \mu g \ g^{-1}$ feces of mice from the 8 mg L⁻¹ treatment, and $19.4 \pm 3.3 \ \mu g \ g^{-1}$ feces of mice from the 32 mg L⁻¹ treatment. Residual CTC could not be detected in feces of mice from the 2 mg L⁻¹ treatment. No inhibitory activity was detected in acidic extracts prepared from feces of mice from the Control treatment.

4.4 DISCUSSION

In the present study, oral administration of non-therapeutic concentrations of CTC and sulfamethazine (i.e. AS700) did not significantly affect the composition of bacterial communities in association with the digesta and mucosa of the cattle intestine, with exception of those associated with the mucosa of the proximal jejunum. Similarly, the highest non-therapeutic dose of CTC (i.e. 32 mg L^{-1}) did not affect the composition of



Figure 4.8. Dendrogram of bacterial community relatedness generated from presence/absence T-RFLP data of the intestinal mucosa of mice not administered CTC. Cluster analysis was performed by Dice's coefficient (>50% mean) and UPGMA algorithm. Acronyms represent the intestinal location of each sample: (DU) duodenum; (JE) jejunum; (IL) ileum; (CE) cecum; (PC) proximal colon; and (DC) distal colon. Symbols represent individual mice that were not administered CTC: (\bullet) mouse 1; (\blacksquare) mouse 2; and (\blacktriangle) mouse 3.

bacterial communities associated with the intestinal mucosa of mice, and the 2 mg L^{-1} and 8 mg L^{-1} did not consistently affect the intestinal microbiota. These findings are not congruent with the microbiota modulation hypothesis as the primary mechanism of action of AGP (Dibner and Richards, 2005).

Consistent with the assumption that little CTC would reach the large intestine due to absorption in the stomach and small intestine, oral administration of non-therapeutic concentrations of AS700 only affected the composition of the mucosa-associated microbiota of the proximal jejunum of cattle in the current study. It has been reported that the administration of non-therapeutic doses of CTC increased the frequency of tetracycline-resistant Campylobacter jejuni and C. hyointestinalis in cattle feces (Inglis et al., 2005), and that the oral administration of CTC concentrations as low as 1 mg L^{-1} to human-flora-associated mice resulted in an increase of cultivable tetracycline resistant bacterial species in the feces (Perrin-Guyomard et al., 2001). In another study, however, non-therapeutic CTC reduced bacterial community diversity in rat feces, but did not affect community abundance or the tetracycline resistance gene copy number in the bacterial community (Brooks et al., 2009). A recent study (16S rRNA gene libraries) reported that oral administration of growth-promoting doses of tylosin and virginiamycin did not affect the fecal community composition of swine (Kalmokoff et al., 2010). These researchers attributed the discrepancy of antimicrobial effects on the fecal microbiota between rats and swine to higher levels of endogenous resistance within the bacterial community of intensively raised swine. All of the above studies examined fecal communities, which are substantially different from bacterial communities found in the digesta or associated with the mucosa of the intestine (Zoetendal et al., 2002). Changes in

the composition of the mucosa-associated bacterial communities in the ilea of piglets due to the administration of non-therapeutic concentrations of CTC (i.e. by 16S rDNA library analysis) have been reported (Rettedal *et al.*, 2009). However, logistical limitations of library analysis, coupled with high inter-animal variability, obscured conclusions as to the real effect on the ileal communities by CTC in this study.

The literature regarding composition of the intestinal microbiota of cattle is scarce. The use of T-RFLP in the current study enabled empirical assessments of AGP effects on the intestinal microbiota composition, as well as a comprehensive characterization of murine and cattle intestinal bacterial communities along the intestinal tract. A salient finding of the current study was the obvious distinct difference in composition of bacterial communities between the digesta- and mucosa-associated microbiota of cattle, which is in concordance with previous findings in pigs (Pryde et al., 1999; Rettedal et al., 2009; Simpson et al., 1999). Differences between fecal- and mucosa-associated microbiota have also been reported in humans (Eckburg *et al.*, 2005; Lepage *et al.*, 2005; Turnbaugh et al., 2009). The current study also revealed a clear distinction in bacterial community composition between the small and large intestine of mice and cattle. The GI tract comprises a series of various distinct anatomic organs with specific functions, which determine the structure and composition of the microbiota (Savage, 1977). The GI tract is subject to several physiologic processes, such as oxygenation, different passage rates of digesta (He et al., 1999), as well as the influence of the gut-associated lymphoid tissue (Cebra *et al.*, 1998), which relate to the substantial heterogeneity of the microbiota along its length. The areas of stagnation in the flowing stream of digesta at the cecum reflect the development of enormous microbial communities at this location, and the decreased

oxygen availability along the intestinal tract is consistent with the presence of mainly anaerobic microbial communities in the large intestine, especially in the colon (Savage, 1977), which distinguishes it from the small intestine. Another important component of the GI tract is the mucus layer, which contains antimicrobial peptides (Meyer-Hoffert et al., 2008), serves as nutrient source, and as protective layer from infective bacteria (McAuley *et al.*, 2007) and mechanical stress, among other functions (Figure 1.3; Atuma et al., 2001). The association of bacteria with the mucus is intrinsic in the intestine, and the difference in thickness of the mucus layer along the intestinal tract (Atuma *et al.*, 2001) may also contribute to the heterogeneity in the microbiota composition in each location of the GI tract. Autochthonous (i.e. indigenous) microorganisms associated with the mucosal surfaces do not exclusively comprise the GI microbiota; allochthonous (i.e. exogenous) transients present in the digesta may also contribute to digest the host's food and to provide products of fermentation that can be absorbed and used as energy sources by the host (Savage, 1987). Unfortunately, community assessments of digesta may not give a good indication of the resident microbiota due to the high level of transient microorganisms, especially in locations with high passage rate. In the current study, the microbiota associated with the small intestine of mice was more variable in composition among replicates than the microbiota associated with the murine large intestine. Furthermore, bacterial communities associated with the mucosa of the murine ileum were most variable, and were distinct from the mucosal microbiota of both the upper small intestine and the large intestine. Relative to the large intestine, it is possible that the microbiota of the upper small intestine in mice is comprised to a greater degree by allochthonous bacteria ingested by coprophagy (Savage, 1987). Accordingly, the majority

of antigenic exposure in the upper small intestine is thought to come from diet components and not bacteria, whereas the ileum and large intestine comprise additional antigenic load from a more abundant microbiota (MacDonald and Monteleone, 2005). Communities of bacteria are thought to be less diverse and with smaller populations in the small relative to the large intestine as a result of the faster passage rate of digesta (Macfarlane and Macfarlane, 2009) and reduced fermentation (Gibson *et al.*, 1996). The largest numbers of bacteria in the small intestine are typically found in the ileum, probably due to an influx of cecal bacteria through the ileo-cecal valve (Savage, 1977).

Varied levels of inter-animal variability were detected in the present study, despite the use of mice and cattle from the same breed, age, gender, and subject to the same diet and experimental conditions. Inter-individual variability has been previously reported in the microbiota of cattle (Durso *et al.*, 2010), swine (Rettedal *et al.*, 2009; Thompson *et al.*, 2008), and humans (Eckburg *et al.*, 2005; Turnbaugh *et al.*, 2009; Zoetendal *et al.*, 2001), which challenges the core microbiome hypothesis (Turnbaugh *et al.*, 2007). Although CTC administration at the dose of 32 mg L⁻¹ had no effect on the composition of the intestinal microbiota of mice in the current study, unexplainably the lowest dose of CTC (i.e. 2 mg L⁻¹) affected the microbiota of the upper small intestine, whereas 8 mg L⁻¹ affected the microbiota of the large intestine. No residual CTC was detected in feces of mice given 2 mg L⁻¹, so it remains to be elucidated whether lower doses of CTC differentially affect mucosa-associated communities in mice, or the inter-animal variability encountered obscured changes in the composition of the microbiota.

In summary, the current study demonstrated that there was no substantive or consistent effects of AGP (i.e. CTC and sulfamethazine for cattle; CTC for mice)

administration on the microbiota of beef cattle intestines, nor on the composition of bacterial communities associated with the mucosa of the murine intestine. These findings are not consistent with the microbiota modulation being the main mechanism of AGP action in mammalian livestock. Thus, alternate mechanisms, including the involvement of host responses to AGP (Niewold, 2007), should be examined.

ACKNOWLEDGEMENTS

I thank Kristen Reti (University of Lethbridge) for conducting the beef cattle T-RFLP analyses; Dr. John Kastelic (AAFC) for assisting with extrapolation of CTC doses; Tara Shelton (AAFC) for assistance with mouse handling and sample collection; Matt Thomas (University of Lethbridge) for assistance with NMS analyses; and Toby Entz (AAFC) for assistance with statistical analyses. This work was funded by an AAFC Peer Review Project Grant to Dr. Doug Inglis, and a Discovery Grant to Dr. Doug Inglis, Dr. Brent Selinger and Dr. John Kastelic from the AFMNet.

CHAPTER FIVE

Non-therapeutic concentrations of orally administered chlortetracycline modulate immune responses to *Citrobacter rodentium* in mice

ABSTRACT

The development of efficacious alternatives to AGP in livestock production is an urgent issue, but is hampered by a lack of knowledge regarding the mode of action of AGP. The belief that AGP modulate the intestinal microbiota has become prominent in the literature; however, there is a lack of experimental evidence to support this hypothesis. Employing a *Citrobacter rodentium*-murine-CTC model, the ability of AGP to modulate the host immune system was investigated. Oral administration of non-therapeutic doses of CTC regulated transcription levels of inflammatory cytokines in *C. rodentium*-infected mice, and minimized weight loss associated with infection. In addition, infected mice that received CTC presented with less pathologic changes in the distal colon than mice that did not receive CTC, relative to untreated mice. Furthermore, CTC administration did not impart profound or consistent effects on the colonic microbiota. The findings of this study supported the hypothesis that AGP function by modulating the host immune system.

5.1 INTRODUCTION

Animal production is an important segment of the global agricultural economy. The growing demand for high quality animal products has necessitated the development of means to enhance animal growth and thus optimize costs of production. An extensively

used method to promote growth of livestock and poultry is the in-feed administration of AGP, which are used at non-therapeutic concentrations. The use of AGP in animal production commenced more than 60 years ago (Moore *et al.*, 1946). The benefits that AGP provide to animal performance and to the food production chain have resulted in their indiscriminate use in agriculture and animal production systems (Khachatourians, 1998), and have contributed to the emergence of AMR in zoonotic pathogens (Conly, 2002; Funk et al., 2006; Inglis et al., 2005). Many AGP, including tetracyclines, are used in human medicine for treatment of various infectious diseases (U.S senate bill S. 2508, 2002). The potential for transmission of resistant bacteria from agricultural environments to humans have resulted in mounting societal concerns regarding AGP use (Khachatourians, 1998). From 1986 to 2006, the European Union implemented a ban on all AGP (Casewell et al., 2003; Castanon, 2007). The European AGP ban has resulted in a number of adverse consequences including a deterioration of animal health, an increase in the therapeutic administration of AMA (Casewell et al., 2003), an increase in the cost of animal production, and a general decline in livestock production (Jensen and Hayes, 2003). The development of efficacious alternatives is an urgent matter, but is hampered by the current dearth of knowledge on the mode of action of AGP. It is clear that the sustainability of livestock production relies on the development of suitable and efficacious alternatives to AGP, and elucidating the mode of action of AGP will facilitate the development of biorationale-based alternatives.

The literature on the mode of action of AGP is scarce, and the most accepted hypothesis is that AGP modulate the intestinal microbiota (Dibner and Richards, 2005; Gaskins *et al.*, 2002). The "microbiota modulation" hypothesis suggests that AGP

reduces microbial competition for nutrients, results in fewer growth-depressing metabolites produced by microbial agents, suppresses opportunistic pathogens, results in a thinner intestinal wall, and thereby increases nutrient assimilation (Dibner and Richards, 2005). The absence of growth-promoting effects by AGP in germ-free animals (Coates *et al.*, 1963) has been used as additional support for the microbiota modulation hypothesis. Despite general acceptance of this hypothesis, there has been no comprehensive assessment that definitively indicates that the administration of AMA in growth-promoting doses imparts substantive modification of intestinal microbiota.

The consistency of growth-promoting effects of AGP on different animals possessing highly dissimilar intestinal microbiota compositions, and that AGP are typically administered at doses that are lower than the minimum inhibitory concentration for most pathogens, challenges the microbiota modulation hypothesis of AGP action (Niewold, 2007). Since many AMA have anti-inflammatory and immunomodulatory properties (Rubin and Tamaoki, 2005), an alternate hypothesis for the mode of action of AGP was recently proposed (Niewold, 2007). This alternate hypothesis indicates that AGP decrease the immunologic stress in the host (Klasing and Korver, 1997). The intestinal mucosa is in a constant state of "physiologic inflammation" (Ma et al., 2003), which is attributable to the close contact of the intestinal mucosa with the microbiota (Biancone et al., 2002). A decrease in the immunologic stress in intestinal mucosa by AGP would reduce the catabolic cost to the host and result in more energy available for muscle development, thereby improving growth performance (Roura et al., 1992). The "immunomodulation hypothesis" for AGP action is consistent with the growth-promotion effects observed when AGP is administered to animals possessing very dissimilar

intestinal microbiota compositions (Niewold, 2007). To my knowledge, the validity of the immunomodulation hypothesis has not been experimentally addressed.

Citrobacter rodentium is a non-invasive, attaching/effacing bacterial pathogen that causes a self-limiting acute colitis in immunocompetent laboratory mice (Barthold *et al.*, 1978; Mundy *et al.*, 2005), and has been widely accepted as a robust *in vivo* model system to assess host-pathogen interactions (Borenshtein *et al.*, 2008; Mundy *et al.*, 2005). *C. rodentium* colonizes the apical surface of the large intestinal mucosa, causing mucosal hyperplasia, localized microvilli loss, and mucosal inflammation (Luperchio and Schauer, 2001), and was used in the present study as an inflammation inciter.

The primary objective of the current study was to test the hypothesis that orally administered non-therapeutic doses of CTC modulate intestinal inflammatory responses in mice infected with *C. rodentium*. The effects of CTC, *C. rodentium* infection, and their interaction, on the composition of the murine intestinal microbiota were also assessed by T-RFLP analysis. To our knowledge, this is the first study to assess a potential interaction of AGP with the host immune responses concomitantly with potential AGP effects in the intestinal microbiota composition. This study furthers our understanding of the mechanisms of action of growth promotion by AGP, and will facilitate the development of efficacious alternatives.

5.2 MATERIALS AND METHODS

Mice and treatments

Forty eight female 4-week-old C57BL/6J SPF mice were purchased from Charles River Laboratories International, Inc. (Montreal, QC). Mice were housed at the small animal facility located at AAFC LRC. All requirements specified by the CCAC were met, and the project was approved by the LRC ACC before commencement of the study (Animal Use Protocol Review 0915). Mice were housed in sterilized, filter-topped cages throughout the experiment, with light cycle specified as in Chapter 4. Mice were randomly assigned to one of the following treatments: (i) no CTC, no inoculation (Control treatment); (ii) 32 mg L^{-1} CTC, no inoculation (CTC treatment); (iii) 32 mg L^{-1} CTC, C. rodentium inoculation (CTC+CR treatment); and (iv) no CTC, C. rodentium inoculation (CR treatment). The 32 mg L^{-1} CTC was derived from the non-therapeutic dose of CTC administered to mammalian livestock (i.e. cattle; Chapter 4). Administration of CTC to mice commenced 3 weeks before inoculation with C. rodentium, and continued for the remainder of the experimental period. CTC was added to autoclaved drinking water, and fresh solutions were provided twice a week. Control and CR treatment mice were provided with autoclaved water alone. Sucrose (5% w/v; Sigma, St. Louis, MO) was added to all water solutions to enhance palatability, and water bottles were covered with aluminum foil.

Citrobacter rodentium

C. rodentium (ATCC 51459) was transformed with a gene that confers resistance to tetracycline. Plasmid pMEK91, a *Campylobacter* shuttle vector that carries the *tet*O gene (Mixter *et al.*, 2003), was modified to remove the green fluorescent protein gene through *Eco*RI digestion and re-ligation. This vector was transferred into *C. rodentium* by electroporation using a protocol described previously (Shuli *et al.*, 2007). Briefly, Luria-Bertani (LB) broth (10 mL; BD, Franklin Lakes, NJ) was inoculated with an overnight culture of *C. rodentium* (5% v/v) and incubated for 2 h at 37 °C and 100 rpm. Cells were

harvested by centrifugation at 7000 x g for 2 min, and washed twice with ice-cold sterile optima water. Cells were suspended in 100 μ L of ice-cold 10% glycerol, and an 80 μ L aliquot was mixed with 10 µL of plasmid DNA, loaded in a 1 mm gap electroporation cuvette (Fisher Scientific Inc., Edmonton, AB), and incubated on ice for 20 min. Electroporation was performed with a model 2510 (Eppendorf Canada Ltd., Toronto, ON) at a pulse of 1.5 kV, followed by the immediate addition of Super Optimal broth with Catabolite repression (SOC; 1 M glucose, 2 M magnesium chloride, 2 M magnesium sulphate and 1 M potassium chloride in 10 mL LB broth). The electroporated cells were incubated for 1 h at 37 °C and 100 rpm, plated on LB agar dishes containing 50 µg mL⁻¹ tetracycline hydrochloride (Sigma, St. Louis, MO), and incubated overnight at 37 °C. To confirm resistance to tetracycline in the transformed strain, a minimum inhibitory concentration test was performed as previously described for tetracycline hydrochloride (Inglis et al., 2005); Escherichia coli (ATCC 25922) was used as the quality control strain. To test the growth abilities of the transformed strain, and to determine the optimal growth time to prepare inocula, a 24 h growth curve was produced for the transformed C. rodentium, and compared to a growth curve produced for the wildtype C. rodentium (Appendix three). To prepare the growth curve, an overnight culture of C. rodentium (LB broth, 37 °C, 100 rpm) was added to LB broth (5% v/v) and incubated at 37 °C and 100 rpm. Culture samples were collected for optical density determination and C. rodentium enumeration every 2 h for the first 12 h, and at 24 h. For each time point, 100 µL of C. rodentium culture was spread onto MacConkey agar, incubated at 37 °C, and enumerated at the dilution yielding 30-300 colony-forming units (CFU) dish⁻¹. The transformed *C. rodentium* was cultured in LB broth with 50 μ g mL⁻¹ of tetracycline.

Inoculation and maintenance of mice

To prepare inocula, transformed *C. rodentium* cells were cultured in LB broth containing 50 μ g mL⁻¹ of tetracycline for 16 h at 37 °C and 100 rpm, centrifuged for 5 min at 1600 x g, the supernatant removed, and the pelleted cells resuspended in sterile phosphate buffer saline (PBS; 10 mM sodium phosphate buffer, 130 mM sodium chloride; pH 7.2) to a final concentration of 10⁹ CFU mL⁻¹. Sodium bicarbonate (2% w/v) was added to the inoculum. Mice (CR and CR+CTC treatments) were inoculated with 100 μ L (10⁸ CFU) of the inoculum by oral gavage on two consecutive days. Uninoculated mice (Control and CTC treatments) were orally gavaged with an equal volume of PBS containing 2% sodium bicarbonate on the same days. Mice were provided with food and water *ad libitum*, and were weighed twice a week. In addition, mice were monitored for signs of disease according to the ACC criteria for stress assessment.

Collection of feces and tissues

Feces were collected aseptically from all mice once a week for estimating residual CTC, and every second day commencing 1 day after the initial gavage inoculation for enumeration of *C. rodentium*. Mice were humanely euthanized by anesthesia with isofluorane (Halocarbon Products Corporation, River Edge, NJ) followed by cervical dislocation 3, 8, 14, and 21 days post-inoculation (p.i), corresponding to early, peak, late infection, and clearance period, respectively (Mundy *et al.*, 2005). Twelve mice were euthanized at each time point (i.e. three mice per treatment; n=48 total). After euthanasia, a midline incision was made, and the entire colon was rapidly harvested and examined for alterations in macroscopic appearance (i.e. thickening of the distal colon; Higgins *et al.*, 1999). Four 1-cm sections from the colon were then collected (distal to cranial) for

histology, quantification of cytokine mRNA expression, characterization of colonic microbiota (i.e. using T-RFLP analysis), and *C. rodentium* enumeration, respectively. Colon samples collected for histology were preserved in 10% buffered formalin. Samples collected for RNA extraction were inserted in sterile microcentrifuge tubes containing RNAlater (Qiagen Inc., Mississauga, ON) and kept at -20 °C until processed; tissues were placed in RNAlater within 2-3 min of euthanasia. For T-RFLP analysis, the colon was cut-open longitudinally, the mucosal surface was gently rinsed with chilled sterile PBS, a tissue sample was aseptically removed with a sterile 3-mm-diameter Biopsy Acu-Punch (CDMV, St. Hyacinthe, QC), and the sample was placed at -20 °C until processed. Colonic samples collected for enumeration of *C. rodentium* were placed on ice immediately after collection, and processed within 2 h.

Estimation of residual CTC in murine feces

Residual CTC in murine feces was measured as described previously in Chapter 4 with the following modification. The diameters of the inhibition zones were measured using a Biomic V3 Image Analyzer (Giles Scientific USA, Santa Barbara, CA), with slight adjustments performed by hand.

Enumeration of C. rodentium

Densities of *C. rodentium* cells were determined by homogenizing feces or colonic samples in Columbia broth, and spreading serial dilutions of the homogenate onto MacConkey agar (BD). Incubation of cultures and enumeration of colonies were performed as described above. *C. rodentium* colonies were identified based on morphology (Vallance *et al.*, 2002), and representative colonies exhibiting characteristic morphology (i.e. an average of three colonies per Petri dish) were subcultured to confirm their identity and the presence of the *tet*O gene by PCR. Reaction mixtures for PCR consisted of 2 μ L of a suspension of cells in 20 μ L of optima water, 1X PCR buffer, 0.2 mM of each deoxynucleoside triphosphate, 0.1 μ g μ L⁻¹ of acetylated bovine serum albumin (Promega, Madison, WI), 0.625 U of Taq DNA polymerase (Qiagen Inc.), 0.5 μ M of each primer, and optima water to a final volume of 20 μ L. For *C. rodentium* identification, the Cr-espB-f (5'-AAGTCTGTCAATACCGCCTC-3') and Cr-espB-r (5'-AATGTGCCAACTGTCTCATC-3') primers were used (Maaser *et al.*, 2004). For *tet*O gene detection, the *tet*O-F-PstI (5'-TAA CTG CAG AGA TTC AGT ATT ATA ACA AGG-3'), and *tet*O-R-PstI (5'-TTA CTG CAG CAT CAT AAT TAT CTC TAA TCC-3') primers were used (Mixter *et al.*, 2003).

Histopathology

Tissue samples were maintained in 10% buffered formalin for a minimum of 4 h and for a maximum of 2 weeks. Tissue samples were dehydrated with ethanol and Histoclear (Fisher Scientific Inc.), and paraffinized with Paraplast Plus (Fisher Scientific Inc.) for 2 h at 60 °C in a vacuum oven. Samples were embedded using a Shandon Histocentre III (Fisher Scientific Inc.), sectioned (4 µm) using a Finesse 325 microtome (Fisher Scientific Inc.), and sections were placed on Superfrost Plus Gold slides (Fisher Scientific Inc.). Sections were deparaffinized with xylene, stained with hematoxylin and eosin (H&E) following a standard protocol, and examined with a Zeiss Axioskop III (Carl Zeiss Canada Ltd., Toronto, ON). Images were captured using an Axiocam camera (Carl Zeiss Canada Ltd.). Histological inflammation scoring was performed in a "blinded" fashion (i.e. as to treatment) by a Veterinary Pathologist (Dr. Richard R. E. Uwiera, University of Alberta), with scoring criteria adapted from previously described

methods (Barthold *et al.*, 1978; Wu *et al.*, 2008) as shown in Table 5.1. The total pathology score was obtained by calculating the sum of scores for all categories for each mouse. Epithelial hyperplasia caused by *C. rodentium* (i.e. crypt height) was quantified by calculating the average of 10 measurements of well-oriented crypts for each section.

Quantification of mRNA expression of cytokine genes

Total RNA was extracted from mouse colonic tissues with TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's recommendations. Residual genomic DNA contamination was removed using a DNA purification protocol from RNeasy kit (Qiagen Inc.). After quantification on an Ultrospect 3100 pro UV/Visible spectrophotometer (General Electric Healthcare, Piscataway, NJ), 1 µg of total RNA was reverse transcribed into cDNA using the RT² First Strand Kit (SABiosciences Corp., Frederick, MD). Quality assurance and control of total RNA was performed with the RT² RNA OC PCR Arrays Kit. A custom RT² Profiler PCR Array System (SABiosciences Corp.) was used to quantify the mRNA expression of the following cytokines: interleukin (IL)-1 β , IL-2, tumor necrosis factor (TNF)- α , interferon (IFN)-γ, IL-6, IL-4, IL-10, IL-17A, IL-22, and transforming growth factor (TGF)-β1. Two housekeeping genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and actin- β were used to provide an estimate of the range of threshold cycles to be expected in subsequent PCR array analyses. The housekeeping gene that presented the least variation of expression among samples with the quality control kit was used to normalize the data. Real-time PCR was performed using an MxPro 3005 thermocycler (Agilent Technologies, Santa Clara, CA). To prepare cytokine mRNA expression data for the comparative cross-threshold (Ct) method and ANOVA, Ct values of the 10 cytokine
Table 5.1. Description	1 of the his	tological inflammation scoring	g criteria applied to cross-sect	tions of the murine d	istal colon	for this study
Scores	0	1	2	3		4
Epithelial cell hyperplasia	None	Mild, crypts with two times the normal amount of cells, focal areas of hyperplasia with areas of normal mucosa	Moderate, three times the normal amount of cells, crowding of cells at the bases of the crypts	Marked, four tim normal amount of	es the f cells	Severe, five times the normal amount of cells, villous distortion, focal dysplasia of epithelial surface
Crypt height	Normal	Mild, 25% increase	Moderate, 50% increase	Marked, 100% in	crease	Severe, more than 100% increase
Epithelial injury	None	Superficial, shedding of less than 10 surface epithelial cells	Moderate, focal erosions, shedding of 11-20 surface epithelial cells	Marked, multi-f erosions of epitheli	ocal al cells	Severe, multi-focal crosions with deep crypt necrosis
Inflammatory infiltrates	Absent	Rare to small numbers of neutrophils within the lamina propria	Moderate numbers within the lamina propria	Large numbers of the lamina proj	within Dria	Large numbers within the lamina propria, submucosa, and possibly muscularis externa and serosa
Scores	0	1	2			3
Mitotic activity of epithelial cells	Normal	Small increase in mitotic acti the basal half of crypt epitheli	ivity in Moderate increase lial cells in the basal half c cel	in mitotic activity of crypt epithelial lls	Promin activity th of (nent increase in mitotic at extends the entire length erypt epithelial cells
Goblet cell depletion	Normal	Low, smaller size of mucin d	droplets Evident, marked d	ecrease of the size droplets		Complete

genes of interest were normalized with GAPDH transcript data (i.e. GAPDH transcript levels, which presented less variation in expression than the β -actin in the quality assurance analysis). Normalized data (Δ Ct values) were then inserted as an exponential function of two, to account for the fold change of amplicons at each PCR cycle. In order to apply the experimental design to the mRNA expression data and to include control values in the analysis, Ct values were normalized only with the housekeeping gene and not with the Control Ct values. All cytokine mRNA expression data was adjusted and log-transformed (i.e. log [2^{- Δ Ct} + 1]) before statistical analysis.

Characterization of bacterial communities

Total genomic DNA was extracted from colonic samples using the Gram-positive bacteria protocol of the DNeasy Blood and Tissue Kit (Qiagen Inc.). Amplification of the 16S rRNA genes for T-RFLP analysis, restriction of amplified rRNA genes, capillary gel electrophoresis, fragment size determination, and analyses were conducted as described previously in Chapter 4.

Statistical Analyses

The experiment was designed as a randomized complete block design with (i) two levels of treatment (i.e. CTC or no CTC, and *C. rodentium* or no *C. rodentium*); (ii) four (i.e. for *C. rodentium* CFU associated with colonic mucosa, cytokine mRNA expression, and colonic crypt height), six (i.e. for murine body weights), or eleven (i.e. for *C. rodentium* CFU in murine feces) levels of time; and (iii) three levels of block as the three replicates were conducted on separate occasions and thus were independent. Data analyses of T-RFLP data were performed as described in Chapter 4. All data were analyzed using SAS software (SAS Institute Inc., Cary, NC). For parametric data (i.e.

CFU counts in feces and colon, murine weight, and cytokine mRNA expression), ANOVA was performed using the MIXED procedure of SAS, with treatment one (i.e. CTC or no CTC), treatment two (i.e. *C. rodentium* or no *C. rodentium*), time, and their interaction, included in the model as fixed effects. Differences among means of interest were compared through the generation of least-square means with Fisher's protected least significant difference test. For *C. rodentium* CFU counts in feces and murine body weights, the repeated-measurement statement was applied, and the proper error structure was determined using Akaike's information criterion and the Bayesian information criterion. In all instances, the UNIVARIATE procedure of SAS was used to produce normal probability plots to confirm normality and to identify outliers, which were removed before completion of the final analysis. For non-parametric data (i.e. histological inflammation scoring data for individual category scores and total scores), the NPAR1WAY procedure of SAS with the Wilcoxon scores (ranks sums) for variable score and one-sided Wilcoxon two-sample test were performed.

5.3 RESULTS

Residual CTC in murine feces

Residual CTC was detected in acidic extracts of feces from mice administered CTC, and no difference was observed between the CTC and CTC+CR treatments (P>0.05). Mean residual CTC value was $63.9 \pm 6.6 \ \mu g \ g^{-1}$ of feces for CTC treatment mice, and $63.3 \pm 9.6 \ \mu g \ g^{-1}$ of feces for CTC+CR treatment mice. No evidence of CTC was observed in acidic extracts prepared from feces of mice that were not administered CTC.

Administration of CTC did not affect C. rodentium colonization

Considerable numbers of *C. rodentium* cells were recovered from feces and colonic mucosa of inoculated mice throughout the experiment (Figure 5.1). No *C. rodentium* cells were recovered from uninoculated mice. Densities of *C. rodentium* cells recovered from feces and mucosal surfaces increased over time (P<0.001); population sizes peaked between 9 and 11 days p.i., and decreased thereafter until no cells were detected 21 days p.i. (Figure 5.1). Relative to the CR treatment, the administration of CTC did not affect (P=0.81) densities of *C. rodentium* CFU in murine feces (P=0.84) at all sample times (Figure 5.1, A). With the exception of the 3 day p.i. sample time (P<0.05), there was also no difference (P>0.05) between treatments in densities of *C. rodentium* CFU associated with colonic mucosa (Figure 5.1, B).

Stability of plasmid containing the tetO gene in transformed C. rodentium

All *C. rodentium* cells recovered and evaluated from the feces of CTC+CR treatment mice possessed the *tet*O gene until day 19 p.i., but did not present the plasmid at day 21 p.i. All *C. rodentium* cells recovered and evaluated from CR treatment mice possessed the *tet*O gene until day 9 p.i.; 50% of cells possessed the *tet*O gene at days 11 and 13 p.i., and no cells possessed the *tet*O gene at days 15, 17, 19, and 21 p.i.

Administration of CTC prevented C. rodentium-induced weight loss

Mice infected with *C. rodentium* (i.e. CR treatment and CTC+CR treatment) did not present *ante mortem* signs of disease. However, CR treatment mice weighed less (P<0.05) than Control treatment mice on days 7, 10, and 14 p.i. (Figure 5.2). Mice infected with *C. rodentium* and administered CTC (i.e. CTC+CR treatment) weighed less (P<0.05) than Control mice only on day 10 p.i., and weighed more (P<0.05) than CR



Figure 5.1. Quantities of *C. rodentium* cells in feces (A) and associated with colonic mucosa (B). Vertical lines indicate standard error of the means (n=3). * Values differ (P<0.05).



Figure 5.2. Change in body weight. Vertical bars associated with means indicate the standard error of the mean. *P<0.05 when compared with Control mice; ** P<0.05 when compared with Control or CTC treatment mice; *** P<0.05 when compared with Control, CTC or CTC+CR treatment mice.

treatment mice on day 14 p.i. There was no difference (P>0.05) in body weight between the Control and CTC treatment mice.

Administration of CTC decreased pathologic changes caused by C. rodentium

Gross observations indicated thickening of the distal colon of approximately two thirds of CR and CTC+CR treatment mice at day 8 p.i. Conspicuous thickening of the distal colon was evident 14 days p.i. in all CR treatment mice and approximately two thirds of CTC+CR treatment mice (Figure 5.3). Histopathological analysis of colonic cross-sections revealed that all C. rodentium-infected mice (i.e. CR and CTC+CR treatments) had pathologic changes relative to Control mice (Table 5.2, Figure 5.4). Levels of inflammation increased throughout the infection period, with the highest scores of inflammation observed 14 days p.i. Notably, transmural inflammation and eosinophilic bacterial colonies were observed only in the colons of CR treatment mice at day 14 p.i. (Figure 5.4). Infection by C. rodentium increased (P<0.05) crypt height, which was first evident 8 days p.i., and crypt height remained significantly different relative to the Control and CTC treatment mice throughout the remainder of the experimental period (Figure 5.5). Crypt height reached a maximum 14 days p.i. (*P*<0.05, Figure 5.5). There was no difference (P>0.05) in crypt height between the CTC+CR and CR treatments, or between the Control and CTC treatments. Total pathology scores and individual category scores representing tissue changes did not differ (P>0.05) between treatments at day 3 p.i. (Table 5.2). At subsequent sample times there was no difference (P>0.05) between Control and CTC treatments, nor between CTC+CR and CR treatments. At day 8 p.i., total score and all individual category scores differed (P<0.05) for the CTC+CR and CR treatments relative to the Control treatment. Although there was no significant difference



Figure 5.3. Gross pathology of the murine large intestine (cecum to rectum). No difference in thickness of the descending colon was observed between Control (A) and CTC (B) treatment mice. All *C. rodentium*-infected mice (D) had conspicuous thickening of the distal colon (arrow), which was not observed in all infected mice that received CTC (C). Horizontal bar corresponds to 1 cm.

Table 5. colon, sh	2. Non-pi	arametric nge of sco	statistical i pres (top) a	analysis of $nd P$ valu	f histo es (bo	logical in ttom, Kr	nflammati uskal Wal	on scorir lis, Chi-s	ig perfoi square)	rmed in	cross-sec	tions of	the mur	ine dista	۲.
Groups	8	Control			C	TC			CTC+	-CR			บ	R	
Days	3	8 14	21	3	8	14	21	3	8	14	21	3	8	14	21
EH	0	0 0	0-1	0-1	0-1	0-1	0	0	2	1-3	0-1	0-1	1-2	2-4	1-2
CH	0	0 0	0	0-1	0	0-1	0	0-1	2-3	1-3	0-2	0-1	5	2-4	1-2
II	0	-1 0	0	0	0	0	0	0	3-4	1-4	0-2	0	2-4	3-4	1-4
EI	0	0 0	0	0-1	0	0	0	0	ю	0-4	0-2	0	3-4	2-3	0-2
MA	0	-1 0	0	0	0	0	0	0-1	2-3	1-3	1-2	0	2-3	С	П
GC	0	0 0	0	0	0	0	0	0	1-2	0-3	0	0	1-2	2-3	0-1
Total	0 0	-1 0	0-1	0-2	0-2	0-2	0-1	0-1	14-16	4-20	1-9	0-1	13-15	16-20	4-12
Groups	Ŭ	ontrol ven	sus CTC+0	CR			Control v	ersus CF	~			CTC+C	R versu	s CR	
Days	3	8	14	21	1 r - ¹	3	8	14	21	ľ	ю	œ		4	21
EH	1.000	0.025*	0.075	0.456		0.317	0.034*	0.076	60.0	0	0.317	0.317	7 0.2	261 (0.197
CH	0.317	0.039*	0.068	0.114		0.317	0.025*	0.076	0.034	*	1.000	0.317	7 0.4	186 (0.796
Ш	1.000	0.043*	0.068	0.121		1.000	0.046^{*}	0.068	0.037	*/	1.000	0.637	0.8	314	0.261
EI	1.000	0.025*	0.197	0.317		1.000	0.034*	0.068	0.11	4	1.000	0.317	3.0 7	316 (0.456
MA	0.317	0.043*	0.068	0.034*		1.000	0.043*	0.045*	0.025	*	0.317	0.456	5 0.3	317 (0.317
GC	1.000	0.034*	0.182	1.000		1.000	0.034*	0.068	0.31	7	1.000	1.000	0.7	96	0.317
Total	0.114	0.046*	0.037*	0.072		0.114	0.046^{*}	0.037*	0.046	*0	1.000	0.261	1.0	000	0.513
* Values (EI) epith for each	represent telial inju nouse.	t significa try, (MA)	ntly diffen mitotic act	ent (P≤0.0 tivity, (GC)5) gro	ups. (EH let cell d	 I) epithelia epletion. 7 	al hyper <mark>ı</mark> Fotal sco	olasia, ((re corre	CH) cry sponds	pt height, to the sum	(II) infl a of scor	ammator es of all	ry infiltu categor	rates, ies



Figure 5.4. Microphotographs (H&E stained cross sections, original magnification 10X) of the murine distal colon. Colonic sections of Control (A) and CTC (B) treatment mice had no pathologic changes. In contrast, colons of CTC+CR (C) and CR (D) treatment mice had submucosal inflammation (arrows) and hyperplasia, particularly 8 and 14 days p.i. Bars indicate 100 μ m.



Figure 5.5. Epithelial crypt height. Vertical lines associated with histograms are standard error of the means (n=3). Bars not indicated with the same letter differ significantly (P<0.05).

between CTC+CR and CR treatments, there was a trend for decreased pathologic changes in CTC+CR treatment at later stages of infection. For example, there was no difference (P>0.05) in mitotic activity scores for the CTC+CR relative to the Control treatment at 14 days p.i., whereas mitotic activity was higher (P<0.05) for the CR treatment relative to the Control treatment (Table 5.2). At day 21 p.i., crypt height, inflammatory infiltrates, and total pathology scores differed (P<0.05) between the Control and CR treatments but did not differ (P>0.05) between the Control and CTC+CR treatments.

C. rodentium infection differentially regulated mRNA expression of cytokines

To assess murine immune responses to *C. rodentium*, mRNA expression of Th1-(IFN- γ , TNF- α , and IL-2), Th2- (IL-4), Treg- (IL-10 and TGF- β 1), and Th17- (IL-17A, IL-22, IL-1 β , and IL-6) related cytokines in colonic tissues was quantified by RT-qPCR at various stages of infection. *C. rodentium* infection resulted in higher (*P*<0.05) expression of cytokine mRNA associated with the Th1 (Figure 5.6, A and B) and Th17 (Figure 5.6, D, E, F and G) pathways 8 and 14 days p.i. Transcript levels of IL-2 were differentially elevated (*P*<0.05) in infected mice at 21 days p.i. only. No significant changes in transcript levels of cytokine genes associated with the Th2 (Figure 5.6, H), and Treg (Figure 5.6, I, J) pathways were observed in infected mice. Mice from the CTC treatment did not show differential mRNA expression for most cytokines relative to the Control treatment. However, a decrease (*P*<0.05) in the expression of TGF- β mRNA and an increase (*P*<0.05) in expression of IL-4 mRNA were observed in CTC treatment mice on day 21 p.i.



Figure 5.6. Relative mRNA expression (log $[2^{-\Delta Ct} + 1]$) of cytokines in colonic tissues. Vertical lines associated with histogram bars are standard error of the means (n=3). (A) IFN- γ ; (B) TNF- α ; (C) IL-2; (D) IL-17A; (E) IL-22; (F) IL-1 β ; (G) IL-6; (H) IL-4; (I) IL-10; (J) TGF- β . Bars not indicated with the same letter differ significantly (*P*<0.05).

Administration of CTC modulated murine immune responses to C. rodentium

In infected mice, CTC administration (i.e. CTC+CR treatment) increased (P<0.05) transcript levels of IFN- γ , TNF- α , IL-2 and IL-22 genes (Figure 5.6, A, B, C, E) and decreased (P<0.05) transcript levels of the IL-1 β gene 8 days p.i relative to the CR treatment (Figure 5.6, F). Furthermore, administration of CTC to infected mice decreased (P<0.05) transcript levels of the IFN- γ , TNF- α , IL-17A, IL-22, and IL-1 β genes 14 days p.i. relative to the CR treatment (Figure 5.6, A, B, C, E).

Temporal treatment effects (i.e. *C. rodentium* inoculation and/or CTC administration) on cytokine mRNA expression are further illustrated in Figure 5.7. Transcript levels of IFN- γ and TNF- α progressively increased over time, with the highest expression observed at day 14 p.i., whereas transcript levels of IL-22 and IL-1 β (Th17related cytokines) peaked at day 8 p.i., and decreased at day 14 p.i. For Th17 cytokines, a trend was observed for an initial increase in transcript levels of IL-17A, IL-22, and IL-6 genes in infected mice administered CTC relative to CR treatment mice. At day 14 p.i., transcript levels of these cytokine genes in CTC+CR treatment mice tended to decrease differentially relative to CR treatment mice. Transcript levels of IL-1 β in CTC+CR mice remained lower relative to CR treatment mice at both the peak and late infection periods.

Colonic microbiota was not consistently affected by C. rodentium or CTC treatment

Diverse bacterial communities were observed in association with mucosal surfaces of the colon of all mice regardless of treatment. Global and pairwise comparisons using the group significance test performed on T-RFLP relative abundance and presence/absence data indicated a high level of inter-animal variability (Figure 5.8). Ordination of T-RFLP profiles by NMS analysis revealed no obvious grouping of



Figure 5.7. Temporal transcript levels of cytokine mRNA in the distal colon of treatment mice. Cytokines are grouped by type of Th response.



Figure 5.8. Non-metric multi-dimensional scaling applied to presence/absence T-RFLP profiles of mucosa-associated bacterial communities in the murine distal colon at days 3 (A), 8 (B), 14 (C), and 21 (D) p.i., showing no treatments effects over time. (\bigcirc) Control treatment; (\bigcirc) CTC treatment; (\blacksquare) CTC+CR treatment; and (\Box) CR treatment. Each data point corresponds to an individual sample. Inter-animal variability is illustrated by the dispersion of data points from the same treatment.

replicate mice within treatments at individual sample times p.i. (Figure 5.8). Global comparisons using the group significance test revealed the formation of unique (P<0.05) community compositions for the Control and CTC+CR treatments at 8 days p.i., and for the CTC+CR and CR treatments at 21 days p.i. (Table 5.3). However, pairwise comparisons indicated that the composition of the colonic microbiota was not consistently affected by CTC or CR treatments over time (Table 5.3). However, when data from all time points were examined collectively, there was a subtle but significant difference in the composition of the between CTC-treated mice and those that did not receive CTC (Figure 5.9). Conversely, there was no difference in the composition of the colonic microbiota between Control and CR treatment mice (Figure 5.9). In summary, infection with *C. rodentium* did not affect (P>0.05) the composition of the colonic microbiota, whereas administration of CTC caused subtle and inconsistent changes in the colonic microbiota (Table 5.3, Figure 5.8).

5.4 DISCUSSION

Research examining the use of AGP in agriculture and animal production have predominantly focused on measuring the prevalence of AMR in human pathogens (Alexander *et al.*, 2008; Berge *et al.*, 2006; Funk *et al.*, 2006; Ghosh and LaPara, 2007; Inglis *et al.*, 2005; Langlois *et al.*, 1978). Modulation of the microbiota by AGP is putatively thought to be the primary mode of action of AGP (Dibner and Richards, 2005; Visek, 1978). However, there is a deficiency of experimental evidence to support this hypothesis. The current study tested the hypothesis that AGP promote growth through an interaction with the immune system of the host. It is noteworthy that CTC administration

Table 5.3. Group significance	analysis of	T-RFLP com	munity profile	es by global (top) and pairw	vise (bottom)	comparisons	8
Global comparisons				V d	alues			
	Col	ntrol		CTC	CTO	C+CR	C	R
Day 3	0.8	888	0	.249	0	069	0.4	.87
Day 8	0.0	03*	0	.708	0.0	033*	0.2	87
Day 14	0.0	391	0	.709	.0	717	0.5	75 .
Day 21	0.0	0 <i>1</i> 0	0	.085	0.0	035*	0.0	35*
Pairwise comparisons				Ρv	alues			
	Da	y 3	Day	/ 8	Day	/ 14	Day	, 21
	A	B	A	B	A	В	А	В
Control (A) vs CTC (B)	0.797	0.021*	0.025*	0.732	0.338	0.542	0.308	0.267
Control (A)'vs CTC+CR (B)	0.807	0.024*	0.053	0.153	0.298	0.599	0.059	0.063
Control (A) vs CR (B)	0.908	0.423	0.023*	0.441	0.570	0.547	0.181	0.151
CTC (A) vs CTC+CR	0.827	0.593	0.738	0.127	0.698	0.746	0.212	0.218
CTC (A) vs CR (B)	0.193	0.633	0.508	0.269	0.808	0.691	0.058	0.065
CTC+CR (A) vs CR (B)	0.042*	0.416	0.008*	0.227	0.653	0.450	0.052	0.057
* Values represent unique grou	sdr							



Figure 5.9. Non-metric multi-dimensional scaling applied to presence/absence (A) and relative abundance (B) of T-RFLP profiles of mucosa-associated bacterial communities in the murine distal colon. (\bullet) Control treatment; (\blacksquare) CR treatment; (\Box) CTC treatment (\bigcirc) CTC+CR treatment; and. Closed-line clusters in A and B are composed predominantly of CTC-treated mice. Dashed-line clusters in A and B are composed predominantly of mice that did not receive CTC.

modulated *C. rodentium*-induced cytokine mRNA expression, prevented *C. rodentium*induced weight loss, and mitigated pathologic changes associated with infection. These findings support the immunomodulation hypothesis of AGP action.

Cell mediated and humoral immunity are required for pathogen clearance in C. rodentium infection (Simmons et al., 2003). The T cell compartment of the immune system can respond to an enormous variety of antigens (Romagnani, 2006), and the type of immune response will depend on the differentiation of effector CD4⁺ T (helper) cells, which are defined according to their pattern of cytokine secretion (Mosmann *et al.*, 1986). In the current study, C. rodentium infection up-regulated Th1 and Th17-related cytokine mRNA expression at the peak (day 8 p.i.) and late (day 14 p.i.) stages of infection, which is in accordance with previous reports (Higgins et al., 1999; Symonds et al., 2009). C. rodentium also induced histopathologic changes that were concomitant with cytokine expression patterns. C. rodentium initially colonizes the cecum, and colonization eventually extends to the distal colon (Wiles et al., 2004) where the bacterium incites acute, self-limited colitis associated with progressive crypt hyperplasia (Barthold *et al.*, 1978). Thickening of the distal colon observed here was attributed primarily to mucosal hyperplasia induced by C. rodentium (Higgins et al., 1999). Severe colitis as observed in C. rodentium-infected mice can also be attributed to transmural inflammation and infiltration of inflammatory cells to the submucosa, muscularis externa, and serosa (Dann et al., 2008; Luperchio and Schauer, 2001).

All infected mice exhibited temporal up-regulation of mRNA expression of Th17related cytokines that peaked at day 8 p.i. In contrast, mRNA expression of IL-17A and IL-6 peaked at 14 days p.i. in infected mice that did not receive CTC. It is noteworthy

that in infected mice administered CTC, the transcript levels of IL-17A, IL-22, and IL-6 genes decreased relative to CR treatment mice at late infection. The Th17 subset of cells were shown to play a crucial role in induction of autoimmune diseases (Kolls and Lindén, 2004), as well as in clearance of extracellular bacterial pathogens that are not adequately handled by Th1 or Th2 responses (Korn et al., 2009; Weaver et al., 2007). C. rodentium is known to induce a strong Th17 response (Korn *et al.*, 2009; Symonds *et al.*, 2009; Zheng et al., 2008), which is mediated largely by IL-22 (Zheng et al., 2008). Nonetheless, clearance of C. rodentium by Th17 differentiation is dependent on an extensive inflammatory tissue response, which is mediated primarily by the proinflammatory cytokine IL-17A (Korn et al., 2009). Here, the observed progressive hyperplasia in infected mice may be attributed primarily to the early differentiation of the Th17 subset of cells. It was observed that the expression of IL-17A was reduced at late infection in C. rodentium-infected mice that received CTC, which may be attributable to the reduced pathologic changes observed in the distal colon of mice from this treatment group. Furthermore, mRNA expression of IL-22 in infected mice that received CTC was down-regulated at the late stage of infection, and up-regulated at the peak stage of infection. IL-22 is crucial in innate immunity against C. rodentium, is required in the early host response, maintains colonic epithelial integrity, and induces production of the Reg family of antimicrobial proteins (Zheng et al., 2008). Therefore, modulation of IL-22 transcript levels by CTC administration observed in the current study would be expected to contribute to pathogen clearance as well as decreased epithelial injury.

It has been shown that TGF- β has a critical role in the commitment of the Th17 lineage of cells in the presence of IL-6 (Korn *et al.*, 2009; Mangan *et al.*, 2006).

However, it was recently reported that Treg efficiently inhibited the proliferation of effector Th17 cells and their production of the pro-inflammatory cytokines IL-22 and IL-17A, as well as the chemokine CXCL8 (Crome *et al.*, 2010). Therefore, the progressive increase in TGF- β expression that was observed over time may be correlated to the decrease of Th17-related cytokines in mice at the late stage of infection and at clearance (i.e. day 21 p.i.). IL-6 induces the development of Th17 cells from naïve cells together with TGF- β , but inhibited TGF- β -induced Treg differentiation (Kimura and Kishimoto, 2010). This may explain the similar mRNA expression pattern between IL-6 and IL-17A, and the decreased transcript levels of TGF- β observed in early and peak stages of infection.

Another interesting observation of the present study was that infected mice presented temporal up-regulation of mRNA expression of Th1-related cytokines, with the highest transcript levels of IFN-g and TNF-a occurring at the late stage of infection, and the highest transcript levels of IL-2 occurring at clearance, regardless of CTC administration. The fact that Th1 and Th2 responses are mutually regulated and antagonistic (Parronchi *et al.*, 1992) explains how mRNA expression of IL-4 (i.e. Th2related cytokine) was not modulated in infected mice, which presented progressive upregulation of Th1-related cytokines. Nevertheless, mRNA expression of IL-4 was increasing by the clearance period in infected mice that received CTC, which can be attributed to the simultaneous decrease in mRNA expression of IFN- γ and TNF- α and the simultaneous increase in mRNA expression of IL-2. The decreased Th1 response at clearance might have contributed to initiate differentiation of Th2 cells, since naïve Tcells can produce sufficient IL-2 and IL-4 to induce their differentiation into Th2 cells

(Ansel *et al.*, 2006). Even so, uninoculated mice that received CTC presented higher concentrations of IL-4 on clearance period thus suggesting that CTC may be contributing directly to the differentiation of Th2 cells. Regarding the increase in mRNA expression of IL-2 at clearance only, it has been reported that *C. rodentium* lysates inhibited IL-2 by mitogen-stimulated lymphoid cells (Malstrom and James, 1998) which may exert critical functions in bacterial pathogenesis. Furthermore, IL-2 is known to inhibit differentiation of Th17 cells, and to promote generation of Treg cells, which might explain the concurrent increasing concentrations of TGF- β at clearance period. The mRNA expression of IL-10 (i.e. Treg-related cytokine), was not altered by *C. rodentium* infection in the current study, likely because IL-10 is not critical for protection against *C. rodentium* (Spahn *et al.*, 2008).

Although *C. rodentium* is a non-invasive bacterial pathogen, translocation of the bacterium into the lamina propria may occur through leaky tight junctions and damaged epithelium, thereby inducing a mucosal Th1 response (Higgins *et al.*, 1999). Moreover, an increase in the production of keratinocyte growth factor occurs in response to proinflammatory cytokines in *C. rodentium* infection (Higgins et al., 1999), resulting in epithelial cell proliferation. The temporal up-regulation of Th1-related cytokines observed in the current study, with highest levels on late infection, is attributed to the progressive and concomitant mucosal hyperplasia and epithelial damage induced primarily by the early differentiation of Th17 cells.

Infected mice that received CTC also presented lower mRNA expression of acutephase response cytokines at the late stage of infection, which may have contributed to pathogen clearance. Transcript levels of IL-1 β were down-regulated at the peak and late

stages of infection, whereas those of TNF- α were up-regulated at early infection and down-regulated at the late stage of infection. An acute-phase response is triggered by an infectious challenge, and is characterized by the release of the pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α , which orchestrate behavioral, cellular, and metabolic adjustments that alter the partitioning of nutrients away from growth and toward processes that support the immune and inflammatory responses (Klasing and Korver, 1997). Furthermore, IFN- γ , IL-1 β and IL-6 facilitate TNF- α -induced muscle cachexia (Pajak et al., 2008). It has been reported that therapeutic administration of tetracycline orally reduced lethality and inflammatory lesions associated with lipopolysaccharide (LPS)-induced septic shock in mice by decreasing serum concentrations of TNF- α , and have inhibited LPS-induced secretion of TNF- α and IL-1 β by human monocytes *in vitro* (Shapira et al., 1996). The non-therapeutic administration of tetracycline to experimentally stressed poultry has been shown to reduce immunologic stress by decreasing plasma concentrations of IL-1 (Roura et al., 1992). These researchers, however, did not attribute the decrease in plasma concentrations of IL-1 to a direct effect of tetracycline on monocytes, which was justified by a previous report indicating that this antimicrobial agent had no direct effect on IL-1 release by monocytes at non-toxic concentrations in vitro (Roche et al., 1988). Conversely, concentrations as low as 0.1 mg mL⁻¹ CTC decreased TNF- α secretion by cultured swine Kupffer cells that were inoculated with LPS (Akunda et al., 2001). In addition, intra-peritoneal injection of 10 mg kg⁻¹ bw tetracycline, and as low as 1.5 mg kg^{-1} bw doxycycline decreased murine serum concentrations of IL-1 α and TNF- α (Milano *et al.*, 1997). In the current study, administration of CTC to infected mice was also observed to decrease the expression of

IL-17A at late infection, which may be attributable to the down-regulation of TNF- α and IL-1 β mRNA at the late stage of infection. It has been reported that Th17 cell response is amplified by TNF- α and IL-1 β (Veldhoen *et al.*, 2006).

Altogether, reduced pathological changes observed at the late stage of infection as well as at clearance, and the absence of weight loss at late infection in infected mice that received CTC is attributed to the up-regulation of IL-22 transcripts at the peak stage of infection, and the down-regulation of TNF- α , IL-1 β , and IL-17A transcript levels at the late stage of infection relative to infected mice not administered CTC.

Infection by *C. rodentium* did not have a profound effect on the mucosa-associated microbiota of the murine distal colon in the current study. In contrast with these findings, partial 16S rRNA gene sequence analysis of mucosa-associated and luminal bacteria in mice infected with *C. rodentium* revealed alterations to the microbiota (Hoffmann *et al.*, 2009). However, they only examined communities in the cecum and proximal colon, and communities were resolved at the phylum level. Furthermore, no assessments of inter-animal variability were performed (Hoffmann *et al.*, 2009). One of the major advantages of T-RFLP is the ability to monitor treatment effects on the microbiota composition by comparing community profiles (Hartmann and Widmer, 2008), which also enables valuable assessments of inter-animal variability. The inter-animal variability observed in the current study may have obscured effects of *C. rodentium* infection on the colonic microbiota. Alternatively, *C. rodentium* may not exert substantive impacts on the microbiota given the bacterium does not colonize the distal colon during the entire infection period (Wiles *et al.*, 2006). The presence of marked hypoxia within the lumen

of the distal colon (He *et al.*, 1999) may partly explain why *C. rodentium* colonization initiates in the cecum (Wiles *et al.*, 2004).

Administration of CTC was observed to affect the composition of the colonic microbiota independent of C. rodentium infection in the present study. However, an effect on the microbiota was only detected when all time points were analyzed together. Thus, CTC exerted subtle effects on the colonic microbiota in the current study, which were inconsistent over time. Administration of non-therapeutic CTC substantially impacted the mucosa-associated microbiota of piglet's ileums, (Rettedal et al., 2009). In contrast, a recent study reported that the administration of virginiamycin and tylosin to intensively raised swine at the grow/finishing phase had no effect on the community composition (Kalmokoff *et al.*, 2010). These researchers also observed a high prevalence of bacteria that carried genes encoding resistance to macrolide-lincosamide-streptogramin B (MLS_B), from which they explained the negative impact of AGP on the fecal community (Kalmokoff et al., 2010). Host genetics and environmental factors can both affect the intestinal microbiota. For example, the fecal microbiota of persons with gene mutations associated with the autoinflammatory disorder, familial Mediterranean fever, presented significant changes in composition, that were also observed at the remission period (Khachatryan et al., 2008). Also, Altered Schaedler Flora (ASF) mice of the same genetic background exhibit significant variation in the intestinal relative cell densities of the eight ASF strains when mice were maintained in separate cages (Alexander et al., 2006).

The inter-animal variability encountered in the current and previous studies (Alexander *et al.*, 2006; Durso *et al.*, 2010; Rettedal *et al.*, 2009) further illustrates how

difficult it is to quantify the impact of a single variable in such a complex system. For example, Rumsey *et al.*, (1999) demonstrated that non-therapeutic CTC reduced the sensitivity of the bovine pituitary gland to a releasing hormone challenge, which may promote improved energy efficiency of tissue deposition. This alternate hypothesis also emphasizes that the direct interaction of AGP with the microbiota is not the main mechanism of growth promotion, and that host responses should not be discounted in studying AGP action.

Whereas CTC administration modulated immune responses in the colon in the current study, it was not possible to definitely conclude whether this effect was a direct or indirect effect of CTC. Considering a direct effect of CTC on the microbiota, it is unclear to how and to what degree subtle changes in the microbiota caused by CTC would induce growth promotion, especially given the complexity of the mechanisms involved and that AGP exert an effect in animals possessing a highly dissimilar microbiota. It has been shown that the indigenous microbiota can influence *C. rodentium* pathogenesis (Luperchio and Schauer, 2001), thus even subtle changes in the microbiota may have influenced immune responses to *C. rodentium*, further illustrating the complexity of the interaction and the difficulty of elucidating specific effects.

In conclusion, the mucosa-associated microbiota of the murine colon was not affected by *C. rodentium*, and was inconsistently affected by oral administration of nontherapeutic CTC in the current study. Furthermore, non-therapeutic CTC administration modulated immune responses to *C. rodentium* infection in mice. Although subtle and inconsistent, CTC administration did alter the composition of the colonic microbiota. Thus, it was not possible to definitively ascertain whether CTC directly or indirectly

modulated the immune system. Nonetheless, that non-therapeutically administered AGP directly or indirectly modulate the intestinal immune system indicates that modulation of the microbiota alone is not responsible for growth promotion. The research presented in this Chapter not only broadens our knowledge of how AGP exert an effect, it also emphasizes the necessity of examining host responses interactively with the intestinal microbiota in future studies to elucidate the mechanisms of action of AGP.

ACKNOWLEDGEMENTS

I thank the following individuals for their invaluable contributions: Dr. Richard Uwiera for conducting histopathological scoring; Tara Shelton, Randy Wilde, and Jenny Gusse (AAFC LRC) for assistance with mouse handling and sample collection; Dr. Lisa Kalischuk, for assistance with *C. rodentium* transformation; Matt Thomas (University of Lethbridge) for assistance with NMS analyses; Toby Entz (AAFC LRC) for assistance with statistical analyses; and Dr. Andre G. Buret (Biological Sciences Department, University of Calgary) for providing the *C. rodentium* strain used in the study. This work was funded by an AAFC Peer Review Project Grant to Dr. Doug Inglis, and a Discovery Grant to Dr. Doug Inglis, Dr. Brent Selinger, Dr. Richard Uwiera, and Dr. John Kastelic from the AFMNet.

CHAPTER SIX

General discussion and future directions

6.1 Characterization of intestinal microbial communities

Elucidating interactions among the host, environmental factors, and microbiota in the mammalian GI tract is remarkably challenging, due to the complexity of this ecosystem. Advances in molecular biology have facilitated the acquisition of new knowledge regarding the structure and function of GI microbiota. Techniques such as T-RFLP allow analyses of numerous samples, making it possible to document spatial and temporal changes in microbial communities with high efficiency (Hartmann and Widmer, 2008). One of the salient advantages of T-RFLP analysis is its utility in empirical studies to evaluate treatment effects on the composition of the microbiota. Nevertheless, T-RFLP has limitations for accurate determination of microbial diversity in highly complex ecosystems (Engebretson and Moyer, 2003), as well as in species identification within communities (Pandey et al., 2007). It is noteworthy that T-RFLP profiles can be used to choose appropriate samples for clone library analysis, and T-RFs can be identified by comparison to clone sequences. In that regard, combining T-RFLP and clone library analysis substantially increases the probability of accurately identifying a particular bacterial species, while retaining the high-throughput advantages of T-RFLP (Moeseneder et al., 2001).

6.2 Variation in intestinal microbiota composition among individuals

Another challenge frequently faced in characterizing temporal and spatial assessments of the mammalian microbiota is the high level of inter-individual variability. The results of recent studies (16S rRNA gene sequencing) characterizing human fecal microbiota now question the existence of a core intestinal microbiota in humans (i.e. commonality amongst individuals; e.g. Turnbaugh *et al.*, 2007). Similarly, recent research in cattle revealed remarkable variation in the fecal microbiota among individuals (Durso *et al.*, 2010), and 16S rRNA gene sequencing of mucosa-associated bacteria from swine also indicated substantial inter-animal variability (Funk *et al.*, 2006). Furthermore, the composition of bacterial communities associated with the intestinal mucosa varies greatly from that present in the digesta and feces (Eckburg *et al.*, 2005; Zoetendal *et al.*, 2002), which suggests that fecal communities may not be representative of biologically relevant communities from a particular location in the intestine, including the colon (Dubos *et al.*, 1967; Savage, 1987).

In the present study, administration of non-therapeutic concentrations of model AGP (i.e. AS700 for cattle; CTC for mice) did not substantially affect the intestinal microbiota of mice and cattle. However, definitive conclusions on the effects of AGP on the intestinal microbiota were obscured by substantial variability among individuals. Although all mice (i.e. a mammalian AGP model) were from the same genetic background, and cattle as well as mice were subjected to similar experimental conditions (i.e. diet, handling, environment), considerable inter-animal variability in the composition of the intestinal microbiota was encountered.

6.3 General conclusions

A primary objective of the current research was to determine if AGP would affect immune responses of the mice to C. rodentium, and if it would induce a significant shift in composition of the intestinal microbiota of cattle and mice. Although administration of CTC modulated murine immune responses to C. rodentium, it was not possible to definitely determine if this effect resulted from a direct action of AGP, shifts in intestinal microbiota composition, or both. Overall, it was concluded that CTC did not cause consistent modifications in the microbial communities in either cattle or mice. This conclusion is consistent with the findings from other studies. For example, a commonly used AGP did not induce substantial changes in abundance of selected bacterial phylogenetic groups of the cecum of chickens (Wise and Siragusa, 2007). Similarly, oral administration of non-therapeutic concentrations of virginiamicin and tylosin to intensively raised pigs did not affect the composition of the fecal microbiota or fecal community resistance to macrolide-lincosamide-streptogramin B (Kalmokoff *et al.*, 2010). These findings are consistent with the immunomodulation hypothesis of AGP action, which would explain the consistency of growth promoting effects observed in animals with highly dissimilar microbiota compositions, as well as in animals administered AGP with distinct spectra of antimicrobial action. Furthermore, it is unlikely that AGP would substantially reduce the microbial load without disrupting colonization resistance, which comprises a complex network of interactions between the microbiota and the intestinal mucosa, and is thought to inhibit colonization by pathogens (Stecher and Hardt, 2008). Nevertheless, findings of this research do not preclude the possibility that AGP administration could exert shifts in microbial composition as a result

of altered immune responses induced directly by AGP. Further evidence that AGP exerts an effect on the immune system of the host is supported by a study in which AGP administration ameliorated weight loss in chickens maintained in an unsanitary environment, whereas no evident growth promoting effect was observed in those maintained in a clean environment (Roura *et al.*, 1992). Therefore, administration of AGP might reduce immune-mediated stress and enable more energy to be used towards growth development (in lieu of the acute-phase response). This warrants examination.

6.4 Restricted use of antimicrobial growth promoters

It is well established that AGP enhance growth of animals maintained in unsanitary environments (Roura *et al.*, 1992); therefore, some producers have come to rely on AGP administration to compensate for poor management practices (HC, 2002). Thus, a ban on AGP should stimulate improvements in management and husbandry practices that reduce stress and promote animal health. However, there are some forms of stress that cannot be entirely prevented with management practices (e.g. stress due to weaning). For instance, the ban on AGP administration in Denmark corresponded with increased mortality in weaned piglets (Jensen and Hayes, 2003).

6.5 Alternatives to antimicrobial growth promoters

The emergence of AMR, and restrictions on the use of AGP have become a driving force to identify alternatives to AGP. Favorable alternatives to AGP are regarded as natural growth promoters, or non-antibiotic growth promoters (NGP), and include: probiotics (Estienne *et al.*, 2005; Opalinski *et al.*, 2007), prebiotics (Chee *et al.*, 2010),

oils, herbal extracts, and organic acids/salts (Huyghebaert *et al.*). To date, research with NGP has been predominantly observational, and has not focused on elucidation of mechanisms. Unfortunately, since many NGP exhibit variable effects on animal growth performance, has promulgated the conclusion that NGP will not be as efficacious as AGP (Huyghebaert *et al.*). However, the use of multiple NGP in concert may be promising. The application of efficacious alternatives to AGP will be greatly facilitated if the mechanisms of action of AGP and NGP are elucidated. In this approach, the search for efficacious alternatives may be targeted (i.e. with host and/or microbiota effects that mimic AGP).

Probiotics are defined as "live microorganisms which, when consumed in adequate amounts as part of food, confer a health benefit on the host" (WHO, 2001). Lactic acid bacteria and its components modulate the immune system, and in many cases enhance the immune response. In that regard, several probiotic effects are mediated through immune regulation, particularly through balance control of pro-inflammatory and antiinflammatory cytokines (Yasui *et al.*, 1999). Moreover, probiotics have distinct regulatory effects on healthy persons, as well as those with inflammatory diseases (Isolauri *et al.*, 2001), suggesting that the immunomodulatory effects of probiotics may depend on the immunologic state of the host. For example, probiotics enhanced growth in weaned pigs, but did not alter the growth performance of pre-weaned pigs (Estienne *et al.*, 2005).

A prebiotic is "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" (Roberfroid, 2007). Prebiotics are administered orally,

usually to monogastric animals, and are regarded as better energy substrates for "favorable bacteria" (e.g. bifidobacteria and lactobacilli), allowing these bacterial species to proliferate at the expense of less beneficial species, such as *Clostridium* spp. and Salmonella spp. (Yang et al., 2009). Currently, not all dietary carbohydrates are considered prebiotics (Roberfroid, 2000). Many types of oligosaccharides (i.e. commonly referred to as a prebiotic ingredient) have been used as feed additives to promote animal growth, including: inulin, fructo-oligosaccharides (FOS) of cereals, trans-galactooligosaccharides of milk products, and mannan-oligosaccharides of yeast cell walls (Falcão-e-Cunha et al., 2007). Similar to probiotics, the effects of oligosaccharides on animal performance lack consistency. For example, administration of 4 g kg⁻¹ of FOS to the basal diet increased average daily weight gain of broilers, whereas administration of 8 g kg⁻¹ FOS to the basal diet did not have any effect (Xu et al., 2003). These inconsistencies in the efficacy of oligosaccharides on animal growth performance may be related to the combined effects of their nutritional impact and the components of the diet, as well as the stimulation of growth and metabolic activities of various bacterial species (Maczulak et al., 1993), including those which are potentially harmful. The combination of prebiotics and probiotics (i.e. synbiotics) may have a synergistic effect, since this combination can improve survival of the probiotic organisms, by providing specific substrates for fermentation (Yang et al., 2009).

Organic acids have been shown to increase animal growth performance through antimicrobial and anti-inflammatory effects. Short-chain fatty acids, such as butyric acid, seem to be an important energy source for intestinal epithelial cells and have stimulated epithelial cell proliferation and differentiation (Dalmasso *et al.*, 2008). Furthermore,

supplementation of feeds with butyric acid decreased the incidence of necrotic enteritis caused by *C. perfringens* in broilers (Timbermont *et al.*, 2010), as well as strengthened the intestinal mucosal barrier by increasing production of antimicrobial peptides in mucus, and by stimulating expression of tight junction proteins (Peng *et al.*, 2007).

Spices, herbs, essential oils, and their extracts possess anti-oxidant, antimicrobial, anti-rheumatic, nephroprotective, antimutagenic, anticancer, gastroprotective, hepatoprotective, lipid-lowering, anti-ulcer, and anti-inflammatory activities (Mofleh, 2010), and are frequently suggested as potential alternatives to AGP. Bio-active components of plants are mostly secondary metabolites, and include terpenoids, phenolics (tannins), glycosides and alkaloids (Huyghebaert *et al.*). In one study, clove and oregano fed together provided growth performance close to that of pigs fed AGP (Costa *et al.*, 2007), which makes the use of herbal extracts a promising alternative to AGP. The challenge is to assess the potential for synergy among various herbal extracts, and to identify and quantify the multitude of actions and claims improving feed utilization, animal physiology and health status when testing herbal extracts as alternatives to AGP (Huyghebaert *et al.*).

6.6 Future research

Further investigations should assess specific mechanisms of immunomodulation by AMA that are commonly used as AGP (Table 1.1). The use of *in vitro* models of intestinal epithelial barrier (Le Ferrec *et al.*, 2001) are expected to provide in depth analysis of bioavailability and absorption of various types of AMA, and of specific metabolic pathways that may be regulated by AMA at the mucosal level. The great

advantages of *in vitro* models include the possibility of undertaking large-scale experiments, and the control of variables and environmental conditions, which will assist in elucidating specific biochemical and physiological pathways. Organotypic models (e.g. intestinal cell lines) enable assessments of specific cellular processes (Spottl *et al.*, 2006). In comparison to intestinal cell lines, isolated and perfused intestinal segments offer the advantage of working with an intact organ, where physiological interactions among cells and the intracellular matrices are preserved (Le Ferrec *et al.*, 2001). The major limitation of perfused intestinal segments is the short duration of the experiments, given the difficulty of maintaining tissue viability for an extended interval.

Regarding interactions between AMA and the immune system, macrolides, quinolones, and tetracyclines are the most investigated AMA to date (Rubin and Tamaoki, 2005). It has been reported that stimulation with TNF- α and IFN- γ has increased the accumulation of azythromycin in macrophages *in vitro* (Bermudez *et al.*, 1991). The uptake of erythromycin, chloramphenicol, rifampin, tetracycline, and lincomycin by alveolar macrophages has also been documented (Hand *et al.*, 1984). The mechanism of AMA accumulation in immune cells is unclear, and is thought to be a protein-mediated process (Rubin and Tamaoki, 2005). Immunomodulatory effects of AMA on phagocytic functions have been widely studied (Labro, 2000). Macrolides prevented leukocyte adhesion, reduced macrophage and leukocyte accumulation, stimulated macrophage chemotaxis, induced phagocytosis, ameliorated neutrophilinduced epithelial damage, and inhibited oxidative burst of neutrophils *in vitro* (Labro, 2000; Rubin and Tamaoki, 2005). Regarding inflammatory responses, macrolides and quinolones reduced the mRNA expression of proinflammatory cytokines, including TNF-
α , IL-1 β , and IFN- γ , *in vivo* and *in vitro*, in a concentration-dependent manner (Rubin and Tamaoki, 2005). To my knowledge, the assessment of immunomodulatory effects of AMA on enterocytes has not been performed. Furthermore, the majority of studies regarding immunomodulatory properties of AMA were performed with therapeutic concentrations (Labro, 2000). Therefore, it would be prudent to conduct assessments of the immunomodulatory effects of AMA on intestinal cell lines in a dose-dependent manner, including the use of non-therapeutic concentrations of AMA.

The use of *in vitro* and *in situ* models will certainly contribute to assessing specific immunomodulatory effects by AGP. Nevertheless, mechanisms of AGP action should also be studied within the dynamic interactions of the mammalian intestine. The intestinal "loop" model, in which an intestinal segment is surgically separated and subdivided in "loops", provides a valuable tool for *in vivo* physiological interactions, and has been used in small ruminants to assess mucosal immune responses (Gerdts *et al.*, 2001), as well as to assess systemic immune responses, e.g. lymphocyte trafficking to the intestine (Meurens *et al.*, 2009). In comparison to the *in situ* model, the intestinal "loop" model provides the advantage of working with an individualized compartment of the intestine, which has normal physiology for an extended interval. The introduction of catheters to the intestinal "loop" model (Uwiera *et al.*, 2009) should enable the assessment of long-term immunological responses to AGP in a ruminant model, given the possibility of introducing multiple treatments over an extended interval.

The use of *in vivo* models for the study of interactions in the intestine is extremely valuable; however, the complexity of the indigenous microbiota, and substantial interanimal variability in microbiota composition may hinder assessments of direct effects of

AGP on the immune system of the host. In this regard, the use of germ-free mice may beneficial for ascertaining the effects of non-therapeutic AGP on host responses by removing inter-animal variability with respect to the intestinal microbiota. However, due to the lack of interaction with microorganisms, germ-free mice have profound alterations in the immune system, intestinal morphology, absorptive function, electrolyte handling, bile metabolism, motility, and enteroendocrine functions (Smith *et al.*, 2007), which would alter the effects of AGP on the host. It is noteworthy that establishment of Altered Schaedler Flora (ASF), consisting of eight murine bacterial species, conferred normal physiological functions within the gut and provide a gnotobiotic model to examine the host-microbiota interaction (Sarma-Rupavtarm *et al.*, 2004). Thus, the use of ASF mice may be advantageous to elucidate the complex interactions among the host, AGP, and microbiota.

As described here, an inflammation inciter is considered necessary to study immunomodulatory effects of AGP in an *in vivo* model. In the current study, *C*. *rodentium* was effective for assessing immunomodulatory effects of AGP in a mouse model. Evaluating immunomodulatory effects by AGP through various inflammatory pathways, such as oxidative stress, is also warranted to elucidate mechanisms of action of AGP.

One of the murine responses to *C. rodentium* infection is the up-regulation of inducible nitric oxide synthases (iNOS), which are central to innate immunity and contribute to host defenses (Vallance *et al.*, 2002). Nitric oxide plays a critical role in several physiological processes in the gastrointestinal tract, such as motility, secretion, digestion, absorption and elimination (Dijkstra *et al.*, 2004). There are two constitutively

expressed NOS, that produce small amounts of NO: neuronal NOS (nNOS – type I) and endothelial NOS (eNOS – type III; Dijkstra *et al.*, 2004). The third, inducible form (iNOS – type II), produces large amounts of NO and its induction occurs in states of inflammation and immune activation, being regulated by nuclear factor kappa-lightchain-enhancer of activated B cells (NF-κB; Dijkstra *et al.*, 2004). High concentrations of NO may act in a negative feedback to block prolonged activation of NF-κB, thereby limiting chronic inflammation (Dijkstra *et al.*, 2004; Korhonen *et al.*, 2001; Meng *et al.*, 1998). There is a combination of cytokines and LPS to activate iNOS, and NO seems to have antimicrobial effects, serving as a microbial killing mechanism in activated macrophages (Korhonen *et al.*, 2001).

Alternative murine enteric inflammation models may also be used to assess mucosal immune responses that are potentially modulated by AGP. Disruption of the murine intestinal epithelial barrier function and colitis may be experimentally induced with the use of chemical agents, such as 2,4,6-trinitro benzene sulfonic acid (TNBS), oxazolone, and dextran sulfate sodium (DSS; Wirtz *et al.*, 2007). Colitis induced by DSS is thought to be mediated by IL-6, IL-12, IL-17, and TNF- α (Lorenz *et al.*, 2005). Model pathogens that cause murine colitis, such as *Salmonella typhimurium* (Hapfelmeier and Hardt, 2005), and *Listeria monocytogenes* (Cossart and Toledoarana, 2008; Lecuit *et al.*, 2007) may also be useful to assess alternative pathways of intestinal inflammation.

To assess the growth promoting effects of AGP, animal catabolism at the muscular level (i.e. protein turnover), and systemic immunologic stress response should be measured. Corticosterone has been described as a mediator of the immunologic stress response (Klasing *et al.*, 1987). Protein radioactivity was effective to determine rates of

protein degradation and synthesis (Costelli *et al.*, 1993). Studies with protein metabolism assessments may be conducted at the whole-body level, or at the regional level (i.e. specific muscles), and require the use of steady-state isotopic techniques, which are logistically challenging (Fouillet *et al.*, 2002).

6.7 Final conclusions

The research conducted here provides valuable new information regarding immune responses that were modulated by the administration of one AGP as a model; this will provide a basis for future studies regarding this alternate mechanism of action. It is noteworthy that alternatives to AGP known to impart effects on the microbiota composition, as well as on components of the immune system, do not exert growth promotion equivalent to that exerted by AGP. A possible explanation for this is that neither the microbiota, nor the immune system, are independently involved in the effects of AGP, but rather an interaction of these two critical aspects contribute to the most favorable growth effects. Therefore, future research should focus on unraveling the mechanisms of action of AGP, and on applying this knowledge to identify alternatives that provide comparable growth-promoting effects to those exerted by AGP. To enable the identification of suitable alternatives to AGP, further investigations should include not only assessments on intestinal microbiota, but on host effects induced by AGP, in comparison with synergistic effects in the host induced by NGP.

REFERENCES

- Aarestrup, F. M., Seyfarth, A. M., Emborg, H.-D., Pedersen, K., Hendriksen, R. S. and Bager, F. (2001). Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. *Antimicrobial agents and Chemotherapy*, **45**(7): 2054-2059.
- Abdo, Z., Schüette, U. M. E., Bent, S. J., Williams, C. J., Forney, L. J. and Joyce, P. (2006). Statistical methods for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. *Environmental Microbiology*, 8(5): 929-938.
- Ahmed, A. M., Furuta, K., Shimomura, K., Kasama, Y. and Shimamoto, T. (2006). Genetic characterization of multidrug resistance in *Shigella* spp. from Japan. *Journal of Medical Microbiology*, **55**(12): 1685-1691.
- Akira, S., Uematsu, S. and Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell*, **124**(4): 783-801.
- Akunda, J. K., Johnson, E., Ahrens, F. A. and Kramer, T. T. (2001). Chlortetracycline modulates acute phase response of ex vivo perfused pig livers, and inhibits TNF-[alpha] secretion by isolated Kupffer cells. *Comparative Immunology, Microbiology and Infectious Diseases*, 24(2): 81-89.
- Alexander, A. D., Orcutt, R., Henry, J., Baker, J., Bissahoyo, A. and Threadgill, D. (2006). Quantitative PCR assays for mouse enteric flora reveal strain-dependent differences in composition that are influenced by the microenvironment. *Mammalian Genome*, **17**(11): 1093-1104.
- Alexander, T. W., Yanke, L. J., Topp, E., Olson, M. E., Read, R. R., Morck, D. W. and McAllister, T. A. (2008). Effect of subtherapeutic administration of antibiotics on the prevalence of antibiotic-resistant *Escherichia coli* bacteria in feedlot cattle. *Applied and Environmental Microbiology*, 74(14): 4405-4416.
- Anonymous. (2002). To preserve the effectiveness of medically important antibiotics by restricting their use as additives to animal feed. Retrieved from http://frwebgate.access.gpo.gov/cgi-bin/getdoc.cgi?dbname=107 cong bills&docid=f:s2508is.txt.pdf.
- Ansel, K. M., Djuretic, I., Tanasa, B. and Rao, A. (2006). Regulation of Th2 differentiation and *Il4* locus accessibility. *Annual Review of Immunology*, 24(1): 607-656.
- Apajalahti, J. H. A., Sarkilahti, L. K., Maki, B. R. E., Heikkinen, J. P., Nurminen, P. H. and Holben, W. E. (1998). Effective recovery of bacterial DNA and percentguanine-plus-cytosine-based analysis of community structure in the gastrointestinal tract of broiler chickens. *Applied and Environmental Microbiology*, 64(10): 4084-4088.
- Atuma, C., Strugala, V., Allen, A. and Holm, L. (2001). The adherent gastrointestinal mucus gel layer: Thickness and physical state in vivo. *American Journal of Physiology and Gastrointestinal Liver Physiology*, **280**(5): 922-929.
- Backhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A. and Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *Science*, **307**(5717): 1915-1920.

- Barthold, S. W., Coleman, G. L., Jacoby, R. O., Livstone, E. M. and Jonas, A. M. (1978). Transmissible murine colonic hyperplasia. *Veterinary Pathology Online*, 15(2): 223-236.
- Barthold, S. W., Osbaldiston, G. W. and Jonas, A. M. (1977). Dietary, bacterial, and host genetic interactions in the pathogenesis of transmissible murine colonic hyperplasia. *Laboratory Animal Science*, 27(6): 938-945.
- Berge, A. C. B., Moore, D. A. and Sischo, W. M. (2006). Field trial evaluating the influence of prophylactic and therapeutic antimicrobial administration on antimicrobial resistance of fecal *Escherichia coli* in dairy calves. *Applied and Environmental Microbiology*, **72**(6): 3872-3878.
- Bermudez, L. E., Inderlied, C. and Young, L. S. (1991). Stimulation with cytokines enhances penetration of azithromycin into human macrophages. *Antimicrobial agents and Chemotherapy*, **35**(12): 2625-2629.
- Besedovsky, H. O. and del Rey, A. (1996). Immune-neuro-endocrine interactions: Facts and hypotheses. *Endocrine Reviews*, **17**(1): 64-102.
- Biancone, L., Monteleone, I., Del Vecchio Blanco, G., Vavassori, P. and Pallone, F. (2002). Resident bacterial flora and immune system. *Digestive and Liver Diseases*, 34(2): 37-43.
- Blackwood, C. B., Hudleston, D., Zak, D. R. and Buyer, J. S. (2007). Interpreting ecological diversity indices applied to T-RFLP data: Insights from simulated microbial communities. *Applied and Environmental Microbiology*, **73**(16): 5276– 5283.
- Borenshtein, D., McBee, M. E. and Schauer, D. B. (2008). Utility of the *Citrobacter rodentium* infection model in laboratory mice. *Current Opinion in Gastroenterology*, **24**(1): 32-37.
- Brooks, S. P. J., Kheradpir, E., McAllister, M., Kwan, J., Burgher-McLellan, K. and Kalmokoff, M. (2009). In-feed administered sub-therapeutic chlortetracycline alters community composition and structure but not the abundance of community resistance determinants in the fecal flora of the rat. *Anaerobe*, **15**(4): 145-154.
- Burdett, V., Inamine, J. and Rajagopalan, S. (1982). Heterogeneity of tetracycline resistance determinants in *Streptococcus. Journal of Bacteriology*, **149**(3): 995-1004.
- Casewell, M., Friis, C., Marco, E., McMullin, P. and Phillips, I. (2003). The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *Journal of Antimicrobial Chemotherapy*, **52**(2): 159-161.
- Castanon, J. I. R. (2007). History of the use of antibiotic as growth promoters in European poultry feeds. *Poultry Science*, **86**(11): 2466-2471.
- Cebra, J. J., Periwal, S., Bhargava, Lee, G., Lee, F. and Shroff, K. E. (1998).
 Development and maintenance of the gut-associated lymphoid tissue (GALT): The roles of enteric bacteria and viruses. *Developmental Immunology*, 6(1-2): 13-18.
- Chapman, H. D. and Johnson, Z. B. (2002). Use of antibiotics and roxarsone in broiler chickens in the USA: Analysis for the years 1995 to 2000. *Poultry Science*, **81**(3): 356-364.
- Chee, S. H., Iji, P. A., Choct, M., Mikkelsen, L. L. and Kocher, A. (2010). Characterisation and response of intestinal microflora and mucins to manno-

oligosaccharide and antibiotic supplementation in broiler chickens. *British Poultry Science*, **51**(3): 368-380.

- Chopra, I. and Roberts, M. (2001). Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews*, 65(2): 232-260.
- Clement, B. G., Kehl, L. E., DeBord, K. L. and Kitts, C. L. (1998). Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. *Journal of Microbiological Methods*, **31**(3): 135-142.
- Compendium of medicating ingredient brochures (2008). Retrieved in June, 2010, from Canadian Food and Inspection Agency website: http://www.inspection.gc.ca/english/anima/feebet/mib/cmibe.shtml
- Coates, M. E. (1975). Gnotobiotic animals in research: their uses and limitations. *Laboratory Animals*, **9**: 275-279.
- Coates, M. E., Fuller, R., Harrison, G. F., Lev, M. and Suffolk, S. F. (1963). A comparison of the growth of chicks in the Gustafsson germ-free apparatus and in a conventional environment, with and without dietary supplements of penicillin. *British Journal of Nutrition*, 17(1): 141-150.
- Conly, J. (2002). Antimicrobial resistance in Canada. *Canadian Medical Association Journal*, **167**(8): 885-891.
- Cossart, P. and Toledoarana, A. (2008). Listeria monocytogenes, a unique model in infection biology: an overview. *Microbes and Infection*, **10**(9): 1041-1050.
- Costa, E., Puhl, N. J., Selinger, L. B. and Inglis, G. D. (2009). Characterization of mucosa-associated bacterial communities of the mouse intestine by terminal restriction fragment length polymorphism: Utility of sampling strategies and methods to reduce single-stranded DNA artifacts. *Journal of Microbiological Methods*, **78**(2): 175-180.
- Costa, L. B., Tse, M. L. P. and Miyada, V. S. (2007). Extratos vegetais como alternativas aos antimicrobianos promotores de crescimento para leitões recém-desmamados. *Revista Brasileira de Zootecnia*, **36**: 589-595.
- Costelli, P., Carbó, N., Tessitore, L., Bagby, G. J., Lopez-Soriano, F. J., Argilés, J. M. and Baccino, F. M. (1993). Tumor necrosis factor-alpha mediates changes in tissue protein turnover in a rat cancer cachexia model. *The Journal of Clinical Investigation*, **92**(6): 2783-2789.
- Crome, S. Q., Clive, B., Wang, A. Y., Kang, C. Y., Chow, V., Yu, J., Lai, A., Ghahary, A., Broady, R. and Levings, M. K. (2010). Inflammatory effects of *ex vivo* human Th17 cells are suppressed by regulatory T cells. *Journal of Immunology*, **185**(6): 3199-3208.
- Culman, S., Bukowski, R., Gauch, H., Cadillo-Quiroz, H. and Buckley, D. (2009). T-REX: Software for the processing and analysis of T-RFLP data. *BioMed Central Bioinformatics*, **10**(1): 171.
- Culman, S. W., Gauch, H. G., Blackwood, C. B. and Thies, J. E. (2008). Analysis of T-RFLP data using analysis of variance and ordination methods: A comparative study. *Journal of Microbiological Methods*, **75**(1): 55-63.

- Dalmasso, G., Nguyen, H. T. T., Yan, Y., Charrier-Hisamuddin, L., Sitaraman, S. V. and Merlin, D. (2008). Butyrate transcriptionally enhances peptide transporter pepT1 expression and activity. *Public Library of Science One*, **3**(6): e2476.
- Dann, S. M., Spehlmann, M. E., Hammond, D. C., Iimura, M., Hase, K., Choi, L. J., Hanson, E. and Eckmann, L. (2008). IL-6-dependent mucosal protection prevents establishment of a microbial niche for attaching/effacing lesion-forming enteric bacterial pathogens. *Journal of Immunology*, **180**(10): 6816-6826.
- Davies, J. (1994). Inactivation of antibiotics and the dissemination of resistance genes. *Science*, **264**(5157): 375-382.
- Davis, C. D. and Milner, J. A. (2009). Gastrointestinal microflora, food components and colon cancer prevention. *The Journal of Nutritional Biochemistry*, **20**(10): 743-752.
- Dempsey, P. W., Vaidya, S. A. and Cheng, G. (2003). The art of war: Innate and adaptive immune responses. *Cellular and Molecular Life Sciences*, **60**(12): 2604-2621.
- Dibner, J. J. and Richards, J. D. (2005). Antibiotic growth promoters in agriculture: History and mode of action. *Poultry Science*, **84**(4): 634-643.
- Dickie, I. and FitzJohn, R. (2007). Using terminal restriction fragment length polymorphism (T-RFLP) to identify mycorrhizal fungi: A methods review. *Mycorrhiza*, **17**(4): 259-270.
- Dijkstra, G., van, G., Jansen, P. L. M. and Moshage, H. (2004). Targeting nitric oxide in the gastrointestinal tract. *Current Opinion in Investigational Drugs*, **5**(5): 529 536.
- Dubos, R., Savage, D. and Schaedler, R. (1967). The indigenous flora of the gastrointestinal tract. *Diseases of the Colon & Rectum*, **10**(1): 23-34.
- Duggar, B. M. (1948). Aureomycin: A product of the continuing search for new antibiotics. Annals of the New York Academy of Sciences, 51(2): 177-181.
- Dunbar, J., Ticknor, L. O. and Kuske, C. R. (2001). Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. *Applied and Environmental Microbiology*, **67**(1): 190-197.
- Dunlop, R. H., McEwen, S. A., Meek, A. H., Friendship, R. A., Clarke, R. C. and Black, W., D. (1998). Antimicrobial drug use and related management practices among Ontario swine producers. *The Canadian Veterinary Journal*, **39**: 87-96.
- Durso, L. M., Harhay, G. P., Smith, T. P. L., Bono, J. L., DeSantis, T. Z., Harhay, D. M., Andersen, G. L., Keen, J. E., Laegreid, W. W. and Clawson, M. L. (2010). Animal-to-animal variation in fecal microbial diversity among beef cattle. *Applied* and Environmental Microbiology, 76(14): 4858-4862.
- Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E. and Relman, D. A. (2005). Diversity of the human intestinal microbial flora. *Science*, **308**(5728): 1635-1638.
- Edwards, I. and Turco, R. (2005). Inter- and intraspecific resolution of nrDNA TRFLP assessed by computer-simulated restriction analysis of a diverse collection of ectomycorrhizal fungi. *Mycological Research*, **109**(2): 212-226.
- Egert, M., de Graaf, A. A., Smidt, H., de Vos, W. M. and Venema, K. (2006). Beyond diversity: Functional microbiomics of the human colon. *Trends in Microbiology*, 14(2): 86-91.

- Egert, M. and Friedrich, M. W. (2003). Formation of pseudo-terminal restriction fragments, a PCR-related bias affecting terminal restriction fragment length polymorphism analysis of microbial community structure. *Applied and Environmental Microbiology*, **69**(5): 2555-2562.
- Egert, M. and Friedrich, M. W. (2005). Post-amplification Klenow fragment treatment alleviates PCR bias caused by partially single-stranded amplicons. *Journal of Microbiological Methods*, **61**(1): 69-75.
- Emborg, H. D., Andersen, J. S., Seyfarth, A. M., Andersen, S. R., Boel, J. and Wegener, H. C. (2003). Relations between the occurrence of resistance to antimicrobial growth promoters among *Enterococcus faecium* isolated from broilers and broiler meat. *International Journal of Food Microbiology*, 84(3): 273-284.
- Engebretson, J. J. and Moyer, C. L. (2003). Fidelity of select restriction endonucleases in determining microbial diversity by terminal-restriction fragment length polymorphism. *Applied and Environmental Microbiology*, **69**(8): 4823-4829.
- Estienne, M. J., Hartsock, T., G. and Harper, A., F. (2005). Effects of antibiotics and probiotics on suckling pig and weaned pig performance. *Journal of Applied Research in Veterinary Medicine*, **3**(5): 303-308.
- Falcão-e-Cunha, L., Castro-Solla, L., Maertens, L., Marounek, M., Pinheiro, V., Freire, J. and Mourão, J. L. (2007). Alternatives to antibiotic growth promoters in rabbit feeding: A review. *World's Rabbit Science Journal*, **15**: 127-140.
- Food and Drug Administration (1980). The effects on human health of subtherapeutic use of antimicrobials in animal feeds. Retrieved in August, 2010, from Food and Drug Administration website:

http://www.fda.gov/ohrms/dockets/dailys/03/Aug03/081403/03n-0324-bkg0001-02-tab1-vol2.pdf

- Food and Drug Administration (2010). The judicious use of medically important antimicrobial drugs in food-producing animals. Retrieved in October, 2010, from Food and Drug Administration website: <u>http://www.fda.gov/AnimalVeterinary/default.htm</u>
- Fouillet, H., Bos, C., Gaudichon, C. and Tomé, D. (2002). Approaches to quantifying protein metabolism in response to nutrient ingestion. *The Journal of Nutrition*, 132(10): 3208S-3218S.
- Fraser, E., Stephen, C., Bowie, W. R. and Wetzstein, M. (2004). Availability and estimates of veterinary antimicrobial use in British Columbia. *The Canadian Veterinary Journal*, **45**: 309–311.
- French, G. L., Shannon, K. P. and Simmons, N. (1996). Hospital outbreak of *Klebsiella pneumoniae* resistant to broad-spectrum cephalosporins and beta-lactam-beta-lactamase inhibitor combinations by hyperproduction of SHV-5 beta-lactamase. *Journal of Clinical Microbiology*, **34**(2): 358-363.
- Frey, J. C., Angert, E. R. and Pell, A. N. (2006). Assessment of biases associated with profiling simple, model communities using terminal-restriction fragment length polymorphism-based analyses. *Journal of Microbiological Methods*, 67(1): 9-19.
- Frostegard, A., Courtois, S., Ramisse, V., Clerc, S., Bernillon, D., Le Gall, F., Jeannin, P., Nesme, X. and Simonet, P. (1999). Quantification of bias related to the extraction of DNA directly from soils. *Applied and Environmental Microbiology*, 65(12): 5409-5420.

- Funk, J. A., Lejeune, J. T., Wittum, T. E. and Rajala-Schultz, P. J. (2006). The effect of subtherapeutic chlortetracycline on antimicrobial resistance in the fecal flora of swine. *Microbial Drug Resistance*, **12**(3): 210-218.
- Gaskins, H. R., Collier, C. T. and Anderson, D. B. (2002). Antibiotics as growth promotants: Mode of action. *Animal Biotechnology*, **13**(1): 29-42.
- Gerdts, V., Uwiera, R. R. E., Mutwiri, G. K., Wilson, D. J., Bowersock, T., Kidane, A., Babiuk, L. A. and Griebel, P. J. (2001). Multiple intestinal 'loops' provide an *in vivo* model to analyse multiple mucosal immune responses. Journal of Immunological Methods, 256(1-2): 19-33.
- Ghosh, S. and LaPara, T. M. (2007). The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. *The International Society for Microbial Ecology Journal*, **1**(3): 191-203.
- Gibson, G., R., Willems, A., Reading, S. and Collins, M. D. (1996). Fermentation of nondigestible oligosaccharides by human colonic bacteria. *Proceedings of the Nutrition Society*, 55: 899-912.
- Goldstein, F. W. (1999). Penicillin-resistant *Streptococcus pneumoniae*: Selection by both β-lactam and non-β-lactam antibiotics. *Journal of Antimicrobial Chemotherapy*, **44**(2): 141-144.
- Gong, J., Forster, R. J., Yu, H., Chambers, J. R., Wheatcroft, R., Sabour, P. M. and Chen, S. (2002). Molecular analysis of bacterial populations in the ileum of broiler chickens and comparison with bacteria in the cecum. *Federation of European Microbiological Societies Microbiology Ecology*, **41**(3): 171-179.
- Gordon, H. A. and Pesti, L. (1971). The gnotobiotic animal as a tool in the study of host microbial relationships. *Bacteriological Reviews*, **35**(4): 390-429.
- Graham, J. P., Boland, J. J. and Silbergeld, E. (2007). Growth promoting antibiotics in food animal production: An economic analysis. *Public Health Reports*, **122**(1): 79–87.
- Hand, W. L., Corwin, R. W., Steinberg, T. H. and Grossman, G. D. (1984). Uptake of antibiotics by human alveolar macrophages. *The American review of respiratory disease*, **129**(6): 933-937.
- Hapfelmeier, S. and Hardt, W.-D. (2005). A mouse model for *S. typhimurium*-induced enterocolitis. *Trends in Microbiology*, **13**(10): 497-503.
- Hartmann, M., Enkerli, J. and Widmer, F. (2007). Residual polymerase activity-induced bias in terminal restriction fragment length polymorphism analysis. *Environmental Microbiology*, **9**(2): 555-559.
- Hartmann, M. and Widmer, F. (2008). Reliability for detecting composition and changes of microbial communities by T-RFLP genetic profiling. *Federation of European Microbiological Societies Microbiology Ecology*, **63**(2): 12.
- Health Canada (2002). Uses of antimicrobials in food animals in Canada: Impact on resistance and human health. Retrieved in July, 2010, from Health Canada website: <u>http://www.hc-sc.gc.ca/dhp-mps/alt_formats/hpfb-dgpsa/pdf/pubs/amr-ram_final_report-rapport_06-27-eng.pdf</u>
- He, G., Shankar, R. A., Chzhan, M., Samouilov, A., Kuppusamy, P. and Zweier, J. L. (1999). Noninvasive measurement of anatomic structure and intraluminal oxygenation in the gastrointestinal tract of living mice with spatial and spectral

EPR imaging. *Proceedings of the National Academy of Sciences of the United States of America*, **96**(8): 4586-4591.

- Higgins, L. M., Frankel, G., Douce, G., Dougan, G. and MacDonald, T. T. (1999). *Citrobacter rodentium* infection in mice elicits a mucosal Th1 cytokine response and lesions similar to those in murine inflammatory bowel disease. *Infection and Immunity*, **67**(6): 3031-3039.
- Hoffmann, C., Hill, D. A., Minkah, N., Kirn, T., Troy, A., Artis, D. and Bushman, F. (2009). Community-wide response of the gut microbiota to enteropathogenic *Citrobacter rodentium* infection revealed by deep sequencing. *Infection and Immunity*, 77(10): 4668-4678.
- Holtz, S. (2009). Reducing and phasing out the use of antibiotics and hormone growth promoters in Canadian agriculture. Retrieved from The Canadian Institute for Environmental Law and Policy website: http://www.cielap.org/pub/pub_AHGPs.php
- Hughes, V. M. and Datta, N. (1983). Conjugative plasmids in bacteria of the 'preantibiotic' era. *Nature*, **302**(5910): 725-726.
- Huyghebaert, G., Ducatelle, R. and Immerseel, F. V. (2010). An update on alternatives to antimicrobial growth promoters for broilers. *The Veterinary Journal*: In Press, Corrected Proof.
- Inglis, G. D., McAllister, T. A., Busz, H. W., Yanke, L. J., Morck, D. W., Olson, M. E. and Read, R. R. (2005). Effects of subtherapeutic administration of antimicrobial agents to beef cattle on the prevalence of antimicrobial resistance in *Campylobacter jejuni* and *Campylobacter hyointestinalis*. *Applied and Environmental Microbiology*, **71**(7): 3872-3881.
- Ingrassia, I., Roques, C. and Prévot, F. (2001). Preliminary experiments for ARDRA validation on flora associated with intestinal mucosa. *Lait*, **81**: 263-280.
- Isolauri, E., Sütas, Y., Kankaanpää, P., Arvilommi, H. and Salminen, S. (2001). Probiotics: Effects on immunity. *American Journal of Clinical Nutrition*, **73**: 444-450.
- Itoh, K., Matsui, T., Tsuji, K., Mitsuoka, T. and Ueda, K. (1988). Genetic control in the susceptibility of germfree inbred mice to infection by *Escherichia coli* O115a,c:K(B). *Infection and Immunity*, 56(4): 930-935.
- Jensen, H. H. and Hayes, D. J. (2003). Antibiotics restrictions: Taking stock of Denmark's experience. *Iowa Ag Review Online*, **9**(3): 4-5.
- Jergens, A. E., Dorn, A., Wilson, J., Dingbaum, K., Henderson, A., Liu, Z., Hostetter, J., Evans, R. B. and Wannemuehler, M. J. (2006). Induction of differential immune reactivity to members of the flora of gnotobiotic mice following colonization with *Helicobacter bilis* or *Brachyspira hyodysenteriae*. *Microbes and Infection*, 8(6): 1602-1610.
- Johansson, M. E. V., Phillipson, M., Petersson, J., Velcich, A., Holm, L. and Hansson, G. C. (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proceedings of the National Academy of Sciences*, **105**(39): 15064-15069.
- Jones, F. T. and Ricke, S. C. (2003). Observations on the history of the development of antimicrobials and their use in poultry feeds. *Poultry Science*, **82**(4): 613-617.

- Kalmokoff, M., Waddington, L., Thomas, M., Leung, K.-L., Kwan, C., Topp, E., Matias, F. and Brooks, S., P. J. (2010). Continuous feeding of subtherapeutic tylosin or virginiamycin during the grow/finishing phase has no apparent effects on fecal community resistance or community structure in commercial swine. *Journal of Applied Microbiology*: (sumbitted).
- Kelly, D., Conway, S. and Aminov, R. (2005). Commensal gut bacteria: mechanisms of immune modulation. *Trends in Immunology*, 26(6): 326-333.
- Kelsall, B. L. (2008). Innate and adaptive mechanisms to control of pathological intestinal inflammation. *The Journal of Pathology*, **214**(2): 242-259.
- Khachatourians, G. G. (1998). Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. *Canadian Medical Association Journal*, **159**(9): 1129-1136.
- Khachatryan, Z. A., Ktsoyan, Z. A., Manukyan, G. P., Kelly, D., Ghazaryan, K. A., Aminov, R. I. and Fraser, J. A. (2008). Predominant role of host genetics in controlling the composition of gut microbiota. *Public Library of Science One*, 3(8): e3064.
- Kimura, A. and Kishimoto, T. (2010). IL-6: Regulator of Treg/Th17 balance. *European Journal of Immunology*, **40**(7): 1830-1835.
- Klappenbach, J. A., Saxman, P. R., Cole, J. R. and Schmidt, T. M. (2001). rrndb: The ribosomal RNA operon copy number database. *Nucleic Acids Research*, **29**(1): 181-184.
- Klasing, K. C. (2007). Nutrition and the immune system. *British Poultry Science*, **48**(5): 525-537.
- Klasing, K. C. and Korver, D. R. (1997). Leukocytic cytokines regulate growth rate and composition following activation of the immune system. *Journal of Animal Science*, **75**(2): 58-67.
- Klasing, K. C., Laurin, D. E., Peng, R. K. and Fry, D. M. (1987). Immunologically mediated growth depression in chicks: Influence of feed intake, corticosterone and interleukin-1. *Journal of Nutrition*, **117**(9): 1629-1637.
- Kolls, J. K. and Lindén, A. (2004). Interleukin-17 family members and inflammation. *Immunity*, **21**(4): 467-476.
- Korhonen, R., Korpela, R., Saxelin, M., Maki, M., Kankaanranta, H. and Moilanen, E. (2001). Induction of nitric oxide synthesis by probiotic *Lactobacillus rhamnosus* GG in J774 macrophages and human T84 intestinal epithelial cells. *Inflammation*, **25**(4).
- Korn, T., Bettelli, E., Oukka, M. and Kuchroo, V. K. (2009). IL-17 and Th17 cells. Annual Review of Immunology, **27**(1): 485-517.
- Kropf, S., Heuer, H., Grüning, M. and Smalla, K. (2004). Significance test for comparing complex microbial community fingerprints using pairwise similarity measures. *Journal of Microbiological Methods*, 57: 187-195.
- Labro, M.-T. (2000). Interference of antibacterial agents with phagocyte functions: immunomodulation or "immuno-fairy tales"? *Clinical Microbiology Reviews*, **13**(4): 615-650.
- Langlois, B. E., Cromwell, G. L. and Hays, V. W. (1978). Influence of chlortetracycline in swine feed on reproductive performance and on incidence and persistence of antibiotic resistant enteric bacteria. *Journal of Animal Science*, **46**(5): 1369-1382.

- Le Ferrec, E., Chesne, C., Artusson, P., Brayden, D., Fabre, G., Gires, P., Guillou, F., Rousset, M., Rubas, W. and Scarino, M.-L. (2001). *In vitro* models of the intestinal barrier. *Alternatives to Laboratory Animals*, **29**: 649-668.
- Lecuit, M., Sonnenburg, J. L., Cossart, P. and Gordon, J. I. (2007). Functional genomic studies of the intestinal response to a foodborne enteropathogen in a humanized gnotobiotic mouse model. *Journal of Biological Chemistry*, **282**(20): 15065-15072.
- Lepage, P., Seksik, P., Sutren, M., Cochetière, M.-F. d. l., Jian, R., Marteau, P. and Doré, J. (2005). Biodiversity of the mucosa-associated microbiota is stable along the distal digestive tract in healthy individuals and patients with IBD. *Inflammatory Bowel Diseases*, **11**(5): 473-480.
- Ley, R. E., Peterson, D. A. and Gordon, J. I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*, **124**(4): 837-848.
- Li, M., Gong, J., Cottrill, M., Yu, H., de Lange, C., Burton, J. and Topp, E. (2003). Evaluation of QIAamp® DNA Stool Mini Kit for ecological studies of gut microbiota. *Journal of Microbiological Methods*, 54(1): 13-20.
- Liu, W. T., Marsh, T. L., Cheng, H. and Forney, L. J. (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology*, 63(11): 4516-4522.
- Lorenz, R. G., McCracken, V. J. and Elson, C. O. (2005). Animal models of intestinal inflammation: Ineffective communication between coalition members. *Springer Seminars in Immunopathology*, 27(2): 233-247.
- Luperchio, S. A., Newman, J. V., Dangler, C. A., Schrenzel, M. D., Brenner, D. J., Steigerwalt, A. G. and Schauer, D. B. (2000). *Citrobacter rodentium*, the causative agent of transmissible murine colonic hyperplasia, exhibits clonality: Synonymy of *C. rodentium* and mouse-pathogenic *Escherichia coli*. *Journal of Clinical Microbiology*, **38**(12): 4343-4350.
- Luperchio, S. A. and Schauer, D. B. (2001). Molecular pathogenesis of *Citrobacter rodentium* and transmissible murine colonic hyperplasia. *Microbes and Infection*, 3(4): 333-340.
- Ma, D., Wolvers, D., Stanisz, A. M. and Bienenstock, J. (2003). Interleukin-10 and nerve growth factor have reciprocal upregulatory effects on intestinal epithelial cells. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 284(5): 1323-1329.
- Maaser, C., Housley, M. P., Iimura, M., Smith, J. R., Vallance, B. A., Finlay, B. B., Schreiber, J. R., Varki, N. M., Kagnoff, M. F. and Eckmann, L. (2004). Clearance of *Citrobacter rodentium* requires B cells but not secretory immunoglobulin A (IgA) or IgM antibodies. *Infection and Immunity*, **72**(6): 3315-3324.
- MacDonald, T. T. and Monteleone, G. (2005). Immunity, inflammation, and allergy in the gut. *Science*, **307**(5717): 1920-1925.
- Macfarlane, G. T. and Macfarlane, L. E. (2009). Acquisition, evolution and maintenance of the normal gut microbiota. *Digestive Diseases*, **27**(Suppl. 1): 90-98.
- Maczulak, A. E., Wolin, M., J. and Miller, T., L. (1993). Amounts of viable anaerobes, methanogens, and bacterial fermentation products in feces of rats fed high-fiber or fiber-free diets. *Applied and Environmental Microbiology*, **59**(3): 657-662.

- Malstrom, C. and James, S. (1998). Inhibition of murine splenic and mucosal lymphocyte function by enteric bacterial products. *Infection and Immunity*, **66**(7): 3120-3127.
- Mangan, P. R., Harrington, L. E., O'Quinn, D. B., Helms, W. S., Bullard, D. C., Elson, C. O., Hatton, R. D., Wahl, S. M., Schoeb, T. R. and Weaver, C. T. (2006). Transforming growth factor-beta induces development of the Th17 lineage. *Nature*, 441(7090): 231-234.
- McAuley, J. L., Linden, S. K., Png, C. W., King, R. M., Pennington, H. L., Gendler, S. J., Florin, T. H., Hill, G. R., Korolik, V. and McGuckin, M. A. (2007). MUC1 cell surface mucin is a critical element of the mucosal barrier to infection. *The Journal* of *Clinical Investigation*, **117**(8): 2313-2324.
- McEwen, Scott A. and Fedorka-Cray, Paula J. (2002). Antimicrobial use and resistance in animals. *Clinical Infectious Diseases*, **34**(3): 93-106.
- Mellon, M., Benbrook, C. and Benbrook, K. (2001). Hogging it: Estimates of antimicrobial abuse in livestock. UCS Publications: www.ucsusa.org/publications.
- Meng, J., Zhao, S., Doyle, M. P. and Joseph, S. W. (1998). Antibiotic resistance of *Escherichia coli* O157:H7 and O157:NM isolated from animals, food, and humans. *Journal of Food Protection*, 61: 1511-1514.
- Meurens, F., Berri, M., Auray, G., Melo, S., Levast, B., Virlogeux-Payant, I., Chevaleyre, C., Gerdts, V. and Salmon, H. (2009). Early immune response following *Salmonella enterica* subspecies *enterica* serovar *Typhimurium* infection in porcine jejunal gut loops. *Veterinary Research*, **40**(1): 05.
- Meyer-Hoffert, U., Hornef, M. W., Henriques-Normark, B., Axelsson, L. G., Midtvedt, T., Pütsep, K. and Andersson, M. (2008). Secreted enteric antimicrobial activity localises to the mucus surface layer. *Gut*, **57**(6): 764-771.
- Miflin, J. K., Templeton, J. M. and Blackall, P. J. (2007). Antibiotic resistance in *Campylobacter jejuni* and *Campylobacter coli* isolated from poultry in the South-East Queensland region. *Journal of Antimicrobial Chemotherapy*, **59**(4): 775-778.
- Milano, S., Arcoleo, F., D'Agostino, P. and Cillari, E. (1997). Intraperitoneal injection of tetracyclines protects mice from lethal endotoxemia downregulating inducible nitric oxide synthase in various organs and cytokine and nitrate secretion in blood. *Antimicrobial agents and Chemotherapy*, **41**(1): 117-121.
- Mixter, P. F., Klena, J. D., Flom, G. A., Siegesmund, A. M. and Konkel, M. E. (2003). In vivo tracking of *Campylobacter jejuni* by using a novel recombinant expressing green fluorescent protein. *Applied and Environmental Microbiology*, 69(5): 2864-2874.
- Moal, V. L.-L. and Servin, A. L. (2006). The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: Mucins, antimicrobial peptides, and microbiota. *Clinical Microbiology Reviews*, **19**(2): 315-337.
- Moeseneder, M. M., Winter, C., Arrieta, J. M. and Herndl, G. J. (2001). Terminalrestriction fragment length polymorphism (T-RFLP) screening of a marine archaeal clone library to determine the different phylotypes. *Journal of Microbiological Methods*, **44**(2): 159-172.
- Mofleh, I. A. A. (2010). Spices, herbal xenobiotics and the stomach: Friends or foes? *World Journal of Gastroenterology*, **16**(22): 2710.

- Moore, P. R., Evenson, A., Luckey, T. D., McCoy, E., Elvehjem, C. A. and Hart, E. B. (1946). Use of sulfasuxidine, streptothricin and streptomycin in nutritional studies with the chick. *Journal of Biological Chemistry*, 165(2): 437-441.
- More, M. I., Herrick, J. B., Silva, M. C., Ghiorse, W. C. and Madsen, E. L. (1994). Quantitative cell lysis of indigenous microorganisms and rapid extraction of microbial DNA from sediment. *Applied and Environmental Microbiology*, 60(5): 1572-1580.
- Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *Journal of Immunology*, 136(7): 2348-2357.
- Mueller, C. and Macpherson, A. J. (2006). Layers of mutualism with commensal bacteria protect us from intestinal inflammation. *Gut*, **55**(2): 276-284.
- Mundy, R., MacDonald, T. T., Dougan, G., Frankel, G. and Wiles, S. (2005). *Citrobacter rodentium* of mice and man. *Cellular Microbiology*, **7**(12): 1697-1706.
- Muyzer, G. and Smalla, K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek*, **73**(1): 127-141.
- National Animal Health Monitoring System (1999). Feedlot report part III: Health management and biosecurity in US feedlots. Retrieved in July, 2010, from National Animal Health Monitoring System website: <u>http://www.aphis.usda.gov/animal_health/nahms/</u>
- National Animal Health Monitoring Service (2006). Swine report part II: Reference of swine health and health management practices in the United States. Retrieved in July, 2010, from National Animal Health Monitoring Service website: http://www.aphis.usda.gov/animal_health/nahms/
- Niewold, T. A. (2007). The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. *Poultry Science*, **86**(4): 605-609.
- Opalinski, M., Maiorka, A., Dahlke, F., Cunha, F., Vargas, F. S. C. and Cardozo, E. (2007). On the use of a probiotic (*Bacillus subtilis* strain DSM 17299) as growth promoter in broiler diets. *Revista Brasileira de Ciência Avícola*, **9**: 99-103.
- Pajak, B., Orzechowska, S., Pijet, B., Pogorzelska, A., Gajkowsa, B. and Orzechowski, A. (2008). Crossroads of cytokine signaling - the chase to stop muscle cachexia. *Journal of Physiology and Pharmacology*, **59**(9): 251-264.
- Pandey, J., Ganesan, K. and Jain, R. K. (2007). Variations in T-RFLP profiles with differing chemistries of fluorescent dyes used for labeling the PCR primers. *Journal of Microbiological Methods*, 68(3): 633-638.
- Parronchi, P., De Carli, M., Manetti, R., Simonelli, C., Sampognaro, S., Piccinni, M., Macchia, D., Maggi, E., Del Prete, G. and Romagnani, S. (1992). IL-4 and IFN (alpha and gamma) exert opposite regulatory effects on the development of cytolytic potential by Th1 or Th2 human T cell clones. *Journal of Immunology*, 149(9): 2977-2983.
- Peng, L., He, Z., Chen, W., Holzman, I. R. and Lin, J. (2007). Effects of butyrate on intestinal barrier function in a caco-2 cell monolayer model of intestinal barrier. *Pediatric Research*, 61(1): 37-41.

- Perrin-Guyomard, A., Cottin, S., Corpet, D. E., Boisseau, J. and Poul, J.-M. (2001). Evaluation of residual and therapeutic doses of tetracycline in the human-floraassociated (HFA) mice model. *Regulatory Toxicology and Pharmacology*, **34**(2): 125-136.
- Perron, G. G., Quessy, S. and Bell, G. (2008). A reservoir of drug-resistant pathogenic bacteria in asymptomatic hosts. *Public Library of Science One*, **3**(11): e3749.
- Pinard, R., de Winter, A., Sarkis, G., Gerstein, M., Tartaro, K., Plant, R., Egholm, M., Rothberg, J. and Leamon, J. (2006). Assessment of whole genome amplificationinduced bias through high-throughput, massively parallel whole genome sequencing. *BioMed Central Genomics*, 7(1): 216.
- Population Reference Bureau (2009). World population data sheet. Retrieved in August, 2010, from Population Reference Bureau website: http://www.prb.org/pdf09/09wpds_eng.pdf
- Pruimboom-Brees, I. M. (2000). Cattle lack vascular receptors for *Escherichia coli* O157:H7 Shiga toxins. *Proceedings of the National Academy of Sciences*, **97**(19): 10325-10329.
- Pryde, S. E., Richardson, A. J., Stewart, C. S. and Flint, H. J. (1999). Molecular analysis of the microbial diversity present in the colonic wall, colonic lumen, and cecal lumen of a pig. *Applied and Environmental Microbiology*, **65**(12): 5372-5377.
- Qiu, X., Wu, L., Huang, H., McDonel, P. E., Palumbo, A. V., Tiedje, J. M. and Zhou, J. (2001). Evaluation of PCR-generated chimeras, mutations, and heteroduplexes with 16S rRNA gene-based cloning. *Applied and Environmental Microbiology*, 67(2): 880-887.
- Rettedal, E., Vilain, S., Lindblom, S., Lehnert, K., Scofield, C., George, S., Clay, S., Kaushik, R. S., Rosa, A. J. M., Francis, D. and Brozel, V. S. (2009). Alteration of the ileal microbiota of weanling piglets by the growth-promoting antibiotic chlortetracycline. *Applied and Environmental Microbiology*, **75**(17): 5489-5495.
- Roberfroid, M. (2007). Prebiotics: The concept revisited. *Journal of Nutrition*, **137**(3): 830-837.
- Roberfroid, M. B. (2000). Prebiotics and probiotics: Are they functional foods? *American Journal of Clinical Nutrition*, **71**(6): 1682S-1687.
- Roche, Y., Fay, M. and Gougerot-Pocidalo, M.-A. (1988). Interleukin-1 production by antibiotic-treated human monocytes. *Journal of Antimicrobial Chemotherapy*, 21(5): 597-607.
- Romagnani, S. (2000). T-cell subsets (Th1 versus Th2). Annals of Allergy, Asthma & Immunology, **85**(1): 9-18, 21.
- Romagnani, S. (2006). Regulation of the T cell response. *Clinical & Experimental Allergy*, **36**(11): 1357-1366.
- Ronaghi, M. (2001). Pyrosequencing sheds light on DNA sequencing. *Genome Research*, **11**(1): 3-11.
- Roncarolo, M. G., Bacchetta, R., Bordignon, C., Narula, S. and Levings, M. K. (2001). Type 1 T regulatory cells. *Immunological Reviews*, **182**(1): 68-79.
- Roura, E., Homedes, J. and Klasing, K. C. (1992). Prevention of immunologic stress contributes to the growth-permitting ability of dietary antibiotics in chicks. *Journal of Nutrition*, **122**(12): 2383-2390.

- Rubin, B. K. and Tamaoki, J. (2005). *Antibiotics as anti-inflammatory and immunomodulatory agents* (1 ed.). Basel, Switzerland: Birkhäuser Verlag.
- Rudi, K., Hoidal, H. K., Katla, T., Johansen, B. K., Nordal, J. and Jakobsen, K. S. (2004). Direct real-time PCR quantification of *Campylobacter jejuni* in chicken fecal and cecal samples by integrated cell concentration and DNA purification. *Applied and Environmental Microbiology*, **70**(2): 790-797.
- Rumbo, M. and Schiffrin, E. J. (2005). Ontogeny of intestinal epithelium immune functions: Developmental and environmental regulation. *Cellular and Molecular Life Sciences*, **62**: 1288-1296.
- Rumsey, T. S., McLeod, K., Elsasser, T. H., Kahl, S. and Baldwin, R. L. ((1999). Effects of oral chlortetracycline and dietary protein level on plasma concentrations of growth hormone and thyroid hormones in beef steers before and after challenge with a combination of thyrotropin-releasing hormone and growth hormonereleasing hormone. *Journal of Animal Science*, **77**(8): 2079-2087.
- Sait, L., Galic, M., Strugnell, R. A. and Janssen, P. H. (2003). Secretory antibodies do not affect the composition of the bacterial microbiota in the terminal ileum of 10week-old mice. *Applied and Environmental Microbiology*, 69: 2100-2109.
- Sakurai, Y., Zhang, X. J. and Wolfe, R. R. (1994). Effect of tumor necrosis factor on substrate and amino acid kinetics in conscious dogs. *American Journal of Physiology-Endocrinology and Metabolism*, **266**(6): 936-945.
- Sarma-Rupavtarm, R. B., Ge, Z., Schauer, D. B., Fox, J. G. and Polz, M. F. (2004). Spatial distribution and stability of the eight microbial species of the altered schaedler flora in the mouse gastrointestinal tract. *Applied and Environmental Microbiology*, **70**(5): 2791-2800.
- Savage, D. C. (1977). Microbial ecology of the gastrointestinal tract. Annual Review of Microbiology, 31(1): 107-133.
- Savage, D. C. (1987). Microorganisms associated with epithelial surfaces and stability of the indigenous gastrointestinal microflora. *Food / Nahrung*, **31**(5-6): 383-395.
- Schauer, D. B., Zabel, B. A., Pedraza, I. F., O'Hara, C. M., Steigerwalt, A. G. and Brenner, D. J. (1995). Genetic and biochemical characterization of *Citrobacter rodentium* sp. nov. *Journal of Clinical Microbiology*, **33**(8): 2064-2068.
- Schiffrin, E. J. and Blum, S. (2002). Interactions between the microbiota and the intestinal mucosa. *European Journal of Clinical Nutrition*, **56**(3): 560–564.
- Shapira, L., Soskolne, W. A., Houri, Y., Barak, V., Halabi, A. and Stabholz, A. (1996). Protection against endotoxic shock and lipopolysaccharide-induced local inflammation by tetracycline: Correlation with inhibition of cytokine secretion. *Infection and Immunity*, 64(3): 825-828.
- Shea, K. M., Committee on Environmental, H. and Committee on Infectious, D. (2004). Nontherapeutic use of antimicrobial agents in animal agriculture: Implications for pediatrics. *Pediatrics*, **114**(3): 862-868.
- Shuli, W., Yanfeng, L. U. O., Xiao, Y. I., Wenwen, Y. U., Zhiwu, X. U., Xiaoqian, M. A., Jianguo, H. E. and Qiuyun, L. I. U. (2007). A highly efficient and highly reliable protocol for transformation of *Escherichia coli* by *electroporation*. *Journal of Rapid Methods & Automation in Microbiology*, **15**(3): 253-258.
- Simmons, C. P., Clare, S., Ghaem-Maghami, M., Uren, T. K., Rankin, J., Huett, A., Goldin, R., Lewis, D. J., MacDonald, T. T., Strugnell, R. A., Frankel, G. and

Dougan, G. (2003). Central role for B lymphocytes and CD4+ T cells in immunity to infection by the attaching and effacing pathogen *Citrobacter rodentium*. *Infection and Immunity*, **71**(9): 5077-5086.

- Simpson, J. M., McCracken, V. J., White, B. A., Gaskins, H. R. and Mackie, R. I. (1999). Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. *Journal of Microbiological Methods*, 36(3): 167-179.
- Skinn, A. C., Vergnolle, N., Zamuner, S. R., Wallace, J. L., Cellars, L., MacNaughton, W. K. and Sherman, P. (2006). *Citrobacter rodentium* infection causes iNOSindependent intestinal epithelial dysfunction in mice. *Canadian Journal of Physiology and Pharmacology*, **84**(12): 1301-1312.
- Smalla, K., Oros-Sichler, M., Milling, A., Heuer, H., Baumgarte, S., Becker, R., Neuber, G., Kropf, S., Ulrich, A. and Tebbe, C. C. (2007). Bacterial diversity of soils assessed by DGGE, T-RFLP and SSCP fingerprints of PCR-amplified 16S rRNA gene fragments: Do the different methods provide similar results? *Journal of Microbiological Methods*, 69(3): 470-479.
- Smith, K., McCoy, K. D. and Macpherson, A. J. (2007). Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. *Seminars in Immunology*, **19**(2): 59-69.
- Smith, T. C., Male, M. J., Harper, A. L., Kroeger, J. S., Tinkler, G. P., Moritz, E. D., Capuano, A. W., Herwaldt, L. A. and Diekema, D. J. (2009). Methicillin-resistant *Staphylococcus aureus* (MRSA) strain ST398 is present in midwestern U.S. swine and swine workers. *Public Library of Science One*, 4(1): e4258.
- Sonnenburg, J. L., Angenent, L. T. and Gordon, J. I. (2004). Getting a grip on things: How do communities of bacterial symbionts become established in our intestine? *Nature Immunology*, 5(6): 569-573.
- Spahn, T. W., Ross, M., Eiff, C. v., Maaser, C., Spieker, T., Kannengiesser, K., Domschke, W. and Kucharzik, T. (2008). CD4+T cells transfer resistance against *Citrobacter rodentium*-induced infectious colitis by induction of Th1 immunity. *Scandinavian Journal of Immunology*, 67(3): 238-244.
- Spottl, T., Hausmann, M., Gunckel, M., Herfarth, H., Herlyn, M., Schoelmerich, J. and Rogler, G. (2006). A new organotypic model to study cell interactions in the intestinal mucosa. *European Journal of Gastroenterology & Hepatology*, **18**(8): 901-909.
- Stackebrandt, E. and Goebel, B. M. (1994). Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic and Evolutionary Microbiology*, 44(4): 846-849.
- Stecher, B. and Hardt, W.-D. (2008). The role of microbiota in infectious disease. *Trends in Microbiology*, **16**(3): 107-114.
- Sternberg, E. M. (2006). Neural regulation of innate immunity: A coordinated nonspecific host response to pathogens. *Nature Reviews Immunology*, 6(4): 318-328.
- Stokes, C. R. and Bailey, M. (2000). The porcine gastrointestinal lamina propria: An appropriate target for mucosal immunisation? *Journal of Biotechnology*, **83**(1-2): 51-55.

- Strober, W. (2006). Immunology: Unraveling gut inflammation. *Science*, **313**(5790): 1052-1054.
- Symonds, E. L., Riedel, C. U., O'Mahony, D., Lapthorne, S., O'Mahony, L. and Shanahan, F. (2009). Involvement of T helper type 17 and regulatory T cell activity in *Citrobacter rodentium* invasion and inflammatory damage. *Clinical & Experimental Immunology*, **157**(1): 148-154.
- Tenover, F. (2006). Mechanisms of antimicrobial resistance in bacteria. *The American Journal of Medicine*, **119**(6): S3-S10.
- Thoma-Uszynski, S., Stenger, S., Takeuchi, O., Ochoa, M. T., Engele, M., Sieling, P. A., Barnes, P. F., Rollinghoff, M., Bolcskei, P. L., Wagner, M., Akira, S., Norgard, M. V., Belisle, J. T., Godowski, P. J., Bloom, B. R. and Modlin, R. L. (2001). Induction of direct antimicrobial activity through mammalian Toll-like receptors. *Science*, 291(5508): 1544-1547.
- Thompson, C. L., Wang, B. and Holmes, A. J. (2008). The immediate environment during postnatal development has long-term impact on gut community structure in pigs. *The International Society for Microbial Ecology Journal*, **2**(7): 739-748.
- Timbermont, L., Lanckriet, A., Dewulf, J., Nollet, N., Schwarzer, K., Haesebrouck, F., Ducatelle, R. and Van Immerseel, F. (2010). Control of *Clostridium perfringens*induced necrotic enteritis in broilers by target-released butyric acid, fatty acids and essential oils. *Avian Pathology*, **39**(2): 117 - 121.
- Tortora, G. J. and Derrickson, B. H. ((2006). Principles of anatomy and physiology. Retrieved from Wiley Higher Education - Student Companion website: <u>http://bcs.wiley.com/he-</u>

bcs/Books?action=contents&itemId=0471689343&bcsId=2287

- Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., Sogin, M. L., Jones, W. J., Roe, B. A., Affourtit, J. P., Egholm, M., Henrissat, B., Heath, A. C., Knight, R. and Gordon, J. I. (2009). A core gut microbiome in obese and lean twins. *Nature*, 457(7228): 480-484.
- Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C. M., Knight, R. and Gordon, J. I. (2007). The human microbiome project. *Nature*, 449(7164): 804-810.
- Turnberg, L. A. (1970). Electrolyte absorption from the colon. Gut, 11(12): 1049-1054.
- Uematsu, S. and Akira, S. (2006). PRRs in pathogen recognition. *Central European Journal of Biology*, **1**(3): 299-313.
- Utiyama, C. E. (2004). Utilização de agentes antimicrobianos, probióticos, prebióticos e extratos vegetais como promotores de crescimento em leitoes recémdesmamados. *Doctor of Philosophy Thesis*: 1-110.
- Uwiera, R. R. E., Kastelic, J. P. and Inglis, G. D. (2009). Catheterization of intestinal loops in ruminants. *Journal of Visualized Experiments*(28): e1301.
- Vallance, B. A. (2002). Mice lacking T and B lymphocytes develop transient colitis and crypt hyperplasia yet suffer impaired bacterial clearance during *Citrobacter rodentium* infection. *Infection and Immunity*, **70**(4): 2070-2081.
- Vallance, B. A., Deng, W., De Grado, M., Chan, C., Jacobson, K. and Finlay, B. B. (2002). Modulation of inducible nitric oxide synthase expression by the attaching and effacing bacterial pathogen *Citrobacter rodentium* in infected mice. *Infection and Immunity*, **70**(11): 6424-6435.

- van de Veerdonk, F. L., Gresnigt, M. S., Kullberg, B. J., van der Meer, J. W., Joosten, L. A. and Netea, M. G. (2009). Th17 responses and host defense against microorganisms: An overview. *Biochemistry and Molecular Biology Reports*, 42(12): 776-787.
- van der Waaij, D., Berghuis-de Vries, J. M. and Lekkerkerk-van der Wees, J. E. C. (1971). Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. *The Journal of Hygiene*, **69**(3): 405-411
- Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M. and Stockinger, B. (2006). TGF[beta] in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity*, 24(2): 179-189.
- Visek, W. J. (1978). The mode of growth promotion by antibiotics. *Journal of Animal Science*, **46**(5): 1447-1469.
- Vollaard, E. J. and Clasener, H. A. (1994). Colonization resistance. *Antimicrobial agents and Chemotherapy*, **38**(3): 409-414.
- Vyas, J. M., Van der Veen, A. G. and Ploegh, H. L. (2008). The known unknowns of antigen processing and presentation. *Nature Reviews Immunology*, **8**(8): 607-618.
- Waksman, S. A. (1947). What is an antibiotic or an antibiotic substance? *Mycologia*, **39**(5): 565-569.
- Weaver, C. T., Hatton, R. D., Mangan, P. R. and Harrington, L. E. (2007). IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annual Review of Immunology*, **25**(1): 821-852.
- Weigent, D. A. and Blalock, J. E. (1995). Associations between the neuroendocrine and immune systems. *Journal of Leukocyte Biology*, **58**(2): 137-150.
- Weiner, H. L. (2001). Induction and mechanism of action of transforming growth factorβ-secreting Th3 regulatory cells. *Immunological Reviews*, **182**(1): 207-214.
- Weisburg, W. G., Barns, S. M., Pelletier, D. A. and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, **173**(2): 697-703.
- Weng, L., Rubin, E. M. and Bristow, J. (2006). Application of sequence-based methods in human microbial ecology. *Genome Research*, **16**(3): 316-322.
- World Health Organization (2000). Global principles for the containment of antimicrobial resistance in animals intended for food. Retrieved in April, 2010, from World Health Organization website:

http://www.who.int/salmsurv/links/en/GSSGlobalPrinciples2000.pdf

World Health Organization (2001). Report of a joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Retrieved in October 15, 2001, from World Health Organization

http://www.who.int/entity/foodsafety/publications/fs_management/en/probiotics.p df

- World Health Organization (2003). 1st Joint FAO/OIE/WHO expert workshop on nonhuman antimicrobial usage and antimicrobial resistance: Scientific assessment Retrieved in June, 2010, from World Health Organization website: <u>http://www.who.int/foodsafety/publications/micro/nov2003/en/</u>
- World Health Organization (2004). Second joint FAO/OIE/WHO expert workshop on non-human antimicrobial usage and antimicrobial resistance: Management

options. Retrieved, from World Health Organization http://www.who.int/foodsafety/publications/micro/en/oslo_report.pdf

- Wiles, S., Clare, S., Harker, J., Huett, A., Young, D., Dougan, G. and Frankel, G. (2004). Organ specificity, colonization and clearance dynamics *in vivo* following oral challenges with the murine pathogen *Citrobacter rodentium*. *Cellular Microbiology*, 6(10): 963-972.
- Wiles, S., Pickard, K. M., Peng, K., MacDonald, T. T. and Frankel, G. (2006). *In vivo* bioluminescence imaging of the murine pathogen *Citrobacter rodentium*. *Infection and Immunity*, 74(9): 5391-5396.
- Wirtz, S., Neufert, C., Weigmann, B. and Neurath, M. F. (2007). Chemically induced mouse models of intestinal inflammation. [10.1038/nprot.2007.41]. *Nature Protocols*, 2(3): 541-546.
- Wise, M. and Siragusa, G. (2007). Quantitative analysis of the intestinal bacterial community in one- to three-week-old commercially reared broiler chickens fed conventional or antibiotic-free vegetable-based diets. *Journal of Applied Microbiology*, **102**(4): 1138-1149.
- Wood, A. J. J., Gold, H. S. and Moellering, R. C. (1996). Antimicrobial-drug resistance. New England Journal of Medicine, 335(19): 1445-1453.
- Wu, X., Vallance, B. A., Boyer, L., Bergstrom, K. S. B., Walker, J., Madsen, K., O'Kusky, J. R., Buchan, A. M. and Jacobson, K. (2008). Saccharomyces boulardii ameliorates Citrobacter rodentium-induced colitis through actions on bacterial virulence factors. American Journal of Physiology and Gastrointestinal Liver Physiology, 294(1): G295-306.
- Xu, Z. R., Hu, C. H., Xia, M. S., Zhan, X. A. and Wang, M. Q. (2003). Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. *Poultry Science*, 82(6): 1030-1036.
- Yang, Y., Iji, P. A. and Choct, M. (2009). Dietary modulation of gut microflora in broiler chickens: A review of the role of six kinds of alternatives to in-feed antibiotics. *World's Poultry Science Journal*, 65(01): 97-114.
- Yasui, H., Shida, K., Matsuzaki, T. and Yokokura, T. (1999). Immunomodulatory function of lactic acid bacteria. *Antonie van Leeuwenhoek*, **76**: 383-389.
- Ye, P., Rodriguez, F. H., Kanaly, S., Stocking, K. L., Schurr, J., Schwarzenberger, P., Oliver, P., Huang, W., Zhang, P., Zhang, J., Shellito, J. E., Bagby, G. J., Nelson, S., Charrier, K., Peschon, J. J. and Kolls, J. K. (2001). Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colonystimulating factor expression, neutrophil recruitment, and host defense. *The Journal of Experimental Medicine*, **194**(4): 519-528.
- Zhang, R., Thiyagarajan, V. and Qian, P.-Y. (2008). Evaluation of terminal-restriction fragment length polymorphism analysis in contrasting marine environments. *Federation of European Microbiological Societies Microbiology Ecology*, 65(1): 169-178.
- Zheng, Y., Valdez, P. A., Danilenko, D. M., Hu, Y., Sa, S. M., Gong, Q., Abbas, A. R., Modrusan, Z., Ghilardi, N., de Sauvage, F. J. and Ouyang, W. (2008). Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nature Medicine*, 14(3): 282-289.

- Zoetendal, E. G., Akkermans, A. D. L., Vliet, W. M. A.-v., Arjan, J., de Visser, G. M. and de Vos, W. M. (2001). The host genotype affects the bacterial community in the human gastronintestinal tract. *Microbial Ecology in Health and Disease*, 13(3): 129 - 134.
- Zoetendal, E. G., Collier, C. T., Koike, S., Mackie, R. I. and Gaskins, H. R. (2004). Molecular ecological analysis of the gastrointestinal microbiota: A review. *Journal of Nutrition*, **134**(2): 465-472.
- Zoetendal, E. G., von Wright, A., Vilpponen-Salmela, T., Ben-Amor, K., Akkermans, A. D. L. and de Vos, W. M. (2002). Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. *Applied and Environmental Microbiology*, 68(7): 3401-3407.

APPENDICES

APPENDIX ONE. SAS program³ for identification of T-RFs in T-RFLP analyses

%let in_file = .txt; * Specify the input file name; % let out file = .html;* Specify the output file name; %let var = : * Specify the variable to be used to find peaks; * The choices are peak height or peak area; %let var mult = ; * Defines peaks - peaks are above VAR MULT * VARIABILITY; %let adjust_for_mean = ; * Used to control how the variability is calculated. Set to YES if you want to adjust for the mean (YES/NO); *** Use these to control what is printed (YES / NO) ***** ****** %let p all data = YES; * Print all the data with peaks identified; %let p only peaks = YES; * Print the results with ID values in columns; * This macro is used to control the printing; %macro print(title=, data=, print=); %if %upcase(&print) eq YES %then %do; title "&title"; proc print data=&data noobs uniform; run: %end: %mend: options nocenter; ods html style=minimal body = "&out file"; ***** Read the data from an external file data file1:

³ Toby Entz (AAFC LRC) designed the present SAS program.

infile "&in_file" expandtabs truncover firstobs=4;

peak = 0;

input id \$ rep peak_num plus_minus frag_size peak_height peak_area; run;

```
* Create a numeric index for the id values so they can be processed
in an IML matrix;
```

```
proc sort data=file1;
 by rep id;
run;
data file1;
set file1 end=eof;
by rep id;
retain index 0;
if first.id then index = index + 1;
if &var ge 0 then
  sq var = &var * &var;
if eof then
  call symput('max index', index);
run;
* Find the peaks based on the variance. PEAK is set to 1 for a peak and
to 0 otherwise;
proc iml;
 use file1;
  read all var (rep index &var sq var peak peak num) into in data;
 rows = nrow(in_data);
 index end = 0;
 do index = 1 to \& max index;
  flag = 1;
  index start = index end + 1;
```

* See where the current ID starts and ends;

```
do i = index start to rows;
   if in data[i, 2] = index then
    index end = index end + 1;
 end;
 do while (flag = 1);
   flag = 0;
   sum sq = 0;
   sum = 0;
   count = 0;
   * Calculate the sum of squares and the cutoff value using
    only values that are not identified as peaks;
   do i = index start to index end;
    if in data[i, 5] = 0 & in data[i, 3] > 0 then do;
     sum sq = sum sq + in data[i, 4];
     if "%upcase(&adjust for mean)" = "YES" then
      sum = sum + in data[i, 3];
     count = count + 1;
    end;
    end;
   cut off = &var mult * (sqrt((sum sq - (sum*sum / count))) / (count - 1)));
   * Set PEAK to 1 from 0 if the cutoff is exceeded;
   do i = index start to index end;
    if in data[i,3] > cut off & in data[i, 5] = 0 then do;
     in data[i,5] = 1;
     flag = 1;
    end;
    end:
    end;
end:
```

create file2 from in_data [COLNAME=(rep index &var sq_var peak peak_num)];
append from in_data;
run;

* Combine the peak identification information with the original data set ***;

```
proc sort data=file1;
 by rep index peak num;
run;
proc sort data=file2;
 by rep index peak num;
run;
data file1 (keep = rep id peak peak num frag size peak height peak area);
 merge file1 (drop = peak)
    file2:
by rep index peak num;
run;
proc sort data=file1;
 by id peak num rep;
run;
%print(title=All the used data: PEAK=1 for identified peaks -
      %upcase(&var) used to identify peaks.
      Peak defining muliplyer = &var mult,
   data=file1, print=&p all data)
* Compare the 2 reps and set PEAK to 1 if both are peaks;
data file2 (keep = id peak num peak1)
  file3 (keep = id peak num peak2);
set file1;
 if rep eq 1 then do;
  peak1 = peak;
  output file2;
 end:
 else if rep eq 2 then do;
  peak2 = peak;
  output file3;
 end;
run;
proc sort data=file2;
 by id peak num;
```

```
run;
proc sort data=file3;
by id peak num;
run;
data file1 (keep = id peak num peak);
merge file2 file3;
by id peak_num;
if (peak1 eq peak2) and peak1 eq 1 then
 peak = 1;
else
  peak = 0;
run;
******
                                                              *****
proc sort data=file1;
by peak num;
run;
proc transpose data=file1 out=file100;
by peak num;
id id;
var peak;
run;
data file100;
set file100;
drop __name_;
run;
%print(title= Peak = 1 No peak = 0 - %upcase(&var) used to identify peaks.
      Peak defining muliplyer = &var mult,
   data=file100, print=&p_only peaks);
```

APPENDIX TWO. Agar diffusion method. Inhibition zones observed around wells on a TSA dish, using *S. aureus* strain ATCC 29213 as the indicator organism. Each well contained known concentrations of CTC: (A) 2 μ g mL⁻¹ CTC solution; (B) 3 μ g mL⁻¹ CTC solution.





