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Adherence Inhibition of Human Pathogens *Campylobacter jejuni* and *Campylobacter coli* by Non-digestible Oligosaccharides

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ADHERENCE INHIBITION OF HUMAN PATHOGENS *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI* BY NON-DIGESTIBLE OLIGOSACCHARIDES

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ADHERENCE INHIBITION OF HUMAN PATHOGEN *CAMPYLOBACTER JEJUNI* AND *CAMPYLOBACTER COLI* BY NON-DIGESTIBLE OLIGOSACCHARIDES

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Adherence is the first and one of the most important steps of bacterial pathogenesis. Natural derived components that inhibit the adherence of pathogens to the surface of epithelial cells have received considered interest.

The goal of this research was to assess the anti-adherence activity of mannan oligosaccharides (MOS), pectic oligosaccharides (POS) and cranberry high molecular weight component (HMW) against *Campylobacter jejuni* and *Campylobacter coli.* First the anti-adherence activity of MOS and their purified fraction (pMOS) was tested against three strains of *C. jejuni* and two strains of *C. coli.* Results shown significant reductions in adherence (up to 70%) of all *C. jejuni* strains and for *C. coli* ATCC 43485 in presence of MOS or pMOS (50 mg/mL). The mannan oligosaccharide fraction appear to be the responsible for the anti-adherence activity of MOS. Adherence inhibition (up to 70%) was also observed

in presence of HMW at the highest concentration of 5 mg/mL of all the strains except for *C. coli* ATCC BAA-1061. Additionally, a blend of MOS-HMW did not shown additive effect to inhibit the adherence of all five *Campylobacter* strains. POS was not effective to inhibit the adherence of any of the strains tested in this study. Invasion inhibition was not observed in the presence of any of the components tested. These results show that naturally derived molecules as MOS and HMW can be used for animal production to reduce pathogens colonization and prevent the onset of human infection.

To God, and to my family.

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Intelligence is the ability to adapt to change.

-Stephen Hawking

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Preface

This thesis is compromised of five chapters. Chapter 1 provides a review of the current literature on the anti-adherence effect of different non-digestible oligosaccharides and plant extracts. Chapter 2 provides a review of the uses of mannan oligosaccharides as animal feed supplement. Chapter 3 describes a study on the effect of Mannan oligosaccharides and Cranberry high molecular weight component on the adherence of *Campylobacter jejuni* and *Campylobacter coli* in HEp-2 cells. Chapter 4 describes the results of testing pectin oligosaccharides as anti-adherence agents against *Campylobacter jejuni* and *Campylobacter coli* to HEp-2 cells. Finally, Chapter 5 provides a conclusion session that summarizes the major research findings presented within this thesis.

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Chapter 1

Non-digestible oligosaccharides as anti-adherence agents against enteric pathogens

Introduction

Non-digestible oligosaccharides (NDO) have received much recent research and commercial attention due to their important functional, physiological, and biological properties. In particular, some NDOs have the ability to modulate the intestinal microbiota by enriching for health-promoting bacteria. In addition, NDO may also interfere with the ability of enteric pathogens to colonize the intestinal tract (Paeschke & Aimutis, 2011).

Many commercial NDO are either found naturally or are derived from plant materials. Others are obtained from microbial sources, including fungi. In addition, shellfish and milk can also serve as a source of NDO. Galactooligosaccharides (GOS), fructooligosaccharides (FOS) and inulin are well studied NDOs that has been recognized for their prebiotic properties. Anew class of NDOs that have gained interest in the past years are derived from plant cell wall polysaccharides. Similar hydrolysis processes are used to obtain oligosaccharides from these sources. For example, arabinogalacto-oligosaccharides can be made from soybeans and sugar beets, rhamnogalacturono-oligosaccharides from apple, mannan-oligosaccharide from yeast, xylo-oligosaccharides from bamboo shoots, pectic oligosaccharides from orange pectin (Mussatto & Mancilha, 2007; Voragen, 1998).

Several of these oligosaccharides are derived from waste materials resulting from food processing and other of manufacturing industries. There is much interest in these products due to their low raw material cost and potential bioactive properties. This interest has led to development of new technologies for

extraction and purification of NDOs, as well as studies to analyze their structural and function properties.

In this review, the composition and structure of several important NDOs and their biological properties will be presented. The primary focus will be on the ability of NDO to reduce adherence of enteric pathogens, especially *Campylobacter*, one of the leading causes of gastroenteritis in the U.S.

Physical-chemical properties of non-digestible oligosaccharides

Non-digestible oligosaccharides (NDOs) are defined as carbohydrates that are not digested (or minimally digested) in the stomach or small intestine and arrive in the large intestine mostly intact, where they might then be utilized by gut microbiota (Paeschke & Aimutis, 2011). This concept was based, in part, on the observation that the anomeric carbon atom (C1 to C2) of monosaccharide units of some dietary oligosaccharides has a configuration that makes their osidic bounds (also called glycosidic linkages) non-digestible to the hydrolytic activity of the human digestive enzymes (Mussatto & Mancilha, 2007; Roberfroid & Slavin, 2000). NDOs are made from one, two or even three different types of monosaccharides (Crittenden & Playner, 1996). They are generally low molecular weight carbohydrates, containing sugar moieties with degrees of polymerization (DP) between 3 and 10 (Patel & Goyal, 2011;Weijers et al., 2008) and water soluble usually between 0.3-0.6 times as sweet sucrose.

NDOs are widely found in nature and are common constituents of many foods such as milk, honey, fruits and vegetables (i.e. onion, Jerusalem artichoke,

chicory, leek, garlic, artichoke, banana, rye, barley) (Crittenden & Playner, 1996; Mussatto & Mancilha, 2007). These naturally-derived components are produced by hydrolyzing polysaccharides or by enzymatic and chemical synthesis from disaccharide substrates (Mussatto & Mancilha, 2007; Sako et al., 1999) (Table 2).

NDOs have been associated with many health benefits, including positive effects on fermentation, mineral absorption, barrier function, fat metabolism, glycemic and insulin responses (Meyer, 2004; Nauta & Garssen, 2013). Hence, they have been introduced as functional food ingredients (Mussatto & Mancilha, 2007; Van Loo et al., 1999).

The *Campylobacter* problem

Campylobacter infection is a leading cause of human bacterial gastroenteritis in the United States, with more than 1.3 million campylobacteriosis cases reported in 2012 (CDC, 2013a). Poultry has been identified as a major reservoir for this pathogen, and contaminated broiler chicken meat is believed to be responsible for 50 - 70% of the human *Campylobacter* cases (EFSA, 2010; Hermans et al., 2011). Indeed, chickens are commonly colonized by *Campylobacter jejuni*, with prevalence rates of 60-80% (EFSA, 2010). Therefore, strategies to prevent *Campylobacter* colonization in chicken are a high priority.

Epidemiology

Campylobacter spp. are commonly found as commensals in the gastrointestinal tract of wild or domesticated cattle, sheep, swine, goat, dogs, cats, and all classes of poultry; (Lee & Newell, 2006; Stanley & Jones, 2003). *C. jejuni* is predominantly associated with poultry (EFSA, 2004; FAO/WHO, 2009; Tauxe et al.,1992) and *C. coli* is found at higher prevalence in pigs (EFSA, 2004; Nielsen et al., 1997; Rosef et al., 1983). The handling and consumption of poultry meat is considered a significant risk for human infection (Corry & Atabay, 2001; Ganan et al., 2009).

The most important foodborne species of *Campylobacter* are *Campylobacter jejuni* and *Campylobacter coli.* Together, they account for over 95% of *Campylobacter* infections in humans (Park, 2002). Surveys have suggested that in the U.S., nearly 70% of chicken carcasses processed on-farm are contaminated with *Campylobacter* (Timble et al., 2013). These authors reported that in the slaughter facility this number increased to 82%. The Foodborne Disease Active Surveillance Network (FoodNet) found a 30% decline in the rates of infection in 2009 for *Campylobacter* compared with previous years. However, low level of contamination is a public health risk, since the reported infectious dose for this pathogen is less than 500 CFU (Black et al., 1988; Park, 2002).

Moreover, it is generally assumed that *C. jejuni* and *C. coli* do not multiply during slaughter, post-processing, transport and refrigeration storage of chicken products, since they require high growth temperature of around 42°C

(FAO/WHO, 2009). However, *Campylobacter* spp. can persist for long times in chilled and frozen products. Proper cooking is sufficient to inactivate *Campylobacter* as they are sensitive to heat (FAO/WHO, 2009; ICMSF, 1996).

Campylobacteriosis

In many industrialized nations, *Campylobacter jejuni* is the most frequently identified pathogen associated with acute diarrheal disease (Acheson & Allos, 2001). *Campylobacter* infections usually involve sporadic cases, or as part of small, family-related outbreaks (FAO/WHO, 2009). The infection due to *Campylobacter* is called campylobacteriosis and are most prevalence in children, elderly people and patients with compromised immune system (Wassenaar & Blaser, 1999; Young et al., 2007). Any individual who has consumed the organism from contaminated food or water is at risk of develop the disease (Altekruse et al., 1999). However, campylobacteriosis is a self-limited disease, and most patients recover almost completely after 1 week (Dasti et al., 2010).

In developing countries, *Campylobacter* species are an important cause of childhood morbidity caused by watery diarrhea predominantly (Acheson & Allos, 2001). In developed countries, campylobacteriosis manifests as bloody diarrhea with mucus, and is usually self-limiting (Young et al., 2007). Although 14 species of *Campylobacter* have been identified, in the United States, more than 99% of reported infections with *Campylobacter* are with *C. jejuni* (Buzby et al., 1997; Friedman et al., 2000). Approximately 0.1% of *C. jejuni* cases are associated with serious ascending motor neuron paralytic disease Guillain-Barré syndrome

(Mandrell et al., 2005; Nachamkin et al., 1998). Studies have reported that 20% to 40% of patients with Guillain-Barré syndrome (GBS) have evidence of recent *Campylobacter jejuni* infection (Buzby et al., 1997; CDC, 2013b), in the 1-3 weeks prior to the onset of neurologic symptoms (Rees et al., 1995). Ang et al., 2004 have suggested that *C. jejuni* probably triggers the GBS through molecular mimicry between core lipooligosaccharides (LOS) in the bacterial cell wall and gangliosides in human peripheral nerve tissue as a consequence cause the paralysis.

Worldwide, the economic loss due to *C. jejuni* infection has been estimated to be in excess of US$ 2 billion per year (CDC, 2013b). Adding to the human and economic costs of *C. jejuni* are the chronic sequelae associated with this infection (Altekruse et al., 1999). In 2012, the Foodborne Diseases Active Surveillance Network (FoodNet) estimated the incidence to be 14.3 cases per 100,000 populations. An estimated 1.3 million persons are affected each year (CDC, 2013b).

Campylobacter **and antibiotics**

Antibiotics are commonly given to chickens to promote growth by increasing weight gain, improving feed efficiencies, reducing mortality, and inhibiting pathogens colonization (Gaskins el al., 2002; Phillips et al., 2004). However, the emergence of antibiotic resistant *Campylobacter* strains has significantly increased in the recent years (Rautelin et al., 1991; White et al., 2002). In 2006, the European Union banned the use of antibiotics in animal feed.

Hence, alternative approaches to reduce *Campylobacter* colonization in livestock animals are required.

Resistance of *Campylobacter* to many common antibiotics, including ampicillin, streptomycin, tetracycline, erythromycin, gentamycin, and fluoroquinolones has been reported (Adzitey et al., 2012; Nobile, et al., 2013). Fluoroquinolone-resistance has especially concerned public health authorities, because this antibiotic is commonly used as first-line therapy for urinary tract infection, enteric infection and gonococcal infection (Cheng et al., 2012; Redgrave et al., 2014). It has been proposed that this resistance is due, in part, for the use of fluoroquinolones to control mortality associated with *Escherichia coli* infection (McDermott et al., 2002), and also to treat colibacilllus and *Mycoplasma* infections in poultry (Wagenaar et al., 2006), as a side effect, the *Campylobacter* spp present in the gastrointestinal tract of the bird become resistance.

Usui et al., 2014 reported that treatment of pigs with fluoroquinolones select to fluoroquinolones-resistance *Campylobacter* and persist in the GIT after the treatment for up to 21 days, becoming a significant risk of potential contamination in humans.

Virulence mechanisms.

The mechanisms of pathogenesis for *Campylobacter* are not well understood. Several proteins have been identified to play an important role in the adherence mechanism of *C. jejuni* (Fouts et al., 2005; Parkhill et al., 2000; Young

et al., 2007). CadF is an outer membrane protein that binds specifically to fibronection, which is located on epithelial cells (Konkel et al., 1999) and it is expressed by all *C. jejuni* and *C. coli* strains (Dasti et al., 2010). Previous studies have shown that *cadF* mutants had significantly reduced capacity to colonize poultry compared with the wild type strain (Monteville et al., 2003; Young et al., 2007; Ziprin et al., 1999). CapA (*Campylobacter ad*hesin protein A), JIpA (*Campylobacter jejuni* surface lipoprotein A), PEB1 (periplasmic binding protein) have been identified and suggested to mediate adherence of the bacterium to epithelial cells (Flanagan et al., 2009; Jin et al., 2003; Pei et al., 1998). Additionally, other proteins have been described (Cj1270c and Cj1349) to contain Fn type III domains and act as an Fn and fibrinogen-binding protein (Flanagan et al., 2009).

The ability of *C. jejuni* and *C. coli* to become established in the gastrointestinal tract of chickens is thought to involve binding and colonization of the intestinal cell surface (Ganan, et al., 2009; Park, 2002). The mechanisms by which *Campylobacter* spp. induce disease in humans is not well understood, but there are some mechanisms postulated for gastrointestinal disease that involve bacterial adherence, invasion and colonization of the intestinal mucosa (Park, 2002). The study of *Campylobacter* pathogenesis is limited by a lack of understanding of the physiology and virulence factors. Hence, *in vivo* and *in vitro* models with live animals and tissue culture cells have been used as a suitable alternative to understand the interactions between *Campylobacter* and the host epithelium cells.

Adherence

Adherence is the first, and one of the most important steps in bacterial pathogenesis (Savage, 1977; Shoaf et al., 2009). Indeed, pathogens that have lost their ability to adhere generally become avirulent (Casadevall & Pirofski, 2001). At a molecular level, adherence is a receptor-mediated process between lectin-like bacterial adhesins and their complementary ligands located on the mucosa surface (Finlay & Falkow, 1989a; Pieters, 2011). Most bacterial adhesins are organized as thin thread-like organelles called fimbriae or pili (Klemm et al., 2010). Importantly, adhesins can be highly specific. Ultimately, the specificity of a bacterial pathogen for a particular host or host tissue depends on the presence of definitive oligosaccharide receptors (Firon et al., 1984; Shoaf et al., 2006). Any specific receptor may contain more than one attachment site for two or more bacterial adhesins (Ofek et al., 2003). Additionally, two different pathogens can express distinct adhesins that have the same receptor specificity (Ofek et al., 2003; Wilson, 2002).

Studies on the bacterial adhesion-host receptor interaction have led researchers to suggest possible strategies to prevent pathogen colonization by blocking the adhesin-receptor interference. Thus, agents that inhibit adherence of foodborne pathogens to the surface of cells have attracted considerable attention in the past years (Shoaf & Hutkins, 2008).

Anti-Adherence

The anti-adherence model is based in the observation of the interaction between bacterial adhesins and host receptors located at the surface of host cell (Klemm et al., 2010b). One of the anti-adhesive mechanisms that have been studied is based on the structural similarity that non-digestible oligosaccharides have to intestinal cell surface receptors (Kunz et al., 2000). Hence, acting as receptor analogs or decoys that result in blocking the adherence process (Shoaf et al., 2008) (Figure 1). As a consequence, pathogens bind to the oligosaccharide decoys and are displaced from the intestinal tract, preventing the onset of infection (Shoaf & Hutkins, 2009). There is significant evidence that supports the use of non-digestible oligosaccharides from natural resources (e.g. human milk oligosaccharides and xyloglucan from berries) and synthetics (e.g. galactooligosaccharides, N-acetyl-galactosamine, chitooligosaccharides) act as molecular decoys to inhibit bacterial adherence (Boehm et al., 2005; Newburg et al., 2005; Quintero et al., 2011; Quintero-Villegas et al., 2013; Shoaf et al., 2006).

Many pathogens utilize monosaccharides or short oligosaccharides as receptors to bind to the surface of intestinal cells (Sharon, 2006) (Table 1).

Pathogen	Carbohydrates Specificity	Target Tissue
Campylobacter jejuni	FucαGalβGlcNAc	Intestinal
Pseudomonas aeruginosa	L-Fuc	Intestinal
Escherichia coli (Type 1-fimbriated)	Manα3Manα6Man	Urinary
Salmonella spp. (Type 1-fimbriated)	Man	Intestinal
Klebsiella pneumoniae	Man	Respiratory and Intestinal
Neisseria gonorrhoeae	Man	Genital
Helicobacter pylori	NeuAc(α2-3)Galβ4GlcNAc	Stomach
Streptococcus pneumoniae	[NeuAc(β2-3)]0,1 Galβ4GlcNAcβ3Galβ4GlcNAc	Respiratory
Neisseria meningitidis	[NeuAc(α2-3)0,1 Galβ4GlcNAcβ3Galβ4GlcNAc	Respiratory
Haemophilus influenza	[NeuAc(α2-3)0,1 Galβ4GlcNAcβ3Galβ4GlcNAc	Respiratory

Table 1. Carbohydrates attachment sites for bacterial pathogens on animal tissues. Adapted from (Ofek et al., 2003; Sharon, 2006)

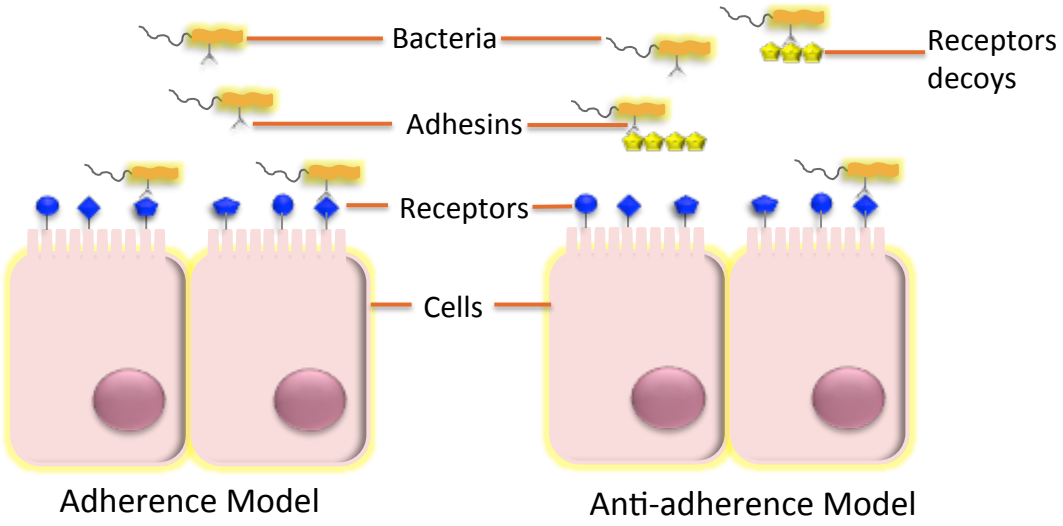

Figure 1. Anti-adherence model involving receptor analogs molecules for reducing adherence of pathogens. Adapted from Ofek et al, 2003.

Hence, future anti-adherence strategies to prevent pathogen infection should first focus on identification and characterization of the molecular interactions between bacterial adhesins and their receptors. It may then be possible to identify the specific targets and ultimately synthesize or produce new molecular decoys that might lead to adherence inhibition.

NDO	Structure	Classification	Sources
β-Glucan	β-(1→3 and 1→4) D-Glc*p* (Backbone); β-(1→36) D-Glc*p* (branches; only on some types)	Soluble, viscous, highly fermentable.	Oat bran, barley four
Chitin	β-(1→4)-D-GlcNAc	Insoluble, poorly fermentable	Shellfish
Chitosan	β-(1→4)-D-GlcNAc and GlcN	Soluble and viscous/gel-forming in acid solutions	Alkali-treated chitin
Pectins	α-(1→4)-DGalA with varying degrees of methyl esterification (backbone) and side groups containing Rha, Gal.	Soluble, viscous, highly fermenatable	Fruits and vegetables
Polydextrose	Random polymer of Glc, sorbitol, and citric acid	Soluble, low viscosity, partially fermentable	Chemically synthesized
Inulin/Fructo oligosaccharides	Fructose oligo- or polymer linked β-(2→1) with Glc at the non-reducing end	Inulin insoluble, FOS soluble and low viscosity; highly fermentable	Onions, Jerusalem artichokes, Enzymatically synthesized
Galactooligo saccharides	Oliginers of galactose	Soluble, low viscosity, highly fermentable	B-galactosidase treatment of lactose

Lactulose	D-Galp-β-(1→4)-D-Fru	Soluble, low viscosity, highly fermentable	Glucose isomerase treatment of lactose
Arabinoxylan	Heteropolymer with a backborne of β-(1→3 or 1→4) –D-Xly complex branches of Ara, Xyl, GlcA and Gal.	Insoluble or soluble and poorly to highly fermentable.	Grain-based materials, most often wheat or corn

Glc, glucose; Gal, galactose; Xyl, xylose; Ara, arabinose; Rha, Rhamnose; GlcA, D-glucuronic acid; GalA, galacruronic acid; GlcN, D-glucosamine; GlcNAc, N-acetyl-D-glucosamine.

Table 2. Main Non-digestible Oligosaccharides structure, classification and sources. (Paeschke & Aimutis, 2011)

Human milk oligosaccharides (HMO)

Oligosaccharides are the major component in human milk, with a concentration range of 8-12 g/L (Boehm et al., 2005). They represent the third largest compound in milk. HMO are formed from 5 monosaccharides: D-glucose (Glc), D-galactose (Gal), N-acetylglucosamine (GlcNAc), L-Fucose (Fuc), and sialic acid (N-acetyl neuraminic acid (Neu5Ac) (Bode, 2006; Engfer et al., 2000). HMO have a variety of biological activities beyond providing nutrition to the infant (Barile & Rastall, 2013). There is abundant evidence that HMO can act as a prebiotic, having a bifidogenic effect in breast-fed infants (Coppa et al., 2006). Recent studies revealed that the catabolism and fermentation of HMO by bifodobacteria have unique preferences on these glycans (Barile & Rastall, 2013; Sela & Mills, 2010; Sela et al., 2011). Moreover, there are several reports

showing that HMO can also serve as anti-adherence agents against pathogens by acting as decoys to prevent binding of pathogens to epithelial cells (Barile & Rastall, 2013; Bode, 2006; Newburg et al., 2004). (Manthey, Autran, Eckmann, & Bode, 2014) HMOs isolated from pooled human milk was effective to inhibit the adherence of EPEC (Enteropatogenic *Escherichia coli*) to cultural epithelial cells in mice. Interestedly, *C. jejuni* binds to intestinal epithelial cells using 2'-fucosyllactosamine receptor (Bode, 2006; Ruiz-Palacios et al., 2003), which is the most prevalent OS in HMO.

Due to the complexity of the HMO, there has been growing scientific and industrial interest in natural derived NDO as galactooligosaccharides, fructooligosaccharides and inulin in the supplementation of infant formulas (Coppa et al., 2006).

Galactooligosaccharides (GOS):

GOS have attracted considerable commercial interest, since the presence of galactose-containing oligosaccharides in human milk has been shown to enhance the establishment of *Bifidobacterium* spp in breast-fed infants (Gibson et al., 2010). Actually, there are several industries that produce GOS from lactose (purified from cow's milk whey) using the galactosyltransferase activity of β-galactosidese to produce several oligomers of different chain lengths (Macfarlane et al., 2008; Prenosil et al., 1987). The galactosylation activity of this enzyme dominates lactose hydrolysis at high lactose concentration (Crittenden & Playner, 1996; Sako et al., 1999; Smart, 1993).

The anti-adherence properties of GOS are now well established. Under *in vitro* conditions, GOS inhibited adherence of EPEC (enteropahogenic *E. coli*) to HEp-2 and Caco-2 cells by 65% and 70% (Shoaf et al., 2006). More recently, Quintero et al., 2011 reported that 16 mg/mL of GOS was also effective against *Cronobacter sakazakii*, inhibiting adherence to HEp-2 cells by 56%. Other studies have shown that adherence of *Salmonella enterica* serovar *typhimurium* to HT-29 cells is inhibited by 5 mg/mL of commercial GOS (Bimuo®) (Searle et al., 2010).

Pectic oligosaccharides (POS)

Pectins are commonly found in cell walls and seed mucilage of plants (Paeschke & Aimutis, 2011). They are commercialized for different industrial applications due to their potential functional properties. Oligosaccharides derived from pectins (POS) have gained attention as a candidate prebiotics (Hothkiss et al., 2003). POS are form by partially methyl esterified homogalacturonan backbone with periodic interruptions by regions of alternating galacturonic acid and rhamnose residues (Rastall, 2010).

POS have been assessed for their ability to stimulate growth of beneficial bacteria, including *Lactobacillus* (Mandalari et al., 2006) and *Bifidobacterium* (Manderson et al., 2005; Olano-Martin et al., 2002). In addition to their putative prebiotic activity, POS have also been recognized for their ability to inhibit the adherence of pathogens (Hotchkiss et al., 2003; Holck et al., 2014), as well as for

their immunomodulatory and anti-carcinogensis properties. (Holck et al., 2014; Morris et al., 2013).

Fructooligosaccharides (FOS):

FOS are a mixture of oligosaccharides composed of glucose and repetitive fructosyl residues in β- (2→1) linkage or β- (2→6) linkage (Boehm et al., 2005). FOS is naturally present in a variety of food products, including onion, garlic, salsify, leek, asparagus root, and Jerusalem artichoke tubers (Molis et al., 1996). There are two main industrial processes to obtain FOS. The first one is produced from sucrose via transfructosylating enzymes and the second one via partial hydrolysis of inulin by endoglycosidases (Coussement, 1999; Hirayama et al., 1989; Molis et al., 1996). FOS is one of the best studies NDO in both *in vitro* and *in vivo* models. Like other NDOs, FOS resists hydrolysis, and is able to reach the colon intact. In the colon, inulin is fermented by resident symbiotic anaerobic bacteria, especially bifidobacteria (Walker & Duffy, 1998) (Roberfroid, 1996)

Chitooligosaccharides (CHOS)

CHOS are produced enzymatically or chemically from chitosan. Structurally, CHOS consist of a linear heteropolymer of β (1→4) linked N-acetyl-D-glucosamine (GLcNAc) and its deacylated counterpart D-glucosamine (GlcN) (Quintero-Villegas et al., 2013). Chitosan is generally produced commercially from crab and shrimp wastes with different degrees of acetylation and molecular

masses, thus presenting a variety of properties (Ganan et al., 2009) . Chitosan has received increased attention for its natural source and bioactivity, and it is now used in different applications for foods and pharmaceuticals (Devlieghere et al., 2004; Ganan et al., 2009). The mechanism behind the bioactivity of CHOS is poorly understood. However, the sequences of GlcNAc and GLcN units in CHOS has been reported to be important for binding affinity, and for ensuring selectivity for pathogens (Aam et al., 2010)

There are several reported studies that have assessed the antimicrobial properties of CHOS against *Campylobacter* spp. One study showed that *C. jejuni* and *C. coli* were highly sensitive to chitosan. The minimal inhibitory concentration (MIC) of chitosan ranged from 0.05% to 0.5% and the most effective antimicrobial fractions were chitosan with a MM of 120 KDa (Ganan et al., 2009). Another study reported that the biological properties of CHOS on *Campylobacter* depended on the composition of the fraction analyzed (Liu et al., 2008). They suggested that the high and medium molecular weight of chitosan obtained from chitosan with a fraction of acetylation (F_A) of 0.09 have stronger inhibition effect compared with the chitosan (F_A 0.25) (Mengíbar et al., 2011).

Mannan oligosaccharides (MOS)

MOS are derived via partial hydrolysis of the polysaccharide, mannan, which consists of α-1,4 linked mannose monomers (Tester & Al-Ghazzewi, 2013). They are obtained commercially either from plant material or from yeast cell walls. Mannose-based carbohydrates could be a promising natural agent for

reducing adherence of enteropathogenic bacteria, since these pathogens rely on adhesins that bind to mannose receptors (Santin et al., 2001). Yeast mannanproteins have also been shown to enhance the growth and persistence of some lactic acid bacteria reach and colonize the gut (Ganan et al., 2012). Extracts rich in mannanproteins also have shown to inhibit the adherence of some foodborne pathogens, such as of *Campylobacter jejuni* (Ganan et al., 2009); *E. coli* (Baurhoo et al., 2007); and *Salmonella* (Fernandez et al., 2002; Posadas et al., 2010).

Arabinoxylan oligosaccharides (AXOS)

Arabinoxylan (AX) are the second most abundant oligosaccharides in hemicellulose fraction of softwoods (Faber, 2012). Arabinoxylan oligosaccharides (AXOS) are derived from wheat bran by extensive hydrolysis of the AX present, but are not commercially available (Van Craeyveld, 2009). AXOS consists of β-1,4 linked backbone of xylose with arabinose side chains.

AXOS are similar to other well know inulin and other prebiotic in that they have shown to have a bifidogenic effect (Grootaert et al., 2007; Paeschke & Aimutis, 2011; Pastell, et al., 2009; Vardakou et al., 2008). Wheat-derived AX polysaccharides fractions have been shown to be fermented *in vitro* by human fecal microbial communities (Hughes et al., 2008). However, more information about the structure-activity of both AX and AXOS, and the development of economically viable process to manufacture these novel oligosaccharides are required (Paeschke & Aimutis, 2011).

Cranberry Extract

Cranberry (V*accinium macrocarpon* Ait., family Eri- caceae) fruit juice is traditionally used to treat and prevent urinary tract infections (Burger et al., 2000; Howell et al., 2005). Most of the studies have been performed *in vitro* with cranberry juice cocktail and concentrated cranberry extract. These studies have shown these cranberry products have bactericidal, bacteriostatic and anti-adhesion properties against different pathogens that colonize the stomach, the mouth and the urinary tract (Sobota, 1984; Steinberg et al., 2005; Watson & Preedy, 2010; Zafriri et al., 1989). A-type proanthocyanidins of cranberry juice cocktail has been shown to inhibit the adherence of P-fimbriated uropathogenic *E. coli* to uroepithelial cells. Therefore, these compounds may be responsible for the beneficial effect on UTI prevention (Howell et al., 2005; Zafriri, et al., 1989).

The high molecular mass constituents of cranberry juice has been shown to inhibit adherence of *H. pylori* BZMC-25 to human erythrocytes by inhibiting the sialic acid-specific adhesion (Burger et al., 2000). The non-dialyzable material (NDM) of cranberry extract had been reported as an anti-adhesion and anti-biofilm agent against *S. sorbinus* (Steinberg et al., 2005). However, an *in-vivo* study showed that dietary supplementation with low (1%) and high (30%) content of proanthocyanidins from cranberry extract had no significant effect in the colonization of *C. jejuni* in young chicks (Woo-Ming, 2012).

Conclusion

The ability of some non-digestible oligosaccharides to prevent bacterial adhesion is a promising strategy to interfere with the initial step of bacterial

pathogenesis. Many well studied NDOs, such as GOS and FOS have been used as food ingredients for their prebiotic effect, especially to stimulate the growth of *Bifidobacterium* spp and to modulate gut microbiota. In addition, other NDOs have been proposed for their prebiotic properties and also to prevent pathogens attachment to epithelial host cells. Other oligosaccharides, including natural plant derived molecules like cranberry extract, have been investigated for their ability to interfere with the adherence of some pathogens.

Antibiotic growth promoters are used in animal production to enhance animal performance and reduce pathogens colonization. However, bans and restrictions on the use of AGPs have led researchers and manufacturers to identify alternative compounds that have similar function to antibiotics. The anti-adherence approach has gained considerable interest for agricultural purposes. Thus, the development of novel natural derived components to prevent pathogens colonization in animals and human is worthy of exploration. However, further investigations are required to understand the structure and functional properties of anti-adherent agents and the interaction of bacterial adhesins with the host cell receptors.

Campylobacter spp. are recognized as one of the leading human foodborne pathogens. Moreover, it is known that this bacterium is well established in the gastrointestinal tract of poultry, and the consumption of contaminated meat is the main significant risk factor for human infection. Therefore, reducing the prevalence of *Campylobacter* spp. in poultry could decrease the incidence of campylobacteriosis in humans. NDOs are promising

agents to be included in poultry diets as a supplement to enhance performance and reduce adherence of pathogenic bacteria.

The objectives of the research described in this thesis were to:

1. Assess the ability of mannan oligosaccharides (MOS) and cranberry high molecular weight component (HMW) to inhibit the adherence and invasion of three strains of *C. jejuni* and two strains of *C. coli* to HEp-2 cells.
2. Determine which component (mannan oligosaccharides or β-glucan fraction) is responsible for the anti-adherence effect.
3. Test the additive effect of a combination of MOS and HMW.
4. Determine the ability of pectic oligosaccharides to inhibit the adherence and invasion of three strains of *C. jejuni* and two strains of *C. coli* to HEp-2 cells.

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Chapter 2

Mannan oligosaccharides as animal feed supplement

Introduction

Naturally derived non-digestible oligosaccharides (NDO) have attracted considerable interest among industrial scientists, due to their functional and biological properties in human food and animal feed. In particular, these oligosaccharides have shown to have beneficial effects on animal health by modulating gut microbiota, suppressing pathogenic bacteria, enhancing immunity, and facilitating mineral absorption (Paeschke & Aimutis, 2011; Patel & Goyal, 2011; Van Loo et al., 1999).

In the past decades, sub-therapeutic levels of antibiotics have been widely used in livestock animals as growth promoters (Dibner & Richards, 2005; Gaggìa et al., 2010). However, the World Health Organization, the American Medical Association, and the American Public Health Association have approved these substances be banned or restricted for animal production applications (Graham & Boland, 2007). The main motivation for restricting GPAs is due to the transmission of antibiotic resistant genes among microorganisms that colonize or infect animals. Ultimately, the spread of pathogenic bacteria that are resistant to antibiotics would pose significant risks to human health.

Recently, NDOs, and mannan oligosaccharides (MOS), in particular, have been reported to serve as alternative agents to replace growth promoting antibiotics in animal production (Patel & Goyal, 2011; Yang et al., 2009, Baurhoo et al., 2007; Ferket et al., 2002; Kim et al., 2011; Miguel et al., 2004; Soren et al., 2013). MOS are derived from the yeast cell walls (Feuillat, 2003), and are inexpensive by-products from industrial processes (i.e. brewers, bakeries).

Several studies have shown beneficial effects of MOS supplementation in animal feed, such as reduce the colonization of some pathogens like *E. coli* and *Salmonella spp*. (Fernandez et al., 2002; Line et al., 1998; Santin et al., 2001), immune modulation (Sang et al., 2009; Staykov et al., 2007), and improve growth performance and welfare in livestock (Sang & Fotedar, 2010; Torrecillas et al., 2011; Zhao et al., 2012).

The present review paper describes the main applications of mannan oligosaccharides in animal feed and their role as alternative components to replace growth-promoting antibiotics.

Mannan oligosaccharides

Mannan oligosaccharides (MOS) are non-digestible oligosaccharides derived via partial hydrolysis of the polysaccharide, mannan (Tester & Al-Ghazzewi, 2013a). The latter consists of α-1,4 linked mannose monomers. Partial hydrolysis yields MOS with degree of polymerization (DP) ranging from 6 to 8 (Bland et al., 2004). Currently, most commercial sources of MOS are derived from the cell walls of bakers' or brewers' yeast strains of *Saccharomyces cerevisiae*. They are used primarily as supplements for animal feed (Halas & Nochta, 2012). The actual composition of yeast-derived MOS depends on several factors, including species, growth phase at harvest, and growth or fermentation conditions.

Other sources of MOS have also been considered. Plant-derived MOS have been studied from hemicellulose plant cell walls, such as konjac (Holck et

al., 2014). Spent coffee grounds, coffee beans and waste coffee mannans have also been studied (Moreno & Sanz, 2014), but no commercial sources for these products currently exist.

MOS from yeast cell wall

The yeast cell wall is a major constituent of the total yeast biomass, accounting for 25 - 30% of dry weight of the cell (Klis et al., 2010; Latgé, 2007) . Yeast cell walls of *S. cerevisiae* are composed of mannoprotein (35%), β-1,3 glucan (25%), β-1,3 glucan bound to chitin (35%), β-1,6 glucan (5%) and small amount of chitin (1-2%) (Feuillat, 2003; Orlean, 2012).

The cell wall proteins exist as glycoproteins that are highly modified with O-and N-linked oligosaccharides. These oligosaccharides are comprised mainly of mannose residues, collectively known as mannan (Gupta et al., 2012). The mannan backbone is homopolymeric, containing mannose residues only or heteropolymeric, with side chains containing other sugars. The latter include mannose, glucose, rhamnose, and galactose, forming glucogalactomannan, rhamnogalactomannan, and galactomanan oligosaccharides (Bowman & Free, 2006; Gupta et al., 2012; Leal et al., 2010).

Many of the proteins found in yeast cell walls are water-or detergent-soluble, and only a few are covalently linked to the polysaccharides. These glycoproteins are classified in two groups: proteins covalently linked to β-1,3 glucan through glutamine reside; and proteins covalently linked by a glycosylphophatidyl inositol

anchor to the β-1,6 glucan of a β-1,3/ β-1,6 glucan core (Gupta et al., 2012; Klis et al., 2006; Latgé, 2007).

Biological properties of MOS

Although non-digestible oligosaccharides, MOS have no known direct nutritive value in animal feed, however, they have been suggested to enhance gut health (Halas & Nochta, 2012). Several properties of MOS that make them useful as nutritional supplements for animal feed products have been described (Table 1) (Ferket et al. 2002 and Soren et al. 2013). In general, MOS should be non-digestible, not absorbed in the stomach and small intestine, and they should reach the colon intact. In the colon, MOS and mannose-based glycans could have prebiotic activity and be fermented by desirable members of the gut microbiota.

The yeast-derived MOS and β-glucan components share similar properties, but are different in several important respects. MOS are polysaccharides-protein complexes, and β-glucans are polymers of glucose. β-glucans have been considered as immunoestimulators of macrophages and other immune effector cells (Chong, 2009). Szymanska-Czerwinska et al., 2009 have reported that the combination of MOS and β- glucans increase cytokine levels and the percentage of lymphocyte subpopulation in calves. More recent study made by Zhang et al., 2012 shown that broiler chickens fed with β-glucans from yeast cell wall enhance cell mediated immune response by modulating the production of cytokines.

Several studies have suggested another important property of MOS. Specifically, mannan oligosaccharides can inhibit the binding of pathogenic bacteria to the mucosa of the gut and therefore reduce colonization and the onset of infections (Klemm & Schembri, 2000; Tester & Al-Ghazzewi, 2013b). In the GIT, MOS act as high affinity ligands, providing competitive binding sites for specific enteric bacteria (Ofek & Beachey, 1978). This binding is usually mediated by bacterial fimbriae that contain terminal adhesin residues. Fimbriae thus serve as adhesive organelles that enable bacteria to target specific host tissues, to which they bind to and colonize (Corrigan et al., 2012). Mannose residues are bound preferentially by type I fimbria, present in several enteric pathogens.

Extracts rich in mannoproteins have been shown to be effective, *in vivo*, against *Campylobacter jejuni* (Ganan et al., 2009; McSweegan & Walker, 1986) , *Escherichia coli* (Baurhoo et al., 2007) and *Salmonella* (Fernandez et al., 2002; Oyofo et al., 1989). Most of these *in vivo* studies have been conducted with broiler chicks, pigs, fish, cattle, and companion animals. Dietary supplementations with MOS from yeast extracts have been used in livestock animals at relatively low concentrations (between 0.5 - 5.0 kg/ton), resulting in enhanced overall performance and reduced inhibition and colonization of enteric pathogens (Baurhoo et al., 2007; Castillo et al., 2008; Torrecillas et al., 2011; Waldroup et al., 2003; Zhao et al., 2012).

MOS production

As noted above, most of the commercial MOS products are derived from various strains of yeast. The most common yeasts used for MOS product are from the genus of *Saccharomyces* and *Candida*. In general, yeast cells are grown using common techniques used in the bakers yeast and brewers yeast industries (Halas & Nochta, 2012). Nutrient-rich media are used to achieve high cell densities, and often include diluted molasses and sulfite waste liquor. Once cell growth has reached a sufficiently high cell density, the yeast cell wall fraction (containing MOS) is extracted. This is usually done by mechanical or enzymatic methods and rely on autolysis, hydrolysis (i.e., via β–mannanases, pectinases, α-galactosidases and cellulases) or physical means (Katz & Brown, 2007)

Autolysis of yeast cell wall is one of the most common methods to extract the MOS fraction. For example, Agrimos®, manufactured by Lallemand Animal Nutrition, is produced by autolysis of yeast cells at high temperatures and at controlled pH. The yeast extract and cell wall is separated by centrifugation, and the cell wall fraction is spray dried and packaged.

Antibiotics in animal production

Antibiotics have been used to promote growth in livestock animals for many decades (Ferket et al., 2002). *In vivo* studies have reported that inclusion of regular low doses of antibiotics in animal diets provides several beneficial performance effects in animals, including increases in weight gain, improved feed efficiencies, reduced mortality, and inhibition of pathogen colonization in

intensive production systems (Gaskins el al., 2002; Phillips et al., 2004). Ultimately, these benefits are suggested to reduce overall animal production costs and to provide economic benefits that are distributed along the food chain (Ferket, 2003). However, the widespread use of antibiotics in animal production has also been suggested to contribute to the emergence and spread of antibiotic resistance bacteria (Phillips et al., 2004).

Controlling the transmission of pathogenic bacteria at the farm level is now a major of concern in animal production. In particular, the poultry industry has identified contamination of raw poultry products to be a consequence of high populations of pathogenic bacteria in the gastrointestinal tract of poultry animals (Ferket et al., 2005).

Mode of action of growth promoters

Although widely used in animal agriculture (Looft et al., 2012), the mechanisms by which antibiotics promote growth are not fully understood (Dibner & Richards, 2005; Graham & Boland, 2007). Many hypothesis have been suggested to explain the mechanisms of action for the growth-promoting effect of antibiotics in production animals (Giguère et al., 2013)

Gaskins et al, 2002 proposed four mechanisms of action of GPAs: (1) inhibition of sub-clinical infections; (2) reduction of growth-depressing microbial metabolites; (3) reduction of microbial use of nutrients; and (4) enhanced uptake and use of nutrients. More recently, Giguère et al., (2013) suggested additional hypotheses to account for the growth-promoting effect of antibiotics in livestock

animals (Table 1), such as: stimulation of intestinal synthesis of vitamins by bacteria; reduction in total number of bacterial in the GIT by reducing competition between microorganism and host animals for nutrients; reducing pathogenic bacterial; inhibition of bacterial urease; improve energy efficiency of the gut; reduced immune stimulation; modification of intestinal enzyme activity; inhibition of bacterial cholytaurin hydrolases activity; improve nutrient absorption from morphological changes to small intestinal epithelium. In contrast, Bedford, 2000 reported that GPAs have no beneficial effect in germ-free chicks, indicating that the direct effect is due to alteration in the gut microbiota composition rather than interact with the physiology of the animal (Conejos et al., 2012).

Effects of antibiotics in animals

After antibiotic administration, animals are more susceptible to pathogens colonization because of the loss of the resident microbiota (Ferket, 2004). The stability of the gut microbiota is important for maintenance of a healthy barrier against pathogen infection in animals (Gaggìa et al., 2010). Administration of competitive exclusion cultures as probiotics (Fedorka-Cray et al., 1999; Sohail et al., 2010) or feeding prebiotic oligosaccharides have been used in livestock to enhance the stability of microbiota (Ferket, 2004; Fernandez et al., 2002). Accordingly, older animals are, in general, more resistant to colonization by enteric pathogens than young animals because they have more stable diverse gut microbiota that compete excluding pathogen colonization (Ferket, 2004).

MOS as new growth promoter agent

Since the use of antibiotics has been banned in Europe and some have been restricted in United States, there are serious concerns about the possibility that antibiotic-resistant genes can be transferred to human pathogens (Chowdhury el al., 2009). Hence, the animal production industry has been seeking to identify or develop alternative products to reduce reliance on antibiotics while maintaining production efficiency (Ferket et al., 2005).

MOS may be an effective alternative to replace antibiotic growth promoters (AGPs), because they have been shown to have similar effect on animal performance (Baurhoo et al., 2007; Castillo et al., 2008; Fritts & Waldroup, 2003). MOS were first introduced as a feed additive for poultry in 1993 (Bio-MOS®, Alttech Inc.). This carbohydrate has been recognized to have potential properties to inhibit the adherence pathogen to the surface of epithelial cells.

Alternatives to antibiotics in poultry production

Although the use of antibiotics as growth promoters in the poultry industry has been widely accepted for many years, there is now considerable concern about their possible effects on public health (Dibner & Richards, 2005). Proponents of this practice have suggested that GPAs are an essential tool to maintain animal productivity by increasing average weight of broilers, reducing the amount of feed required to reach market weight, and ultimately decreasing the cost of chicken products for consumers (Graham & Boland, 2007; Smith,

2002). However, alternative inexpensive and natural agents are now being used for pathogen control and to enhance the overall performance of the animal and replace the use of GPAs. These include probiotics and prebiotics, herbal products, vaccines, and sanitation practices to prevent vertical transmission of pathogens and promote gut health (Stanley & Jones, 2003; Yano et al., 2013).

MOS in poultry production

Mannan oligosaccharides products, especially those derived from the cell wall of *S. cerevisiae*, are extensively used as natural feed additives in poultry due to the beneficial effect in the performance and gastrointestinal health (Xiao et al., 2012). However, studies have shown contradictory results in chickens and turkeys. In some studies, supplementation of MOS in the diets had significant effects on the performance (i.e., increase in body weight, feed conversion, feed intake) of the animal (Baurhoo, et al., 2007; Fernandez et al., 2002; Waldroup et al., 2003). Similarly, decreases in pathogenic bacteria in the gut, stimulation of the immune response, and elevating the strength of the intestinal mucosa have also been reported (Corrigan et al., 2012; Fernandez et al., 2002; Fritts & Waldroup, 2003). In contrast, other studies that reported that there was no significant effect when MOS was included in the diets of poultry compared with a normal diet or with diet with antibiotic supplementation (Table 1).

MOS in swine production

In swine nutrition, supplementation of feed with prebiotics and probiotics have been reported to have beneficial effects on growth performance, gastrointestinal functional, and health of pigs (Zhao et al., 2012). Previous studies have demonstrated that mannan oligosaccharides improved the daily rate of weight gain and/or feed efficiency during early stages of nursery pigs (Davis et al., 2002; Miguel, Rodriguez et al., 2004; Zhao et al., 2012) and in sow diets during late gestation and lactation (Halas & Nochta, 2012). However, there are other studies that reported less consistent results, specifically, that MOS had no effect in the performance of young pigs (Castillo et al., 2008; Wenner et al., 2013). Although, there are some conflicting results with the effect of MOS as a growth promoter in pigs, other studies have shown that the supplementation with MOS in the diet reduce the total coliforms counts in the gut and appears to have immune modulation effect in the post weaning-period in pigs (Davis et al., 2004; Kim et al., 2011) (Table 2).

Weaning is a critical period in piglets that is associated with stress, diarrhea, and depressed growth rates (Liu et al., 2008). Antibiotics have long been used to control and prevent these post weaning symptoms. MOS as a feed additive are suggested to be an alternative of the GPAs in swine, based on the relevant literature that have shown these products maintain gut health, interfering with pathogen colonization and enhancing the defense mechanisms in post weaning pigs and in other livestock animals.

MOS in fish production

Aquaculture production have been recognize as a growing industrial sector that have a huge socio-economic impact in developing and emerging countries (Yousefian & Amiri, 2009). Indeed, one of the most important priorities in the aquaculture industry is to produce fish and seafood under sustainable and safe conditions. As for poultry and swine, feed supplements are needed to improve animal health, reduce mortality, improve growth performance, and reduce or eliminate antibiotics in the diets (Torrecillas et al., 2007). The use of non-digestible oligosaccharides have shown promise as preventive and environmentally friendly alternatives to antibiotics in aquaculture, and to improve overall health especially for fishes and crustacean (Bachere, 2003; Soltanian et al., 2007) that may lead to expand and maintain this industry.

The mechanism of action of MOS in fish production appears to be similar to other animal applications. These products may prevent pathogen colonization, enhance overall health and welfare, and promote gut maturation and growth (Genc et al., 2007; Sado et al., 2008; Torrecillas et al., 2011). Incorporation of MOS in the diet has been reported to promoted resistance to bacterial infection in Cobia (*Rachycentron canadum)*(Salze et al., 2008)*,* rainbow trout *(Salmo gairdneri irideus)* (Staykov et al., 2007)*,* sea bass *(Dicentrarchus labrax)* (Torrecillas et al., 2011)*,* channel catfish (*Ictalurus punctatus*) (Peterson et al., 2010), marron (*Cherax tenuimanus*) (Sang et al., 2009). However, other studies showed no effect of MOS on growth performance of aquatic species (a Dimitroglou et al., 2009; Pryor, 2003). For example, investigations with Gilthead

sea bream *(Sparus aurata)* (A. Dimitroglou et al., 2010), Atlantic salmon (Grisdale-Helland, et al., 2008) revealed no significant effect when supplementing diets with MOS.

Conclusion

The worldwide trend toward eliminating the uses of antibiotic growth promoters for animal production has led researchers to seek new strategies to enhance growth, increase feed efficiency, reduce pathogen colonization, and improve overall performance in livestock animals. Mannan oligosaccharides are naturally derived molecules that may have the properties necessary to provide benefits similar to antibiotics. However, further research must be conducted to identify the function and mechanism of action of these substances. Ultimately, the use of MOS and other similar material to inhibit specific pathogens in livestock animals may have important implications for improving food safety.

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Table 1. *In vivo* uses of mannan oligosaccharides in poultry

Diet	Performance	Gut Health	References
0.2% MOS BioMOS	Significant improvement on feed conversion. No effect on body weight, mortality and breast meat yield.	Increased the population of bifidobacteria and lactobacilli in the ceca, after 42 days of treatment	(Baurhoo et al., 2007)
0.1% MOS BioMOS	Higher body weight gain, better feed conversion in broiler chickens and higher villus height at 7 days of age.	No reported	(Waldroup et al., 2003)
0.2% MOS	No reported	*S. enteritidis* colonization decreased over time in chick fed with MOS diet.	(Santin et al., 2002)
2.5 % MOS	Body weight gain, feed intake and feed conversion ratio of broilers by adding MOS are not significant different (6 weeks)	Increased *Bifidobacterium* spp., while decreasing member of *Enterobacteriaceae* and *Enterococcus* spp.,	(Fernandez et al., 2002)
0.15% MOS	Body weight and feed conversion ratio are no significantly affected by the MOS diet.	No reported	(Yalcinkaya et al., 2008)
0.10 % BioMOS	Significant improvement in feed conversion after the 20 week of inclusion of MOS. Body weight gain and mortality are no significantly affected by the treatment.	No reported	(Fritts and Waldroup, 2003)
0.22% MOS BioMOS	No reported	Identified 672 genes expressed in the jejunum by	(Xiao et al., 2012)

		MOS supplementation that are involved in the diverse biological functions including energy production, cell death, and protein translation.	
0.1% Yeast *S. boulardii*	No significant differences in body weight, and feed conversion ratios.	Significant reduction in the colonization of challenged *Salmonella typhimurium* in chickens fed with MOS diet. *Campylobacter jejuni* colonization was not significantly affected with the diet.	(Line et al., 1998)
0.1% MOS (BioMOS)	No reported	MOS supplementation displayed significantly altered bacterial community structure in young turkeys.	(Corrigan et al., 2012)
0.3 and 0.5% MOS (BioMOS)	The MOS supplement let to minor improvement in body weight but no improvement in feed conversion ratio.	No reported	(Iji et al., 2001)

Table 2. *In vivo* uses of mannan oligosaccharides in pigs

Diet	Performance	Gut Health	References
0.2 % MOS BioMOS	Improve efficiency of weight gain during starter period of piglets.	Decreased enterobacteria counts in jejunum.	(Castillo et al, 2007)
0.1% MOS	Significant improvement in average daily gain and average daily gain intake in pigs fed with MOS during 28 days.	No reported	(Zhao et al, 2013)
0.2% MOS BioMOs	No statistical difference in growth rate, average daily feed intake and feed conversion in weaning pigs fed with MOS diet.	No reported	(Wenner et al, 2013)
0.2% MOS	Improve in daily feed intake during the starter period (2 weeks). Feed conversion was improved.	Pigs fed with MOS diet decreased enterobacteria counts in the jejunum.	(Castillo et al., 2007)
0.2% MOS	Increased average daily gain and average daily feed intake of nursery pigs between 12 to 14 days of MOS diet.	No reported	(Lemieux et al., 2003)
3 % brewers dried yeast	None effect in the growth performance of nursery pigs.	Total coliforms, *Escherichia coli*, and *Clostridium perfringens* in feces were not affected by diet. *Bifidobacteria* spp. counts were lower in pigs fed the yeast diet and lactobacilli counts were higher in pigs fed yeast.	(White et al., 2002)

0.2% MOS	Average daily gain, average daily feed intake and gain: feed ratio increased with MOS diet from day 0 to 10 in weanling pigs.	No reported	(Davis et al., 2002)
0.3 % MOS	Increased the average daily gain and average daily feed intake in the pigs fed with MOS diet	No reported	(Rozeboom et al, 2005)
0.3% MOS	No reported	Increases of apparent coefficient of digestibility, and in villi height of the animals fed with MOS.	(Conejos et al, 2012)

Table 3. *In vivo* uses of mannan oligosaccharides in fish and crustacean.

Diet	Performance	Gut Health	References
0.2% MOS	No reported	Viable intestinal bacterial population was significant reduced (approx. 2 Logs) like *Aeromonas/Vibrio spp*. in juvenile trout fed with MOS.	(Staykov et al., 2007)
0.2% and 0.4% MOS BioMOS	Fish fed with 0.2% and 0.4% MOS shown significant increase body weight and growth rate.	European sea bass intestinal villi length was no affected by MOS diet. 0.4% MOS significantly improved head kidney macrophages phagocytic activity. Significant reduction in the challenge of *V. alginolyticus* in presence of 0.4%.	(Torrecillas et al., 2007)
0.2% and 0.4% MOS	No significant effect in the body weight, feed conversion, and specific growth rate and protein efficiency ratio in sea bream fed with MOS diet for 9 weeks.	MOS diet increased microvilli density in both the anterior and posterior. intestinal regions. No significant effect in the gross of villi structure. MOS diet alters the intestinal microbiota and morphology of gilthead sea bream.	(Dimitroglou et al., 2010)
	Fish fed diets with 0.4% MOS had best weight gain but it is no significant different from the	No reported	(Sado et al., 2008)

	control. Feed conversion decrease with the increase levels of MOS.		
0.3% MOS	Enhanced growth performance and feed conversion ratio was observed in shrimp fed with MOS for 48 days.	No reported	(Genc et al., 2011)
0.4 % and 0.6 % MOS	Feed consumption was improved in fish feed with MOS. Reduction in feed intake was found in fish with the inclusion of MOS.	Enhancement in the number of cells secreting acid mucins in posterior gut of fish fed MOS.	(Torrecillas et al., 2011)
0.3% MOS	No significant effect in the growth performance in fish fed with MOS.	No significant effect in the gastrointestinal morphology or spiral valve villi structure was found in fish fed with MOS supplementation.	(Pryor, 2003)
0.4% MOS	No reported	Gut mucosal folds height, width and folds surface area were increased in fish fed with MOS diet during 8 weeks Enhancement in the number of cells secreting acid mucins and in gut mucus lysozyme activity.	(Torrecillas et al., 2011)
0.4% MOS	Significant higher growth rate and average weekly gain in lobster fed with MOS for 56 days	Lower mortality of lobster fed with MOS when *Vibrio spp* was challenge for 7 days.	(Sang and Fotedar, 2010)

Chapter 3

Adherence inhibition of *Campylobacter jejuni* and *Campylobacter coli* to HEp-2 cells by mannan oligosaccharides and cranberry high molecular weight component.

Abstract

Campylobacter infections are a leading cause of human bacterial gastroenteritis in the United States and a major cause of diarrheal disease throughout the world. Poultry has been identified as a major reservoir for this pathogen, and contaminated chicken meat is considered a significant risk for human infection. Colonization and subsequent infection and invasion of *Campylobacter* require that the organism adheres to the surface of host cells as the first and one of the most important steps in bacterial pathogenesis. Agents that inhibit adherence could therefore be used prophylactically to reduce *Campylobacter* carriage and infection. Yeast mannan oligosaccharides (MOS) have been used as a feed supplement in livestock animals to replace growth-promoting antibiotics. However, some non-digestible oligosaccharides as MOS may also have the ability to prevent gastrointestinal infections by inhibiting pathogen adherence. Cranberry juice has been used to treat and prevent urinary tract infections, due in part to a high molecular weight (HMW) fraction that has anti-adherence activity against pathogens. The goal of this study was to assess the ability of MOS, purified MOS, cranberry HMW and the combination of MOS and HMW to serve as anti-adherence agents against *C. jejuni* ATCC 29428, *C. jejuni* ATCC 33291, *C. jejuni* ATCC 700819, *C. coli* ATCC 43485 and *C. coli* ATCC BAA-1061. Adherence experiments were performed using a human epithelial tissue cell line (HEp-2) in the absence or presence of MOS, pMOS, HMW and MOS/HMW. Significant reductions in adherence of *C. jejuni* ATCC 29438, *C. jejuni* ATCC 700819, *C. jejuni* ATCC 3329 and *C. coli* ATCC 43485 were observed in the

presence of MOS/pMOS (up to 40 mg/mL) and with HMW (up to 3 mg/mL). There was no significant effect in the adherence of *C. coli* ATCC BAA-1061. There was no additive effect in adherence of all the strains with the blend of MOS/HMW. The results obtained in this work suggest that MOS, pMOS and HMW may have an anti-adhesive effect on specific strains of *C. jejuni* and *C. coli* and directly inhibit the adherence to HEp-2 cells. These results suggest that both components MOS and HMW could be used to reduce *Campylobacter* colonization in livestock animals and prevent the onset of human infections.

Introduction

Mannan oligosaccharides (MOS) are non-digestible oligosaccharides derived via partial hydrolysis of the polysaccharide, mannan (Tester & Al-Ghazzewi, 2013). The latter consist of α-1,4 linked mannose monomers and are obtained commercially either from plant material or from yeast cell walls. In particular, brewers' and bakers' yeast strains of *Saccaromyces cerevisiae* are widely used to produce MOS products for animal nutrition applications (Halas & Nochta, 2012). Yeast cell walls of *S. cerevisiae* are composed of mannoprotein (35 %), β-1,3 glucan (25%), β-1,3 glucan bound to chitin (35%), β-1,6 glucan (5%) and small amount of chitin (1-2%) (Feuillat, 2003; Orlean, 2012).

Mannose-based carbohydrates could be a promising natural agent for reducing adherence of enteropathogenic bacteria, since because bacterial adherence in the gut is often mediated by binding of bacterial lectins to receptors containing D-mannose (Ganan et al., 2012; Shoaf et al., 2006). MOS as non-

digestible oligosaccharide have no direct nutritive value in animal feed, however they have been shown to maintain or promote gut health (Halas & Nochta, 2012). Extracts rich in mannanproteins have been shown to have a bioprotective effect against some pathogens such as *Campylobacter jejuni* (Ganan et al., 2009; Mcsweegan, 1986; Russell & Blake, 1994), *Escherichia coli* (Baurhoo et al, 2007) and *Salmonella* (Fernandez et al., 2002; Oyofo et al., 1989). Most studies have been conducted *in-vivo* with broiler chicks and pigs, and to a less extend in cattle and swine. Dietary supplementations with mannan oligosaccharides from crude yeast extract have been used in livestock animals, resulting in enhance overall performance and inhibiting colonization of enteric pathogens.

Another natural source of anti-adherence substances is derived from cranberry juice. Cranberry fruit juice has traditionally been used to treat and prevent urinary tract infections (UTI) (Vinson et al., 2008; Zafriri et al., 1989). In particular, two main components in cranberry juice have been shown to reduce adherence of *E. coli* to uroepithelial cells. Fructose inhibits adherence of *E. coli* phenotypes possessing type 1 (mannose-sensitive) fimbriae (Foo et al., 2000; Zafriri et al., 1989), and type-A proanthocyanidins (PACs) inhibits adherence of P-fimbriated *E. coli* in vitro (Howell et al., 2005). Thus, these compounds may be responsible for the beneficial effect of cranberry juice on UTI prevention.

Cranberry PACs have unusual A-type linkages compared to the more common B-type linkages found in PACs from other tannin-rich foods (Foo et al., 2000; Howell et al., 2005). There are no existing data about the anti-adherent effect of B-linkage proanthocyanidins against pathogenic microorganism.

Cranberry PACs consists of epicatechin unids (epicatechin-(4β-8, 2β-O-7)-epicatechin-(4β-8)) (Duarte et al., 2006) with degree of polymerization (DP) of 4 and 5 and at least one A-type linkage (Seeram et al., 2004). The mode of action of the cranberry type-A PACs is still unknown, however, it has been suggested they act by hydrophobic interactions, which modify bacterial surface macromolecules like fimbria and lipopolysaccharides (Steinberg et al., 2005), reducing adherence to epithelial cells.

Steinberg et al., 2005 observed anti-adherence and anti-biofilm formation activity against *Streptococcus sobrinus* by cranberry high molecular weight component (15000 MW cutoff) also known as nondialyzable material (NDM). Another study reported that cranberry juice inhibited the adherence of *Helicobacter pylori* to human gastric mucus and to human erythrocytes (Burger et al., 2000).

Campylobacter jejuni and *Campylobacter coli* are among the leading human foodborne pathogens (Leblanc-Maridor et al., 2011) and have been implicated as the most common cause of gastroenteritis in developed countries. The consumption of chicken contaminated with *Campylobacter* is considered a major risk factor for human infection (Friedman et al., 2000). Although *Campylobacter* infections have low mortality, about 76 people die each year from *Campylobacter* infections (CDC, 2013). In addition to causing gastroenteritis, Guillain-Barré syndrome (GBS) is the most significant implication of *Campylobacter jejuni* infections (Buzby et al., 1997; Mawla et al., 2014). It is a rare autoimmune disease that affects the peripheral nervous system causing

muscle weakness and paralyses. Around 40% of the cases of GBS in United States are due to previous campylobacteriosis (Buzby et al., 1997; CDC, 2013). It is estimated that approximately one every 1,000 cases of reported *Campylobacter* illness leads to GBS (CDC, 2013).

The mechanisms of pathogenesis for *Campylobacter* are not well understood. However, adherence to host epithelial cell is the first and perhaps the most important step for bacterial colonization and infection. Thus, there is a considerable interest in identifying preventive strategies to mitigate *Campylobacter* infection in humans. One approach would be to reduce colonization of this organism in poultry, and the anti-adherence approach using non-digestible oligosaccharides and naturally derived molecules could be a potential solution. MOS, in particular, have been suggested to be effective anti-adherence agents.

Although anti-adherence strategies have been proposed for several human and animal applications, this approach has one important limitation. Specifically, it is known that pathogenic bacteria often encode and express genes for more than one type of adhesin, a process known a phase variation (Henderson et al., 1999). Hence, it may be more effective to target more than one adhesion, by using a mixture of different anti-adherence agents to provide a broad spectrum effect (Ofek et al., 2003; Shoaf & Hutkins, 2009).

The main goal of this research was to assess the ability of both mannan oligosaccharides whole soluble fraction (MOS), purified mannan oligosaccharides (pMOS), cranberry high molecular weight component (HMW)

and the combination of MOS and HMW to inhibit adherence of *C. jejuni* and *C. coli* to HEp-2 tissue culture cells.

Materials and Methods

Organisms and growth conditions. Strains of *C. jejuni* subsp. *jejuni* ATCC 29428, *C. jejuni* subsp. *jejuni* ATCC 700819, *C. jejuni* subsp. *jejuni* ATCC 33291, *C. coli* ATCC 43485 and *C. coli* ATCC BAA-1061 were obtained from W. Miller (Agricultural Research Service, Albany, CA) and used for all the adherence experiments. Prior to each experiment, frozen stock cultures of each organism were thawed, plated onto Columbia Blood Agar Base (Oxoid) with 7% of horse blood defibrinated (BBL^{TM}) and grown for 48 hours at 42°C in modular incubator chamber under microaerophilic environment (5% O_2, 10% CO_2, and 85% N). A single colony was inoculated into 10 ml of Brucella Broth (BBL^{TM}; BD) supplemented with 250 µL of Fetal Bovine Serum defined (FBS; HyClone®) and grown in semi-solid media using the biphasic system (5 mL of Brucella agar and 10 mL of Brucella broth in sterile 25 cm^2 C/N tissue culture flasks (Corning) in horizontal position) and incubated under microaerophilic environment at 42°C during 24 hours (late exponential phase). After 24 hours incubation, cultures were harvested by centrifugation (4000 x *g* for 8 minutes). The cells were washed twice with Phosphate-Buffered Saline (PBS) pH 7.4 and re-suspended in Minimal Essential Medium (MEM/EBSS, HyClone, Thermo Fisher Scientific Inc, Utah, USA) supplemented with 10% fetal bovine serum (FBS; HyClone®). MEM was pre-equilibrated overnight at tissue culture conditions (5% CO_2, 95% relative humidity at 37°C).

Yeast mannan oligosaccharides (MOS). Yeast mannan oligosaccharide was obtained from Lallemand (Ontario, Canada) as a powdered material. According to the supplier, the starting material contained 24% MOS and 25% β-glucan. To fractionate this material, a stock solution was prepared dissolving 100 mg of powder per mL of distilled water. The solution was centrifuged at 1000 x g for 10 minutes to remove insoluble material. The soluble supernatant fraction was collected, filtered through a 0.4-µm filter, and freeze dried. A stock solution was prepared in distilled water at a final concentration of 100 mg/ml (pH 7.0).

To separate the mannan oligosaccharides from the β-glucan, the stock solution was mixed with 100% ethanol at a ratio of 3:1. After 1 hour, two phases were observed, allowing for separation. The precipitated β-glucan was freeze dried, and the supernatant containing the MOS was collected and the ethanol was removing using a rotary evaporator. The final component was then freeze dried.

Cranberry extract. Cranberry concentrate was obtained from Ocean Spray Cranberries, Inc (Lakeville-Middleboro, MA). The concentrate was also fractionated by dialysis to yield a high molecular weight component (HMW). Briefly, approximately 50 mL of concentrate was dispensed into dialysis tubing (12,000 – 14,000 MW cutoff). The material was dialyzed for 4 days at 4°C against distilled water with agitation. The non-dialyzable material was then freeze dried. A stock solution was prepared by resuspending the freeze-dried material in distilled water to a concentration of 10 mg/ml (pH 7.0). The solution was filter sterilized using 0.45 µm filters and stored at -20°C.

Tissue culture cells. HEp-2 cells were obtained from the American Type Culture Collection (ATCC; Manassas, Virginia). Cells were grown in 150 cm^2 sterile tissue culture flasks (Corning) containing 50 mL of MEM with 10% Fetal Bovine Serum (FBS) and incubated (5% CO_2, 95% relative humidity at 37°C). Confluent HEp-2 cells were washed once with PBS (pH 7.4) and harvested by adding 3.5 ml of Trypsin-EDTA Solution (Sigma) and incubating for 10 minutes at tissue culture conditions. Then, trypsin was inactivated with 3.5 ml of FBS. After that, cells were seeded onto 12-mm diameter wells in 24-well tissue culture plates (Thermo Scientific) at approximately 3.6 x 10^5 viable cells per well, and 500 µL of Minimal Essential Media (MEM) supplemented with 10% FBS was added to each well. Plates were incubated under tissue culture conditions for one day prior to the start of each experiment.

Adherence assays. Cells suspension of *C. jejuni* and *C. coli* were prepared as described above. The MOS, pMOS, or HMW were mixed with bacterial cultures (in MEM supplemented with 10% FBS and pre-incubated for 1 hour in microaerophilic conditions at 42°C) prior to being added to the tissue culture cells. As a control, water was added to the bacterial cultures at the same volume as the agent. The plates were incubated for 3 hours at tissue culture conditions. The wells were then washed 5 times with PBS (pH 7.4) to remove non-adhered bacterial cells. Experiments were replicated 6 times (n = 6) for analysis by quantitative plating. Adherence was also observed microscopically (Quintero et al., 2011)

For other experiments, MOS and HMW were prepared as concentrated stock solutions with distilled water at a final concentration of 100 mg/mL and 10 mg/mL. A 1:1 blend of MOS and HMW was prepared by mixing equal volumes to give a 50 mg/mL and 5 mg/mL of each one respectively. A negative control was prepared with distilled water and a positive control was made with the combination of distilled water and the component at the same concentration of the blend. The solutions were filter sterilized using 0.45-µm.

Invasion assays. The same procedure described above was followed for cell infection. Cell monolayers were washed 5 times with PBS (pH 7.4), and then 250 µL of pre-equilibrated MEM (supplemented with 10% FBS) with 150 µg/mL of gentamycin sulphate (Sigma) and 250 µL of 100 mg/mL of MOS were added. Cell monolayers were incubated 2 hours at 37°C in a 5% CO_2 humidified atmosphere chamber. Monolayer cells were then lysed with 1 mL Triton X-100 at 1% v/v in PBS (pH 7.4). Invaded bacteria were determined by serial dilutions and cultured on plates of Brucella Agar (BBL™; BD). The minimum inhibitory concentration (MIC) of each agent needed to inhibit the invasion of *C. jejuni* and *C. coli* to HEp-2 tissue cell lines were expressed as the percentage of invasive bacterial relative to an invasion control.

Culture Enumeration: Cells were washed as described above and detached by addition of 0.1% Triton X-100 in PBS and repeated pipetting. The cells were collected and enumerated on Brucella Agar (BBL™; BD) after incubating at 42°C

for 48 hours in microaerophilic conditions. Adherence inhibition was calculated as the average number of adhered bacteria per ml in the control minus the average number of adhered bacteria per ml in the treatment divided by the number of adhered bacteria per ml in the control.

PCR screening for adhesin identification:

Campylobacter strains were growth as described above. Cells were harvested from Brucella broth after 24 hours of incubation and DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Instructions provided in the manufacturer's manual for DNA extraction were followed for Gram negative bacteria.

An internal fragment of each gene was amplified via PCR using the primers listed in Table 1. Genes were amplified using the following parameters: 94°C for 2 min (1 cycle): 94°C for 45 s, 60°C (-1° per cycle) for 30 s, 70° for 1.5 min (10 cycles); 94°C for 45 s, 50°C for 30 s, and 70°C for 1.5 min (25 cycles); and 70°C for 1.5 min (25 cycles); and 70°C for 8 min (1 cycle) (Flanagan et al., 2009). PCR amplified products (8 µL) were resolved in 1.0% agarose gel (60 V, 90 min) and visualized by staining with ethidium bromide.

Table 1. Specific primers used in this study to identify presence of suspected adhesin genes.

Locus tag	Gene product	Primer*	Sequence
Cj1478c (cadF)	CadF	cadF-F	TATTTCTATGGTTTAGCAGGTGGAG
		cadF-R	GCTCTACCTTCTTTAGTGTCATTGC
Cj0628 (capA)	CapA	capA-F	TGAATCGAAGTGGAAAAATAGAAG
		capA-R	CCCATTTTTGTATCTTCATAACCT
Cj10983 (JlpAF)	JlpA	jlpA-F	TCTCAGGACTCTGGAATAAAGATTG
		jlpA-R	GTGTGCTATAGTCACTAACAGGGATG
Cj1279c (cadF)	Cj1279c (FlpA)	Cj1279c-F	TCAGAAGATGGCAAGGTTATAGAAG
		Cj1979c-R	GTTATTGCTATTGATTCAGCTGGAC
Cj0921c (peb1A)	PEB1	peb1-F	TCTAGGTGCTTGTGTTGCATTTAG
		peb1-R	TGTCTACAGAAAACGCATCAACTC

* Primer used to amplify a DNA fragment for the present/absent identification of specific adhesin gene.

Statistical analysis: Significant differences between the treatments was determined by analysis of variance (ANOVA) and Tukey's test to compare all pairs of columns were used to determine differences between the different concentrations. GraphPad Prism 5 Software (Version 5.03) was used for the statistical analysis.

Results

Mannan oligosaccharides reduce adherence of *Campylobacter jejuni* and *Campylobacter coli* to HEp-2 cells. Adherence assays with all strains of *C. jejuni* and *C. coli* were conducted in the presence and absence of MOS. Bacteria adherence to HEp-2 cells was measured by cultural enumeration (Figure 1). The results showed that high concentration of MOS (up to 40 mg/ml) were necessary to achieve an anti-adherence effect of the strains used for these studies. Thus, 40 mg/mL and 50 mg/mL inhibited adherence of *C. jejuni* ATCC 29428 by 94%

and 94%, *C. jejuni* ATCC 700819 by 73% and 94%, *C. jejuni* ATCC 33291 by 71% and 82%, and *C. coli* ATCC 43485 by 91% and 92%, respectively. However, there was no significant effect on the adherence of *C. coli* ATCC BAA-1061, suggesting that the effect found in MOS may be *Campylobacter* strain-specific. Microscopic observations provided qualitative evidence of the anti-adherence activities of the MOS (Figure 2).

Adherence of *C. jejuni* and *C. coli* in the presence and absence of purified MOS gave similar results as for the whole soluble MOS (Figure 3). Experiments revealed that the minimum inhibitory concentration of the purified MOS was also 40 mg/mL and that adherence of *C. coli* ATCC BAA-1061, was not affected by MOS at any of the concentrations.

Mannan oligosaccharides does not reduce invasion of *Campylobacter jejuni* **and** *Campylobacter coli* **to HEp-2 cells.** Invasion assays were conducted for all five strains of *Campylobacter* on HEp-2 cells. Invasion was measured by cultural enumeration. The results revealed that there were no significant reductions in the invasion of *C. jejuni* ATCC 700819, *C. jejuni* ATCC 33291, *C. jejuni* ATCC 29428, *C. coli* ATCC 43485 and *C. coli* ATCC BAA-1061 in presence of MOS (50 mg/mL) (data not shown).

High Molecular weight of cranberry extract reduces adherence of *Campylobacter jejuni* **and** *Campylobacter coli* **to HEp-2 cells.** Adherence assays with all five strains of *C. jejuni* and *C. coli* were conducted in the presence and

absence of cranberry HMW component to HEp-2 cells. Adherence was measured by cultural enumeration (Figure 4). The results revealed that the adherence of *C. jejuni* strains was inhibited by greater than 90% at the highest concentration of 5 mg/mL of HMW. However, the minimum concentration to achieve more than 50% of inhibition for four of five of the strains tested was 3 mg/mL. There was no significant effect in the adherence of *C. coli* ATCC BAA-1061.

High Molecular weight of cranberry extract does not reduce invasion of *Campylobacter jejuni* **and** *Campylobacter coli* **to HEp-2 cells.** Invasion assays of *C. jejuni* and *C. coli* in presence and absence of cranberry HMW component were measured by numerical enumeration (data no shown). The results revealed that there was no effect in the invasion process in presence of the highest dose 5 mg/mL of HMW with all the strains tested in this study.

Combinations of MOS and HMW are not additive. Adherence assays with all five strains of *C. jejuni* and *C. coli* were conducted in the presence and absence of a blend (1:1) of cranberry HMW and MOS. Bacteria adherence to HEp-2 cells was measured by cultural enumeration (Figure 5). Although, both HMW and MOS were effective at reducing adherence of *C. jejuni* ATCC 700819, *C. jejuni* ATCC 33291 and *C. jejuni* ATCC 29428 to HEp-2 cells, the results revealed that there was not additive effect of the blend HMW/MOS. That is, the extent of the inhibition was neither additive nor augmented by the mixture of the HMW/MOS.

Results with *C. coli* ATCC 43485 showed as before that MOS is effective against the adherence to HEp-2 cells, but HMW was not. There was no significant effect on the adherence of *C. coli* ATCC BAA-1061, suggesting that the effect found in MOS and HMW may be *Campylobacter* strain-specific.

***Campylobacter jejuni* expresses all of the adhesins screened in this study.** PCR was conducted to amplify adhesin genes using specific primers (Table 2). All *C. jejuni* strains were positive for the presence of all five adhesins tested in this study. However, only two adhesin genes were identified for *C. coli* strains.

Table 2. Presence/Absence of adhesin genes for the *Campylobacter* strains used in this study.

	Target Adhesin genes				
Strains	***cadF***	***peb1A***	***jlpA***	***capA***	**Cj1279**
C. coli ATCC BAA-1061	+	+	-	-	-
C. coli ATCC 43485	+	+	-	-	-
C. jejuni ATCC 700819	+	+	+	+	+
C. jejuni ATCC 33291	+	+	+	+	+
C. jejuni ATCC 29428	+	+	+	+	+

(+ Presence / - Absence)

Discussion

Over the past several years, different beneficial effects have been attributed to MOS (Davis et al., 2002; Ferket et al., 2005; Hanning et al., 2009; Staykov et al., 2007). Because they are also easy to produce at a relatively low cost, MOS have gained significant interest in agricultural applications. In

particular, MOS have been widely used in animal production as a feed supplement to replace antibiotics and other growth promoting agents (Baurhoo et al., 2009; Castillo et al., 2008). Most studies have been conducted *in-vivo* with broiler chicks, pigs, and fish and less extended in cattle and pets. In these reports, the population of pathogenic bacteria such as *Salmonella, E. coli*, *Campylobacter* and *Clostridium* was reduced after supplementation with MOS in the animal diet. Additionally, MOS have been shown to enhance growth performance of livestock animals.

Cranberry is a widely consumed fruit in United States and it has been identified to have a diverse biological properties (Duarte et al., 2006; Ofek et al., 1991; Sobota, 1984), among them the ability to inhibit the adherence and biofilm formation of some pathogens to epithelial cells (Burger et al., 2000; Foo et al., 2000; Zafriri et al., 1989). A high molecular weight component isolated from cranberry has been found to present the anti-adherent capacity (Burger et al., 2000), acting as inhibitor or by altering bacterial surface hydrophobicity (Steinberg et al., 2005)

In this study, adherence inhibition of three strains of *C. jejuni* and two strains of *C. coli* to HEp-2 cells in the presence of MOS, pMOS, cranberry HMW and a blend of MOS/HMW was determined. The results showed that both fractions MOS and pMOS at 40 mg/ml and 50 mg/mL have the property to inhibit adherence of some strains of *Campylobacter*. However, there were no significant differences between the whole soluble mannan oligosaccharide and purified fractions. This suggests that the anti-adherence effect is due to the mannan

oligosaccharides fraction and that the β-glucan fraction does not inhibit adherence. The mannan oligosaccharides material used in this study is derived from yeast cell walls produced from *S. cerevisiae* and was composed mainly by mannan oligosaccharides, β-glucan and others unknown ingredients. However, the exact amount of mannan oligosaccharides in both fractions (MOS and pMOS) is unknown. Efforts to understanding and identifing the mode of action of each of the commercial MOS compounds is worthy of exploration.

Results shown that the adherence of *C. jejuni* strains and *C. coli* ATCC 43485 was significantly reduced by cranberry HMW at a minimum concentration of 2 mg/mL and 3 mg/mL, respectively. However, at higher concentrations (up to 5 mg/mL) further reductions in adherence were not observed. Adherence of *C. coli* ATCC BAA-1061 was not inhibited by any dose of HMW.

The results with the blend of MOS/HMW showed that there was not an additive effect with these components. This suggests that some of the strains used in this study express a particular adhesin that is target for both MOS and HMW, or one of the anti-adhesive agents have a broad spectrum to target different *Campylobacter* adhesins.

Although the adherence mechanisms of *Campylobacter* are not well understood, several adhesins have been described. Importantly, adherence to host epithelial cells appears to be crucial for *C. jejuni* colonization of chickens (Flanagan et al., 2009; Ziprin et al., 1999). Adhesins as CadF (*Campylobacter ad*hesin to fibronection) are involved in the adherence of *C. jejuni* to fibronection (Konkel et al., 1999). Previous studies have suggested that CadF promotes

adherence in cell culture systems and during chicken colonization (Mourik, 2011: Flanagan, 2009; Konkel et al, 1997). Moreover, *C. jejuni cadF* mutants were shown to have reduced adherence (50%) to intestinal human cells compared with the *C. jejuni* wild-type strain (Flanagan et al., 2009; Monteville, Yoon, & Konkel, 2003). Two other major outer membrane proteins, CapA (*Campylobacter ad*hesin protein A) and JllpA (*Campylobacter jejuni* surface lipoprotein A) have been identified and suggested to mediate adherence of the bacterium to epithelial cells (Flanagan et al., 2009; Jin, Song et al., 2003). However, Flanagan et al, (2009) reported that CapA is not conserved among the strains of *C. jejuni* tested in the study, suggesting this adhesin may not be essential for *Campylobacter* colonization in chickens. Nevertheless, this particular protein may contribute during the initial steps of the adherence process. Jin et al, (2003) reported that JlpA is a surface exposed lipoprotein that is crucial for HEp-2 cell binding. Previous work has suggested that JlpA is located predominately in the bacterial inner membrane and can be found loosely associated with the outer membrane (Flanagan et al, 2009). PEP1 is a periplasmic binding protein associated with ABC transports (ATP-binding cassette (ABC) transports). Disruption of *peb1A* has been shown to reduce *C. jejuni* adherence to human HeLa epithelial cells (Kervella et al, 1993), and *C. jejuni* mutant strains failed to colonize the intestinal tract of mice (Pei et al, 1998). There are still open questions to be resolved about *Campylobacter* pathogenesis. In particular, it will be necessary to understand the importance and contribution of each known

adhesin for colonization, and additionally to identify the specific receptors for these adhesins.

There are few *in vitro* studies that have compared MOS preparations in an anti-adherence model. Previously, *in vitro* analysis with yeast-derived mannoproteins suggested that the yeast cell wall is effective at inhibiting adherence of *C. jejuni* to Caco-2 cells, but it had no significant effect on invasion (Ganan et al., 2009). Mannose derived from *Candida albicans* cell walls was shown by Oyofo et al., (1989) to inhibit the *in vitro* adherence of *Salmonella typhimurium* to intestinal cells of day old chickens. Another study reported that mannose was effective at blocking adherence of *C. jejuni* on Caco-2 cells by (Russell & Blake, 1994). These results indicated that the mannose content in the mannoprotein fraction appeared to be crucial for inhibiting the adherence process of some pathogenic microorganisms.

Mannose is a component of *N*-glycan structures (Parry et al., 2006), and many pathogens bacteria bind to mannose residues on the host tissue. Most of the mannose-binding lectins that have been identified in bacteria contain FimH-like adhesins commonly located at the tip of type I fimbriae (Firon et al., 1984; Ganner & Schatzmayr, 2012). However, these extracellular adhesins have not been recognized in *C. jejuni*. In contrast, *E. coli* and *Salmonella* are known to possess these mannose-sensitive type 1 fimbriae (Day et al., 2009; Firon et al., 1984; Neeser et al., 1986). In a previous study, Day et al. (2009) reported that *C. jejuni* 11168-O has a mannose-binding lectic whose expression was dependent on the environmental temperature and atmospheric conditons. Aditionally, Day et

al. (2009) suggested that mannose recognition by *C. jejuni* may not require mannose for initial adherence to host tissues, but may be unnecessary for subsquent colonisation (Thomas et al., 2004; Thomas et al., 2002).

Finally, it was previously reported that *C. jejuni* does not ferment or oxidase carbohydrates as a carbon source (Weingarten et al., 2009). Therefore, any observed reduction of binding can be attributed to direct interactions involved in adherence and not as an indirect metabolic effect (Day et al., 2009).

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Figure 1. Adherence of *C. jejuni* and *C. coli* to HEp-2 cells in the presence of mannan oligosaccharides (0, 10, 20, 30, 40 and 50 mg/mL) analyzed by cultural enumeration (n=6). Statistically significant effects are indicated by an asterisk.

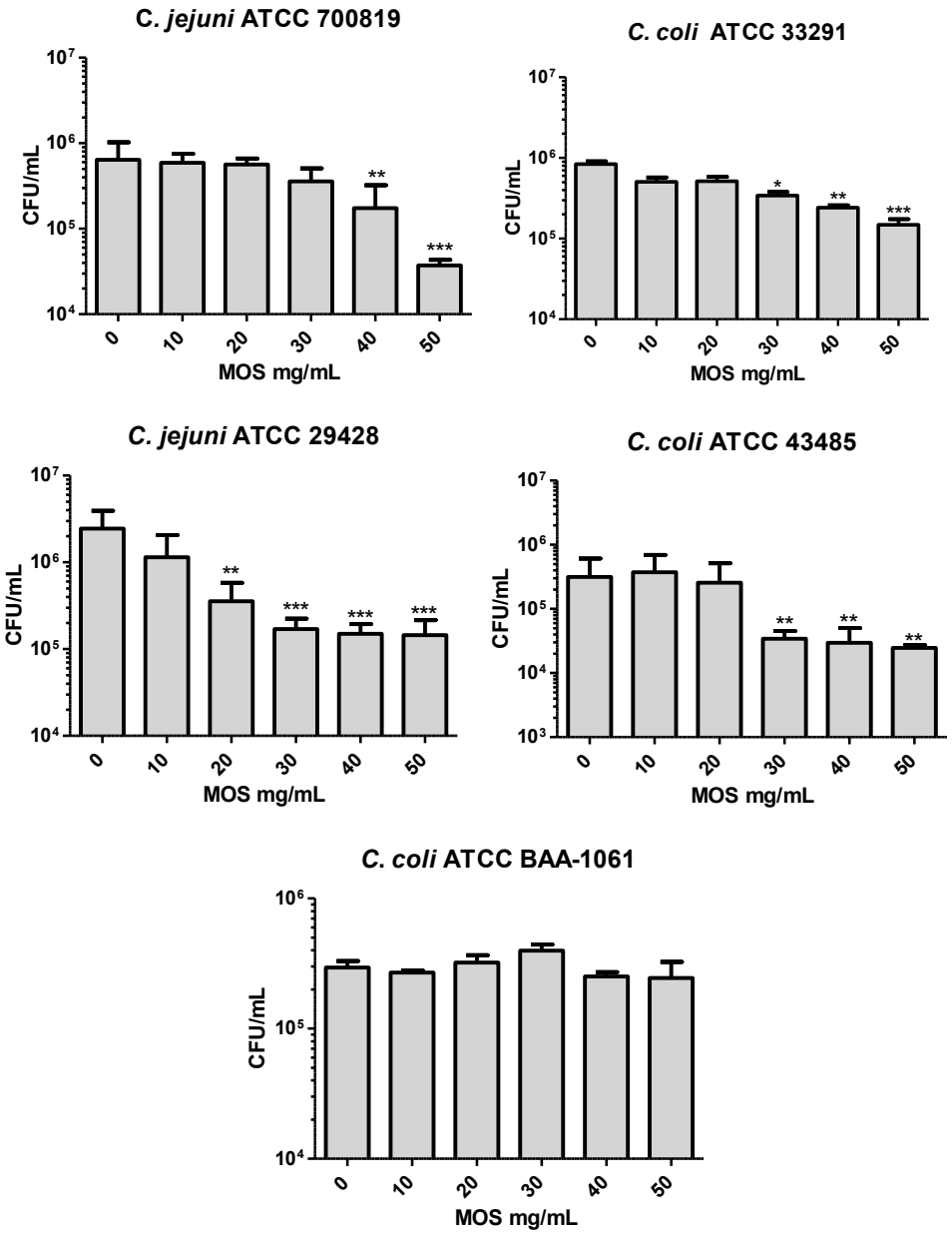

C. jejuni ATCC 700819
CFU/mL
MOS mg/mL
C. coli ATCC 33291
CFU/mL
MOS mg/mL
C. jejuni ATCC 29428
CFU/mL
MOS mg/mL
C. coli ATCC 43485
CFU/mL
MOS mg/mL
C. coli ATCC BAA-1061
CFU/mL
MOS mg/mL

Figure 2. Microscopy observation 1000 x magnification of *C. coli* ATCC 43485 adhere to surface of HEp-2 cells. Control (A) and 50 mg/mL of MOS (B).

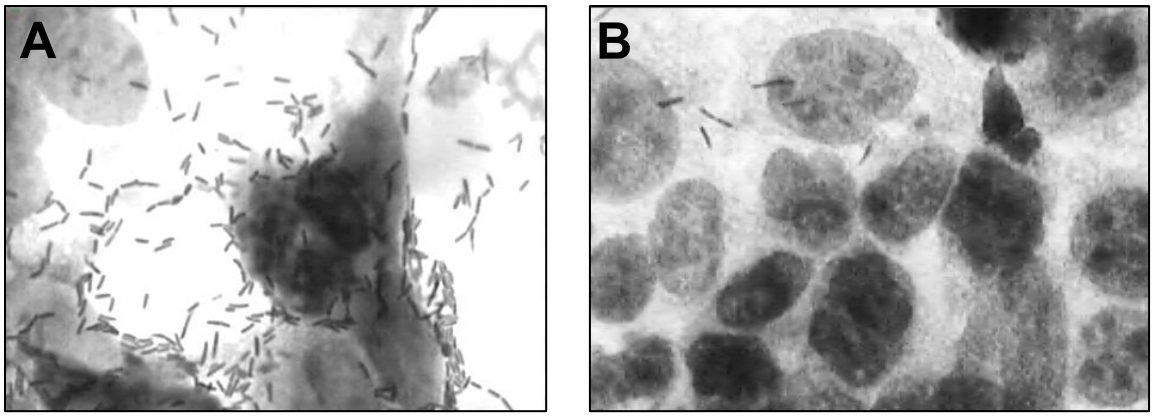

A
B

Figure 3. Adherence of *C. jejuni* and *C. coli* to HEp-2 cells in the presence of purified mannan oligosaccharides (0, 10, 20, 30, 40 and 50 mg/mL) analyzed by cultural enumeration (n=6). Statistically significant effects are indicated by an asterisk.

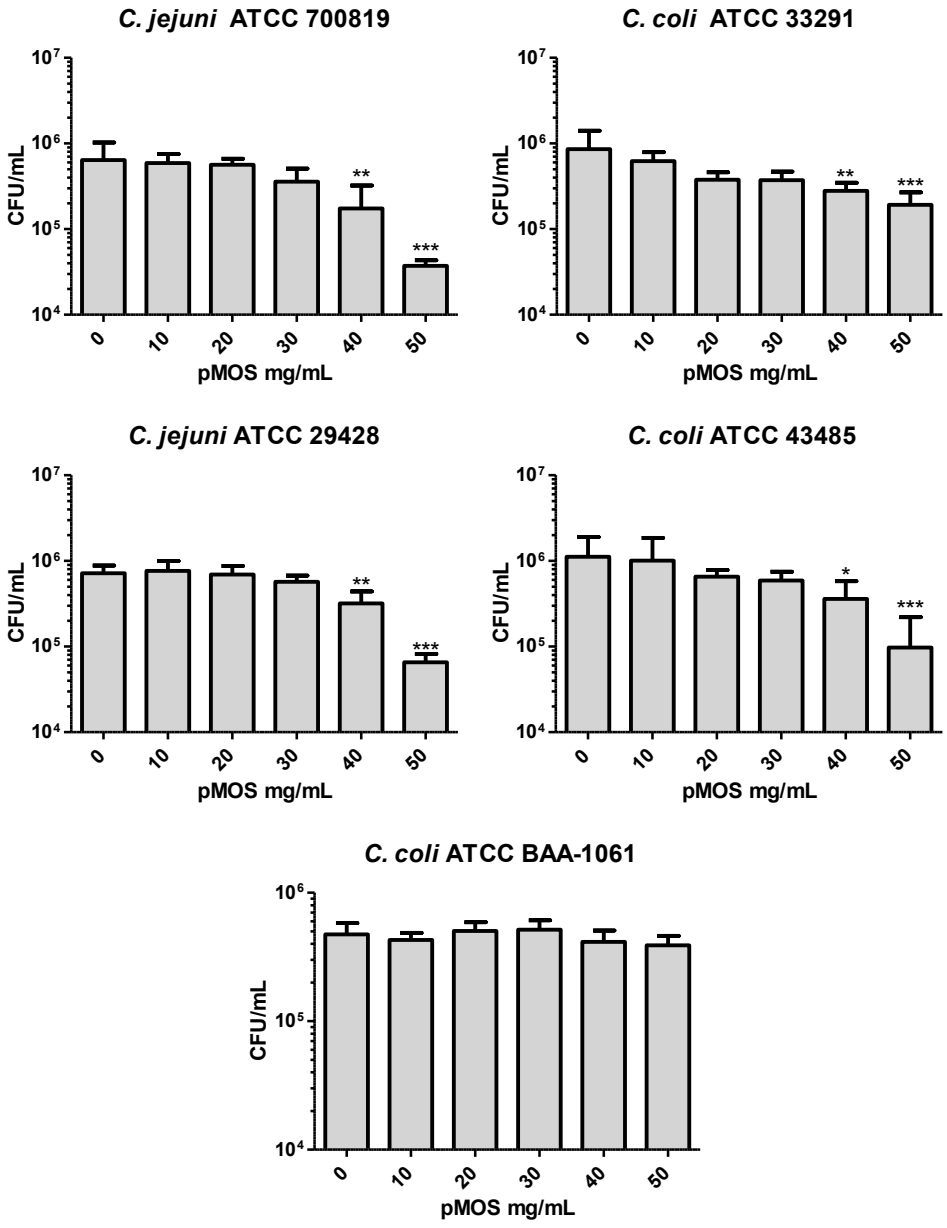

C. jejuni ATCC 700819
C. coli ATCC 33291
C. jejuni ATCC 29428
C. coli ATCC 43485
C. coli ATCC BAA-1061
CFU/mL
pMOS mg/mL
0
10
20
30
40
50
**
***
*

Figure 4. Adherence of *C. jejuni* and *C. coli* to HEp-2 cells in the presence of high molecular weigh of cranberry extract (0, 1,2,3,4 and 5 mg/mL) analyzed by cultural enumeration (n=6). Statistically significant effects are indicated by an asterisk.

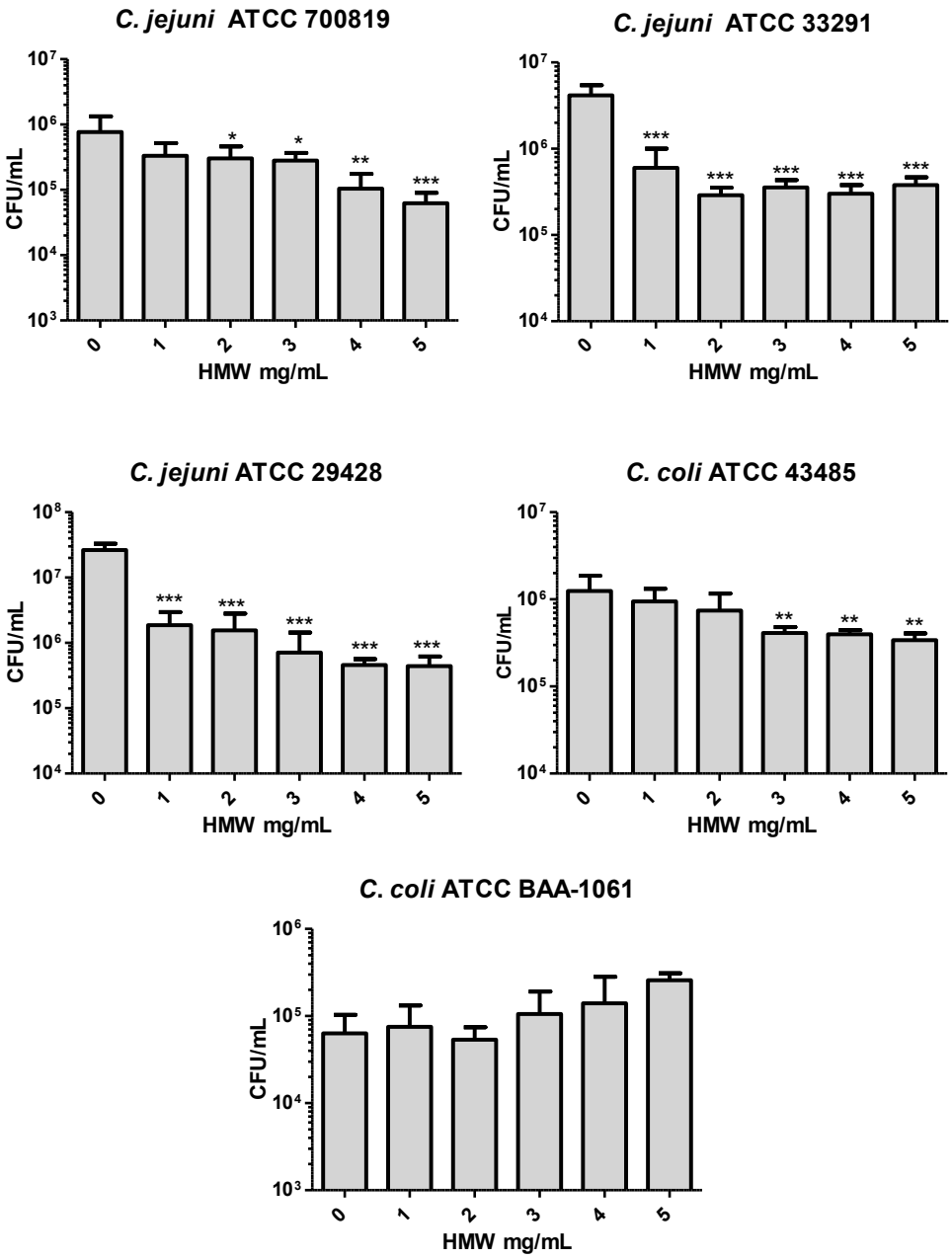
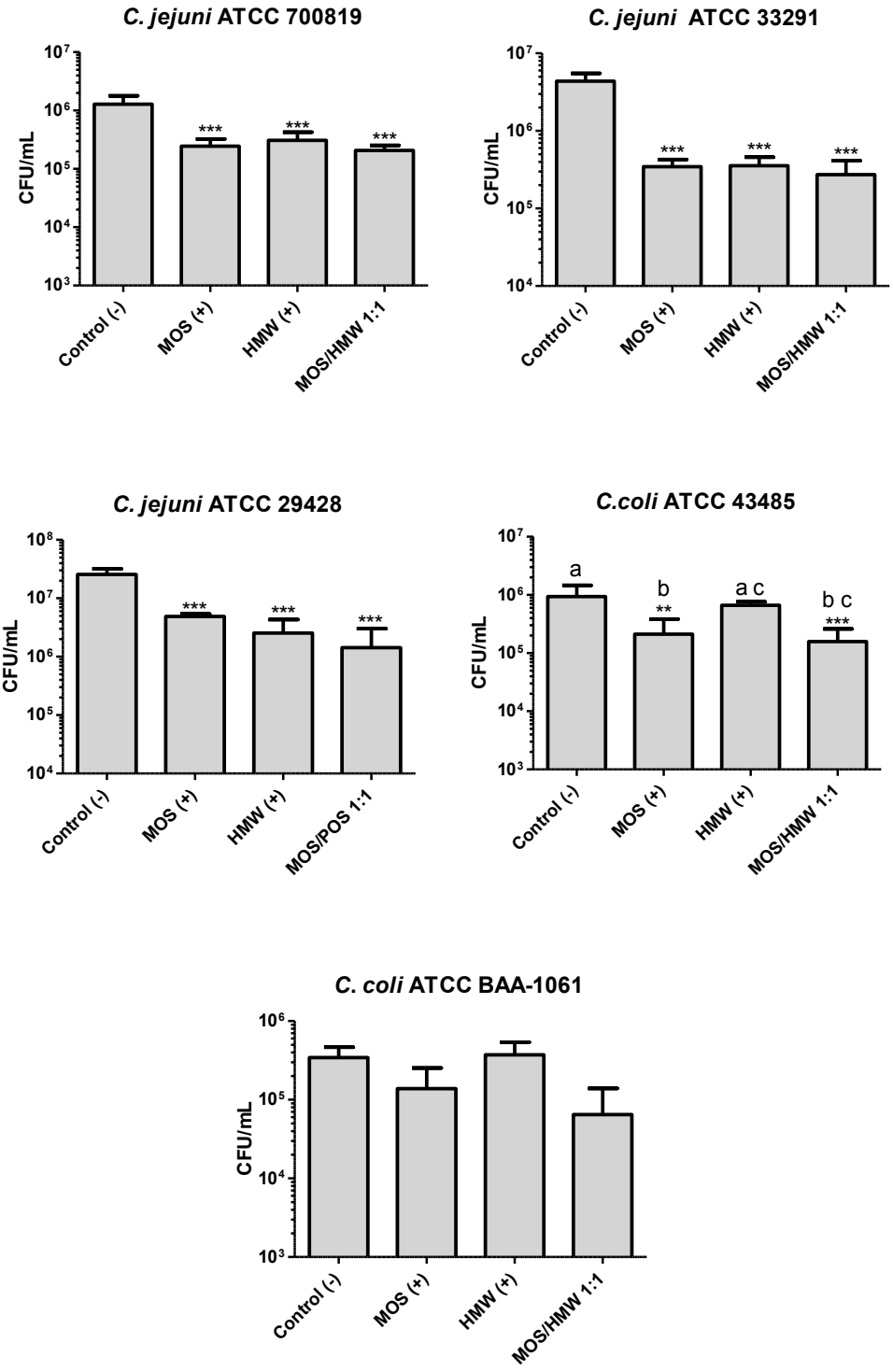

C. jejuni ATCC 700819
CFU/mL
HMW mg/mL
C. jejuni ATCC 33291
CFU/mL
HMW mg/mL
C. jejuni ATCC 29428
CFU/mL
HMW mg/mL
C. coli ATCC 43485
CFU/mL
HMW mg/mL
C. coli ATCC BAA-1061
CFU/mL
HMW mg/mL

Figure 5. Adherence of *C. jejuni* and *C. coli* to HEp-2 cells in the presence of high molecular weight of cranberry extract and yeast mannan oligosaccharides (1:1, HMW 5 mg/mL, MOS 50 mg/mL) analyzed by cultural enumeration (n=6). Statistically significant effects are indicated by an asterisk, the letters significant the differences among the treatments.


C. jejuni ATCC 700819
CFU/mL
Control (-)
MOS (+)
HMW (+)
MOS/HMW 1:1
C. jejuni ATCC 33291
C. jejuni ATCC 29428
MOS/POS 1:1
C.coli ATCC 43485
a
b
a c
b c
C. coli ATCC BAA-1061

Chapter 4

Adherence inhibition of *Campylobacter jejuni* and *Campylobacter coli* to HEp-2 cells by pectin oligosaccharides

Abstract

Many recent studies have described the beneficial effects and mechanisms of action of prebiotics. However, there is now active interest in prebiotic oligosaccharides derived from suitable bioresources. Pectic oligosaccharides (POS) are non-digestible oligosaccharides commonly found in fruit and vegetables. POS have been recognized for their potential prebiotic properties. In addition to their ability to modulate the gut microbiota, POS have also been suggested to inhibit attachment of some pathogens to epithelial cells. The goal of this research was to assess the ability of orange pectic oligosaccharides to inhibit the adherence of *C. jejuni* ATCC 29428, *C. jejuni* ATCC 33291, *C. jejuni* ATCC 700819, *C. coli* ATCC 43485 and *C. coli* ATCC BAA-1061. Adherence experiments were performed using a human epithelial tissue cell line (HEp-2) in the absence or presence of POS. There were no significant reductions in adherence of any *Campylobacter* strains. However, adherence tended to increase as the concentration of POS increases. The results obtained in this work suggest that POS does not have an anti-adhesive effect on the specific strains of *C. jejuni* and *C. coli* tested in this study. Further investigation is necessary to determine the mechanisms of interaction of POS and *Campylobacter* strains in an *in vitro* model.

Introduction

Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or

limited number of bacterial species in the colon (Gibson et al., 1995). The commercial prebiotic products currently on the market are produced by enzymatic synthesis or degradation of materials such as lactose, sucrose, inulin, starch and xylan (Hotchkiss et al., 2003; Crittenden & Playner, 1996).

Non-digestible oligosaccharides (NDO) have been suggested as potential agents for improving gut health and well-being by maintaining a balanced intestinal microbiota (Roberfroid, 2007). Recently, some NDO have been recognized to have other biological properties. Specifically, they may block the adherence of some pathogenic bacteria, preventing these pathogens from attaching to target cells in the gut (Shoaf et al., 2006). Since adherence is the first and more important step in bacteria pathogenesis (Savage, 1977; Shoaf & Hutkins, 2009), strategies based adherence inhibition could be very effective. NDOs are among the agents being considered for this function (Klemm et al., 2010; Shoaf et al., 2006). Specifically, pectic oligosaccharides (POS) have been proposed to have this activity (Paeschke & Aimutis, 2011).

Pectin is an acidic polysaccharide found in fruit and vegetable processing residues (Poli et al., 2011). Pectin consists of homogalacturonan and rhamnogalacturonan I (RGI) in the backbone with arabinan, galactan and arabinogalactan neutral sugar-chains attached to rhamnose in RGI regions (Carpita & Gibeaut, 1993; Holck et al., 2014; Ridley et al., 2001). POS are complex polysaccharides that represent one of the major components of plant cell wall of dicotyledonous plants (Mandalari et al., 2006; Willats et al., 2000). POS are formed via enzymatic hydrolysis of pectins (DP 2-20) (Holck et al.,

2014), resulting mainly in monomers of α-1-4-linked galactosyluronic acid residues (Alonso & Parajo, 2010).

Pectin-derived oligosaccharides from plant cell walls are produced by several chemical and physical steps. The cell wall material is pre-treated and the pectin polysaccharides are extracted. The oligosaccharides are released and separated and then purified by chromatography (Holck et al., 2014). Commercial pectin for food additive purposes depends of molecular weight, degree of esterification and intrinsic viscosity (Hotchkiss et al., 2003).

POS have been proposed to be a potential prebiotic (Ganan et al., 2010). POS have been evaluated to stimulate the growth of beneficial bacteria, including *Lactobacillus* (Mandalari et al., 2006b) and *Bifidobacterium* (Manderson et al., 2005; Olano-Martin et al., 2002). In addition to their putative prebiotic activity, POS have also been recognized for their ability to inhibit the adherence of pathogens (Hotchkiss et al., 2003; Holck et al., 2014).

The main goal of this research, therefore, was to asses the ability of pectic oligosaccharides (POS) to inhibit the adherence of *C. jejuni* and *C. coli* in HEp-2 tissue culture cells..

Materials and Methods

Organisms and growth conditions. Strains of *C. jejuni* subsp. *jejuni* ATCC 29428, *C. jejuni* subsp. *jejuni* ATCC 700819, *C. jejuni* subsp. *jejuni* ATCC 33291, *C. coli* ATCC 43485 and *C. coli* ATCC BAA-1061 were obtained from W. Miller (Agricultural Research Service, Albany, CA) and used for all the adherence experiments. Prior to each experiment, frozen stock cultures of each organism

were thawed, plated onto Columbia Blood Agar Base (Oxoid) with 7% of Horse Blood defibrinated (BBL™) and grown for 48 hours at 42°C in modular incubator chamber under microaerophilic environment (5% O_2, 10% CO_2, and 85% N). A single colony was inoculated into 10 ml of Brucella Broth (BBL™; BD) supplemented with 250 µL of Fetal Bovine Serum (FBS; HyClone®) and grown in semi-solid media using the biphasic system (5 mL of Brucella agar and 10 mL of Brucella broth in sterile 25 cm^2 C/N tissue culture flasks (Corning) in horizontal position) and incubated under microaerophilic environment at 42°C during 24 hours (late exponential phase). After 24 hours incubation, cultures were harvested by centrifugation (4000 x *g* for 8 minutes). The cells were washed twice with Phosphate-Buffered Saline (PBS) pH 7.4 and re-suspended in Minimal Essential Medium (MEM/EBSS, HyClone, Thermo Fisher Scientific Inc, Utah, USA) supplemented with 10% fetal bovine serum (FBS; HyClone®). MEM was pre-equilibrated overnight at tissue culture conditions (5% CO_2, 95% relative humidity at 37°C).

Orange pectic oligosaccharide (POS): Citric pectic oligosaccharide was obtained from Agricultural Research Service Eastern Regional Research Center (Wyndoor, PA), as a powdered material. A stock solution of 10 mg/ml was prepared in phosphate buffer solution (PBS) at pH 7.4, filtered through a 0.45µm filter and stored at -20°C.

Tissue culture cells. HEp-2 cells were obtained from the American Type Culture Collection (ATCC; Manassas, Virginia). Cells were grown in 150 cm^2 sterile tissue culture flasks (Corning) containing 50 mL of MEM with 10% Fetal Bovine Serum (FBS) and incubated (5% CO_2, 95% relative humidity at 37°C). Confluent HEp-2 cells were washed once with PBS (pH 7.4) and harvested by adding 3.5 ml of Trypsin-EDTA Solution (Sigma) and incubating for 10 minutes at tissue culture conditions. Then, trypsin was inactivated with 3.5 ml of FBS. After that, cells were seeded onto 12-mm diameter wells in 24-well tissue culture plates (Thermo Scientific) at approximately 3.6 x 10^5 viable cells per well, and 500 µL of Minimal Essential Media (MEM) supplemented with 10% FBS was added to each well. Plates were incubated under tissue culture conditions for one day prior to the start of each experiment.

Adherence assays. Cells suspension of *C. jejuni* and *C. coli* were prepared as described above. The POS were mixed with bacterial cultures (in MEM supplemented with 10% FBS and pre-incubated for 1 hour in microaerophilic conditions at 42°C) prior to addition to the tissue culture cells. As a control, water was added to the bacterial cultures at the same volume as the agent. The plates were incubated for 3 hours at tissue culture conditions. The wells were then washed 5 times with PBS (pH 7.4) to remove non-adhered bacterial cells. Experiments were replicated 6 times (n = 6) for analysis by quantitative plating. Adherence was also observed microscopically (Quintero et al., 2011).

Invasion assays. The same procedure described above was followed for cell infection. Cell monolayers were washed 5 times with PBS (pH 7.4), and then 250 µL of pre-equilibrated MEM (supplemented 10% FBS) with 150 µg/mL of gentamycin sulphate (Sigma) and 250 µL of 10 mg/mL of POS were added. Cell monolayers were incubated 2 hours at 37°C in a 5% CO_2 humidified atmosphere chamber. Monolayer cells were then lysed with 1 mL Triton X-100 at 1% v/v in PBS (pH 7.4). Invaded bacteria were determined by serial dilutions and cultured on plates of Brucella Agar (BBL™; BD). The minimum inhibitory concentration (MIC) of each agent needed to inhibit the invasion of *C. jejuni* and *C. coli* to HEp-2 tissue cell lines were expressed as the percentage of invasive bacterial relative to an invasion control.

Culture enumeration: Cells were washed as described above and detached by addition of 0.1% Triton X-100 and repeated pipetting. The cells were collected and enumerated on Brucella Agar (BBL™; BD) after incubating at 42°C for 48 hours in microaerophilic conditions. Adherence inhibition was calculated as the average number of adhered bacteria per ml in the control minus the average number of adhered bacteria per ml in the treatment divided by the number of adhered bacteria per ml in the control.

Statistical analysis: Significant differences between the treatments were determined by analysis of variance (ANOVA) and Tukey's test to compare all pairs of columns were used to determine differences between the different

concentrations. GraphPad Prism 5 Software (Version 5.03) was used for the statistical analysis.

Results

Pectic oligosaccharides do not reduce adherence of *Campylobacter jejuni* **and** *Campylobacter coli* **to HEp-2 cells.** Adherence of *C. jejuni* and *C. coli* in presence and absence of pectic oligosaccharides to HEp-2 cells was measured by cultural enumeration (Figure 1). The results revealed that POS did not inhibit the adherence of all five strains of *Campylobacter* tested in this study (Figure 2). Interestingly, adherence tended to increase as the POS concentration increases. We suggest that POS might function as a bridge to enhance binding of some strains of *Campylobacter* to epithelial cells. However, *C. jejuni* ATCC 33291 was the only strain that showed adherence reduction but it was not statistically significant compared with the control.

Pectic oligosaccharides does not reduce invasion of *Campylobacter jejuni* **and** *Campylobacter coli* **to HEp-2 cells.** Invasion assays of *C. jejuni* and *C. coli* were conducted in presence and absence of POS by numerical enumeration (Figure 3). The results revealed that there was no inhibitory effect on invasion in the presence of the highest dose 5 mg/mL of POS of all the strains to HEp-2 cells.

Discussion

The anti-adherence strategy against pathogens was proposed several years ago (Andersson et al., 1986; Cravioto et al., 1991; Ofek & Beachey, 1978). Since then, many studies have evaluated the activity of several oligosaccharides that may act as molecular decoys and block the bacterial attachment to epithelial cells. Prebiotic oligosaccharides are of considerable interest because, in addition to their health promoting properties, they have been suggested to inhibit the adherence of some pathogens and reduce bacterial colonization and infection. Oligosaccharides from pectin (POS) are proposed to be in the group of potential new generation of prebiotics (Paeschke & Aimutis, 2011).

In this report, we compared the ability of pectin oligosaccharides to inhibit the adherence of *C. jejuni* and *C. coli* to HEp-2 cells. The results showed that POS had no effect on adherence or the invasion of any of the *Campylobacter* strains tested in this study. Adherence to HEp-2 was increased in some strains.

Pectic oligosaccharides are a co-product of pectin manufacturing. They are a significant component of the supernatant remaining after pectin precipitation (Ganan et al., 2010). Therefore, pectin is a suitable raw material for the production of oligosaccharides with promising prebiotic potential (Alonso & Parajo, 2010). The most common commercial sources of POS are citrus peel, apple pomace, sugar beet pulp and potatoes pump (Holck et al., 2014). Specially, orange pectin oligosaccharides were shown to prevent adhesion of several *E. coli* strains, including *E. coli* NTCC 12900, *E. coli* NTCC 13127, *E. coli* NTCC 13128, *E.coli* NTCC O111:H27, and *Desulfovibrio desuldurincans.*

Adherence inhibition of over 80% of inhibition were reported (Rhoades et al., 2008) and invasion of *C. jejuni* in Caco-2 cells (Ganan et al., 2010; Olano-Martin et al., 2003). POS, have also shown protective effects against *E. coli* verocytototixins in HT29 tissue culture cells (Olano-Martin et al., 2003; Paeschke & Aimutis, 2011). Guggenbichler et al., 1997 reported that digalacturonic acid (α-D-Gal*p*A-(1-4)-D-Gal*p*A) derived from acid pectin oligosaccharides inhibited adherence of P-fimbriated *E. coli* to uroepithelial cells.

The methyl-esterified oligogalacturonic acids component in POS appear to be responsible for the anti-adherent property and anti-toxin attachment to specific epithelial receptors (Holck et al., 2014; Rhoades et al., 2008). However, it is clear that the effect of POS may be strain specific.

Holck et al., 2014 reported that differences in POS sources and composition could alter the results of bacterial adherence and invasion to specific epithelial cells. Thus, a better understanding of the POS structure, function and relationship with bacterial pathogens is required.

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Shoaf, K., & Hutkins, R. W. (2009). Adherence, anti-adherence, and oligosaccharides preventing pathogens from sticking to the host. *Advances in Food and Nutrition Research*, 55,101–161.

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Figure 1. Adherence of *C. jejuni* and *C. coli* to HEp-2 cells in the presence of Pectic Oligosaccharide (0, 1, 2, 3, 4 and 5 mg/mL) analyzed by cultural enumeration (n=6). An asterisk indicates statistically significant effects.

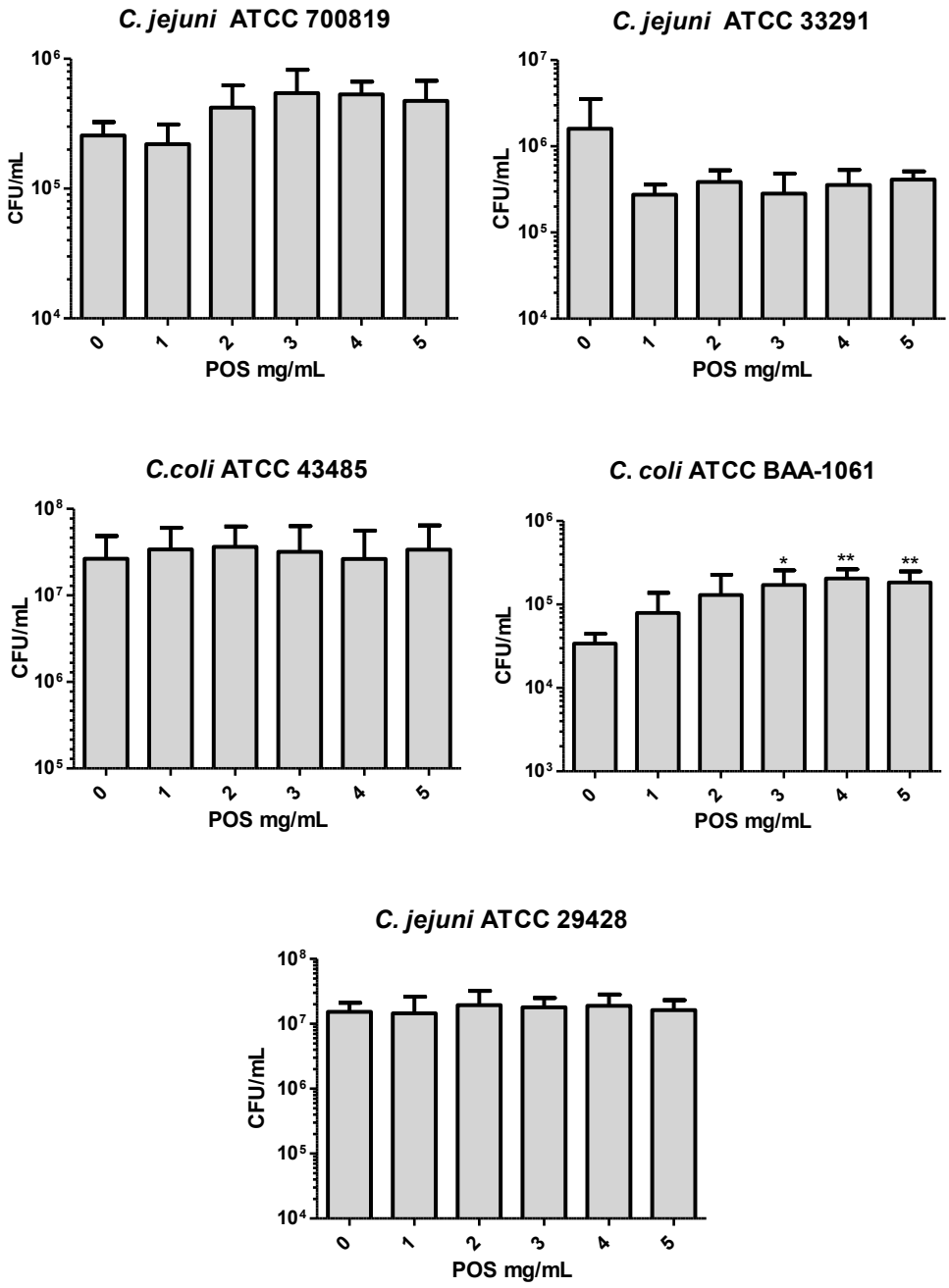

C. jejuni ATCC 700819
CFU/mL
POS mg/mL
C. jejuni ATCC 33291
CFU/mL
POS mg/mL
C.coli ATCC 43485
CFU/mL
POS mg/mL
C. coli ATCC BAA-1061
CFU/mL
POS mg/mL
C. jejuni ATCC 29428
CFU/mL
POS mg/mL

Figure 2. Microscopy observation 1000 x magnification of *C. coli* ATCC 43485 adhere to surface of HEp-2 cells. Control (A) and 5 mg/mL of POS (B).

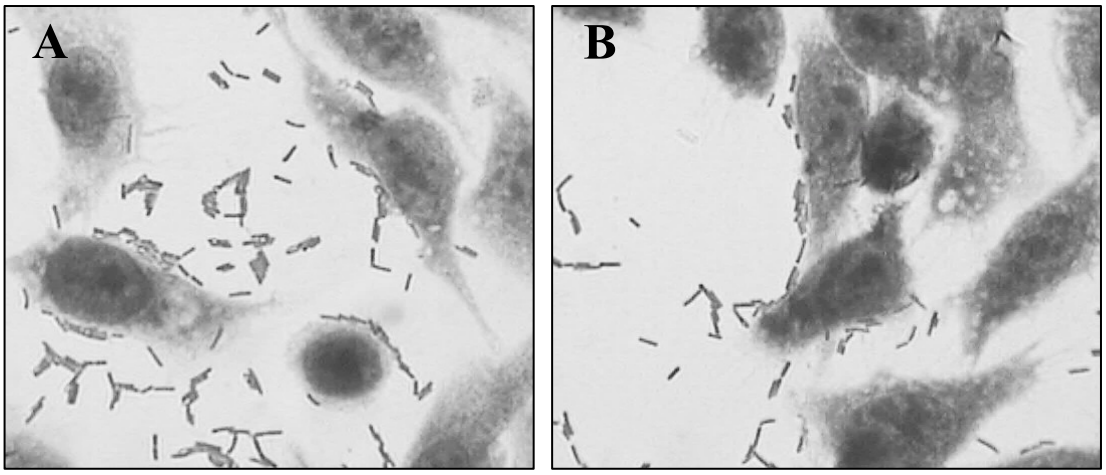
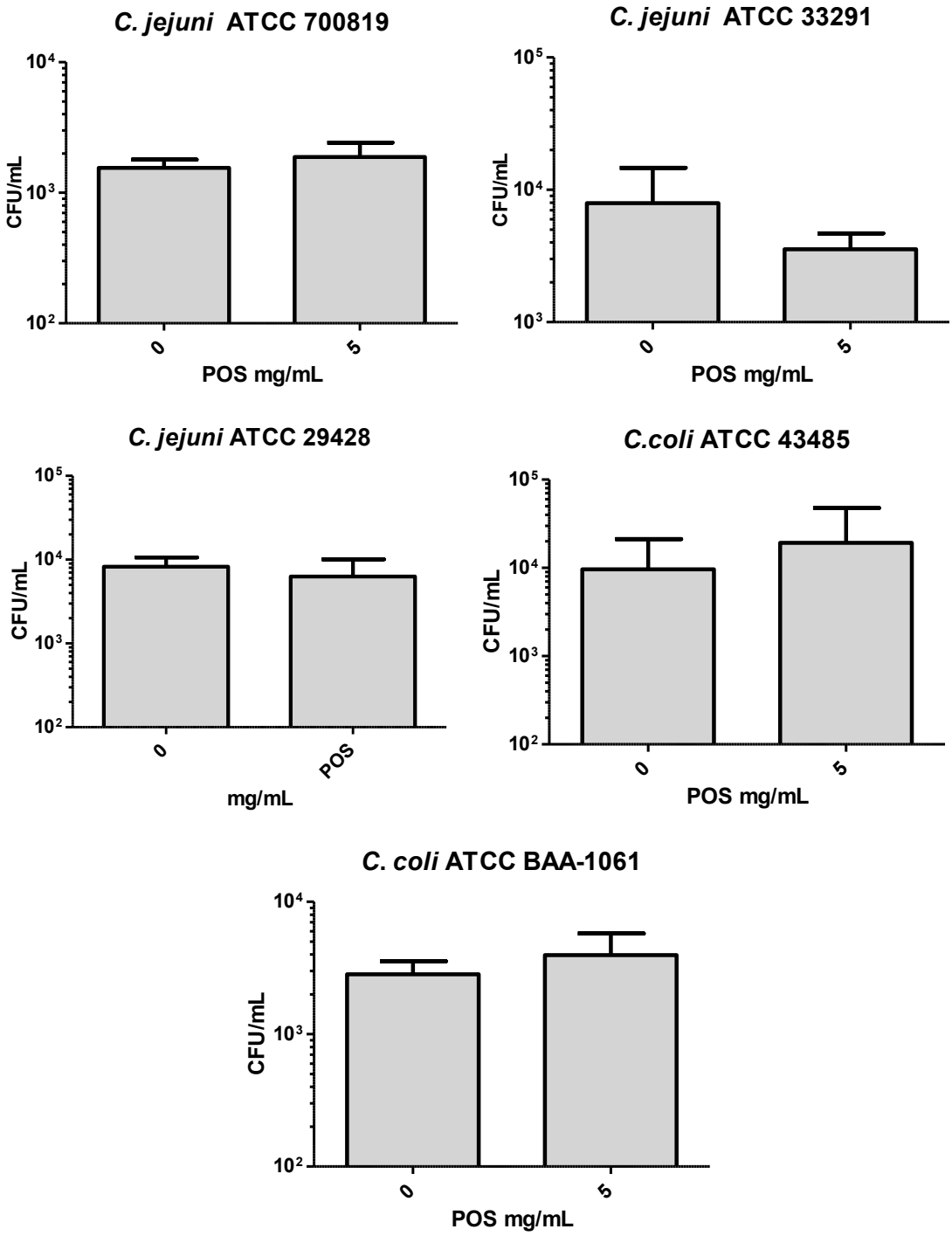

A
B

Figure 3. Invasion of *C. jejuni* and *C. coli* to HEp-2 cells in the presence of POS 5 mg/mL) analyzed by cultural enumeration (n=6). Statistically significant effects are indicated by an asterisk.


C. jejuni ATCC 700819
CFU/mL
POS mg/mL
C. jejuni ATCC 33291
CFU/mL
POS mg/mL
C. jejuni ATCC 29428
CFU/mL
POS
mg/mL
C.coli ATCC 43485
CFU/mL
POS mg/mL
C. coli ATCC BAA-1061
CFU/mL
POS mg/mL

Chapter 5

Conclusion

In this research we proposed that mannan oligosaccharides and cranberry high molecular weight components could be used to inhibit adherence of *C. jejuni* and *C. coli* strains to epithelial cells. Specifically, we established that a mannan oligosaccharides fraction was responsible for the anti-adherent effect against the *Campylobacter* strains used in this studied. Furthermore, it was determined that pectic oligosaccharides derived from orange did not inhibit the adherence of *C. jejuni* and *C. coli* strains to epithelial cells. The major findings of this research are described below.

- MOS and pMOS effectively reduce adherence of *C. jejuni* and *C. coli* strains in tissue culture experiments.
- MOS are not effective at reducing the invasion of *C. jejuni* and *C. coli* strains to epithelial cells.
- The minimum MOS and pMOS concentration required for *C. jejuni* and *C. coli* strains adherence inhibition was 40 mg/mL; higher concentrations did not exhibit significantly higher adherence inhibition.
- The activity of the effective MOS was attributed to the mannan fraction and not to the β-glucan fraction.
- Cranberry high molecular weigh component effectively reduce the adherence of *C. jejuni* and *C. coli* strains in tissue culture experiments.
- Cranberry high molecular weigh component is not effective for reducing invasion of *C. jejuni* and *C. coli* strains to epithelial cells.

- The minimum HMW concentration required for inhibiting adherence of *C. jejuni* and *C. coli* was 2 and 3 mg/mL, respectively; however, higher concentrations did not enhance adherence inhibition.
- There was no additive effect observed by using a combination of MOS and HMW against adherence of *C. jejuni* and *C. coli* strains to epithelial cells.
- The anti-adherence effect of MOS and HMW was strain-specific.
- Pectin oligosaccharides did not inhibit adherence or invasion of *C. jejuni* and *C. coli* strains.
- Collectively, the results obtained from this study provide evidence that mannan oligosaccharides and cranberry extract have anti-adherence activity and may be useful act as prophylactic agents against pathogenic bacteria that infect production animals.