ABSTRACT

Title of Dissertation: REDUCED CAMPYLOBACTER INFECTION AND ENHANCED PERFORMANCE IN POULTRY WITH BIOACTIVE PHENOLICS THROUGH EPIGENETIC MODULATION OF THE GUT MICROBIOME

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Doctor of Philosophy
2017

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Campylobacter jejuni, a major enteric pathogen and a natural resident in the poultry gut, causes gastrointestinal illness followed by severe post-infection complications, including Guillain-Barré syndrome, reactive arthritis, myocarditis, and ulcerative colitis in humans. Risk assessment studies have projected a 30-fold reduction in human campylobacteriosis cases with only a 100-fold reduction in the number of *C. jejuni* colonizing the poultry gut. Current commercial poultry production practices involve use of antibiotic growth promoters (AGP); modulation of gut microbiota with AGPs for food safety and enhanced performance in poultry can be justified until acquisition of antibiotic resistance in zoonoses through inter-bacterial transfer of antibiotic resistance genes (ARGs) in a complex microbial community is considered. As an

alternative, natural phenolics extracted from by-products of berry juice industry, with antimicrobial, anti-inflammatory, anticarcinogenic, antioxidant and vasodilatory activities, demonstrate promising prospects. In this study, we adopted mass-spectrometry, microbiological, phylogenetic, and metagenomic approaches to evaluate bioactive phenolic extracts (BPE) from blueberry (*Vaccinium corymbosum*) and blackberry (*Rubus fruticosus*) pomaces as AGP alternative. We detected that major phenolics in BPE included, but were not limited to, apigenin, catechol, chlorogenic acid, cinnamic acid, coumarin, ellagic acid, eugenols, flavan, gallic acid, gingerol, glucosides, glucuronides, myricetin, phenols, quercetin, quinones, rhamnosides, stilbenol, tannins, triamcinolone, and xanthine. BPE reduced *C. jejuni* growth and motility *in vitro*, resulting in lower adherence and invasiveness to chicken fibroblast cells. Anti-inflammatory effects of BPE significantly reduced the expression of pro-inflammatory cytokine genes in chick macrophage cell line *ex vivo*. Furthermore, BPE reduced the colonization of *C. jejuni* in broiler cecum by 1 to 5 logs while increasing broiler weight by 6% compared to 9.5% with commercial AGPs. Metagenomic analysis of broiler gut indicated that BPE caused an AGP-like pattern in bacterial communities with a comparative increase of Firmicutes and a concomitant reduction of Bacteroidetes in broiler ceca. AGP supplementation clearly caused phage induction and a richer resistome profile in the cecal microbiome compared to BPE. Functional characterization of cecal microbiomes revealed a significant variation in the abundance of genes involved in energy and carbohydrate metabolism. Our findings established a baseline upon which mechanisms of plant based antimicrobial performance-enhancers in regulation of animal growth can be investigated.

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by

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2017

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Preface

This dissertation is original, independent work by the author, Serajus Salaheen under supervision of Dr. Debabrata Biswas.

Dedication

To my parents, who taught me the value of hard-work and sacrifice

Acknowledgements

I like to thank my mentor, Dr. Debabrata Biswas, for his constant support and flawless guidance. I also like to thank my PhD dissertation committee members for their constructive criticism which helped me to be a better researcher.

Finally, a special thanks to friends and family!

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List of Abbreviations

AGP, Antimicrobial Growth Promoter

ARG, Antibiotic Resistance Gene

BPE, Berry Pomace Extract

GAE, Gallic Acid Equivalent

Scholarly Articles

Findings from this dissertation have been published in the following peer-reviewed articles:

1. Salaheen, S., Nguyen, C., Hewes, D., and Biswas, D. (2014). Cheap extraction of antibacterial compounds of berry pomace and their mode of action against the pathogen *Campylobacter jejuni*. Food Control, 46, 174–181.
2. Salaheen, S., Almario, J. A., and Biswas, D. (2014). Inhibition of growth and alteration of host cell interactions of *Pasteurella multocida* with natural byproducts. Poultry Science, 93, 1375–1382.
3. Salaheen, S., Nguyen, C., Mui, C., and Biswas, D. (2015). Bioactive berry juice byproducts as alternative and natural inhibitor for *Salmonella* Gallinarum and *Salmonella* Pullorum. Journal of Applied Poultry Research, 24 (2), 186-197.
4. Salaheen, S., Jaiswal, E., Joo, J., Peng, M., Ho, R., OConnor, D., Aranda-Espinoza, J., and Biswas, D. (2016). Bioactive extracts from berry byproducts on the pathogenicity of *Salmonella* Typhimurium. International Journal of Food Microbiology, 237, 128–135.

Chapter 1: Literature Review

Campylobacteriosis. Campylobacter is one of the major enteric pathogens and is the causative agent of major foodborne bacterial illnesses in the US. *Campylobacter*iosis, an infectious disease of the gastrointestinal tract, is caused by this pathogen. Diarrhea with presence of mucus and blood in stool, abdominal cramp, fever, nausea, vomiting and loss of appetite are the major identifying symptoms of the infection with *Campylobacter*. *Campylobacter* is responsible for 845,025 infections, 8,463 hospitalizations and 76 deaths in the US annually (Scharff, 2012). According to the World Health Organization, approximately 5%-14% of all diarrhoea worldwide is thought to be caused by *Campylobacter*. In 2012, Foodborne Diseases Active Surveillance Network (FoodNet) identified 14.3 *Campylobacter*iosis cases for every 100,000 inhabitants of the twelve states of the US and this rate was 14% higher than the rates found in 2006-2008. This is regarded as the highest rate of *Campylobacter* infections in the century. More importantly, according to the Center for Disease Control and Prevention (CDC), for every laboratory confirmed *Campylobacter*iosis case, 30 more cases go unrecorded and/or unreported in the US. On an average, each laboratory confirmed *Campylobacter*iosis case result in an expanse of 1,846 US Dollars, which make up a total of more than 1.5 billion US Dollars average annual cost in the US (Scharff, 2012). As a result, *Campylobacter* is considered a serious threat to public health as well as national and global economy.

Classification and ecology of Campylobacter. Campylobacter, a microaerophilic, spiral-shaped, Gram-negative bacterium, is a major cause of bacterial gastroenteritis worldwide. *Campylobacter* genus includes 17 species and *C. jejuni* and *C. coli* are the most common isolates and involved in human gastrointestinal infection. *Campylobacter* generally reside in the intestinal mucosa of worm blooded animals. Once they are spilled or excreted from the gut of the host, *Campylobacter* encounter various hostile environmental factors, such as abnormalities in osmolarity, high concentration of atmospheric oxygen, high/low temperature, and insufficient nutrition in the environment. Survival in such hostile situation necessitates infection into another

Kingdom:	Bacteria
Phylum:	Proteobacteria
Class:	Epsilonproteobacteria
Order:	*Campylobacter*ales
Family:	*Campylobacter*aceae
Genus:	*Campylobacter*

host or adoption of stress-response mechanisms. One of the stress response in *Campylobacter* is conversion into Viable but Non-Culturable (VBNC) state where bacterial cells become coccoid instead of spiral shaped. *Campylobacter* can also develop mechanisms for survival in unfavorable environment by expressing SoxRS and OxyR proteins against oxidative stress, BetAB, GbsAB, OtsAB and ProP proteins as osmoprotectants or cold shock protein CspA (Murphy et al. 2006).

Optimum growth temperature for *Campylobacter* is around 42ºC, as a result poultry can serve as a major host of this pathogen due to its relevant body temperature. Elevated temperature help *Campylobacter* for proper gene expression, regulation of energy/nutrient metabolism and further proliferation. As a result, transmission from *Campylobacter* colonized chicks to humans is an important possible risk factor and possibility of which often increases as the number of interaction between them increases. Broiler flocks can be colonized by *Campylobacter* which depends on the age of the birds. The shedding of this pathogen starts about 2-3 weeks after hatch and over time virtually all the birds become contaminated. It has been found that the spread of the infection from a single bird to a flock size of 30,000 birds can happen within 3 days. Once colonized, bacterial load can reach upto 10^7 CFU per g of cecum content (Stern et al., 1995). *Campylobacter* can colonize the whole gut but the colonization of this bacteria is mostly favorable in cecum and colon of poultry though they are also found in the crop and the lower intestine. Intermittent colonization of duodenum, jejunum, ileum, spleen and liver has also been reported.

Genotypic similarity has been found among *Campylobacter* isolates from human and poultry where majority of the birds were found to be colonized with *Campylobacter* (Nadeau et al., 2002). Others report that the colonization of *Campylobacter* is host-specific which limit the occurrence of common serotype among humans, poultry or other animals. There are several factors that are important in transmission of *Campylobacter* from bird to bird, the most important may be physical proximity. However, in some cases, flocks residing at close proximity was also found free from *Campylobacter* contamination which indicated the involvement of factors other than physical proximity in the transmission of *Campylobacter* from bird to bird. Berndtson et al., (1996) reported that *Campylobacter* was not able to transmit from one pen to another if the pens were divided with a physical barrier, which raise the question on the possibility of *Campylobacter* transmission through air. The capability of *Campylobacter* to enter the eggshell under certain conditions has been reported which support the vertical transmission of this pathogen, but there are still valid debates on the vertical transmission. Pests, wild birds and animals are often contributed as

reservoir or vector for *Campylobacter* transmission to *Campylobacter*-free flocks. After establishment of initial colonization, even with a small number of viable or VBNC *Campylobacter* cells in an individual bird, horizontal transmission causes the entire flock to be infected with this pathogen at a very short period of time. Initial colonization of specific subtype of *Campylobacter* differ from the predominant subtypes prevalent at the time of slaughter which indicates further transmission of new subtypes from the environment on the later stage of poultry lifecycle (Bull et al., 2006). The reason behind the migration from one dominant subtype or species to another is still unknown, but may result from seasonal variations, environmental alterations or later subtype being genetically more favorable to replace the older subtype.

Campylobacter can also reside in the intestinal tract of production animals, like cattle, pig, sheep and goats. Like poultry, *C. jejuni* is the major species recovered in the cattle (Bae et al., 2005; Harvey et al., 2005; Inglis et al., 2004) although it has been reported that the prevalence of *C. coli* was nearly as high as *C. jejuni* (20% *C. coli*, 23.8% *C. jejuni*) in operations that rear calves (Bae et al., 2005). The presence of *Campylobacter* in cattle rumen is not supported by many investigators, who suggest their presence is more likely due to recent ingestion rather that active infection with the bacteria. Other organs of the cattle, such as, gallbladder, bile and mucosal tissue have been reported to harbor *Campylobacter*. Unlike poultry, *C. coli* is more prevalent in swine and in some studies, *C. coli* was isolated in 99% cases. Jensen et al., (2013) found severe fluctuations in the number of *C. jejuni* or *C. coli* colonized in the intestine of swine though *C. coli* was always more prevalent than *C. jejuni*. Infection of various hosts with various species implies the requirement for certain condition for the survivability of specific species of *Campylobacter*. The use of synthetic antibiotics also alter the composition of *Campylobacter* species. Swine grown without antibiotic was shown to contain 50% more *C. coli* compared to the farms where antibiotic were not used. *C. jejuni* was found to be more resistant to environmental stresses such as chlorinated water treatment or chilling. Thereby these inhibiting stresses might reduce the level of *C. coli* than *C. jejuni*.

Wild birds and animals can also be colonized with *Campylobacter* at a catastrophic level. *Campylobacter* serotypes from the wildlife have been shown to be similar to the human clinical isolates. As a consequence, wild birds, pests, flies, and other small wildlife animals have been considered as possible vectors for *Campylobacter* transmission to farm animals (Hald et al., 2004; Meerburg et al., 2006). Although the survivability of *Campylobacter* on fresh produce is not well

documented, several outbreaks of *Campylobacter*iosis associated with raw vegetables indicate the survival ability and transmission of *Campylobacter* through produce (Harris et al. 2003; Jacobs-Rietsma 2000; Mandrell and Brandl 2004). However, the pathogen showed susceptibility to air-drying on abiotic surfaces (Kusumaningrum et al., 2003), UVB exposure (Obiri-Danso et al., 2001), hydrostatic pressure (Solomon and Hoover, 2004), and acidic pH (Waterman and Small 1998). It was found that *Campylobacter* could not survive or converted to VBNC state in absence of sufficient water activity on produce, hence contamination of produces with animal feces might be the reason of *Campylobacter* on produce surface. *Campylobacter* was also isolated from both free flowing environmental streams as well as stagnant water (Hörman et al., 2004), so water may also be linked to the transmission of *Campylobacter* in wild and domesticated animals.

Campylobacter in food and environment. Products including raw and undercooked poultry meat, unpasteurized milk and some other food products were found to harbor *Campylobacter*. Due to cross contamination during evisceration process, *Campylobacter* primarily transmits to the skin of infected birds from contaminated equipment, gut and cecum content at the poultry processing plants (Berrang et al., 2001). *Campylobacter* spp. occupies a liquid film on the carcass and are entrapped in the channels and crevices of skin (Chantarapanont et al., 2003) which provides a favorable microenvironment for survival and growth. This suitable microenvironment foster the growth and survival of *Campylobacter* (Chantarapanont et al., 2003) and *Campylobacter* spp. are capable to persist in the carcass even under freezing temperature or refrigerated storage condition. Contamination in muscle tissue has also been reported during storage in retail shops. It was found that almost half of all packaged chicken legs were contaminated on the skin alone, less than 1% of samples were positive within the muscle alone in retailers (Scherer et al., 2006). The prevalence of *Campylobacter* in meat samples were 87.5%, 71.43% and 33.33% in farmers markets, organic and retail supermarkets in Maryland and the DC metropolitan area, respectively (Salaheen et al., unpublished data). Scheinberg et al. (2013) reported that 90%, 28% and 52% of chicken meat samples collected from farmers market, organic and non-organic sources, respectively, were positive for *Campylobacter* in Pennsylvania. The prevalence of *Campylobacter* in the poultry meats from organic and conventional retail supermarkets ranges from 43% to 89% in the US (Smith et al. 1999; Zhao et al. 2001; Cui et al. 2005; Price et al. 2005; Luangtongkum et al. 2006; Price et al. 2007; Han et al. 2009). Cui et al. (2005) reported 76% and 74% contamination rate in

organic and conventional retail meats, respectively in Maryland. At similar period, Price et al. (2007) reported 67 to 97% contamination rate in Maryland. So, it is evident that post-harvest poultry products are highly contaminated with *Campylobacter*.

Infection, complications and post infection sequelae. The infection and virulence strategies exploited by *Campylobacter* is still far from being elucidated. Genomics, proteomics, and metabolomics tactics show high inter- and intra-strain variation in *Campylobacter*. This diverse nature facilitates adaptive mechanism by which *Campylobacter* survive in various environmental conditions and interacts with the mucus layer of the gut. Studies suggest that *Campylobacter* starts the infection by actively penetrating the intestinal mucosa layer followed by discharging toxin (cytolethal distending toxin, *cdt* A, B and C) or proteins (*Campylobacter* invasion antihgen, *cia*) via flagellar apparatus which serve as Type III secretion system in this bacteria. Being modulated by the proteins, epithelial cells engulf *Campylobacter* which disrupts the integrity of the epithelial lining. Pro-inflammatory cytokines, chemokines and effector molecules of the innate immunity are highly induced when *Campylobacter* antigens are presented by the antigen presenting cells. Complications related to *Campylobacter* infections arise at this stage which include pancreatitis, cholecystitis, peritonitis, and substantial gastrointestinal hemorrhage. Transient bacteremia arises occasionally in immunocompetent patients. Though, serious systemic illness caused by *Campylobacter* can lead to sepsis and death, which is rarely reported. Rather than food poisoning and local *Campylobacter* enteritis, post infection sequelae of *Campylobacter*iosis, such as Guillain-Barre´ syndrome, rewactive arthritis, cardiac problem, and uncreative colitis are more important in terms of health hazard and economic burden.

The most important post-*Campylobacter*iosis complication is the Guillain-Barre´ syndrome (GBS). GBS is considered as an acute demyelinating disease of the peripheral nervous system that result in < 1 patient per 1000 cases affecting 1-2 persons per 100,000 people annually in the US. It has been speculated that the risk of developing GBS is amplified after infection with certain *Campylobacter* serotypes, for example, in the US, Penner type O:19 is commonly linked to GBS development.

Reactive arthritis associated with *Campylobacter* infection and prevalence varies extensively due to lack of diagnostic criteria, case ascertainment differences, exposure differences,

and genetics and ages of exposed patients (Pope et al., 2007). Five percent of *Campylobacter*-associated reactive arthritis were found to be chronic or revert with musculoskeletal symptoms.

Incidences of *C. jejuni*-associated myocarditis and myopericarditis have been reported, all of which involved immunocompetent hosts (Uzoigwe, 2005). Typical clinical symptoms involved transitory acute pain in chest, with concomitant electrocardiogram variations and increased secretion of cardiac enzymes, in association with antecedent or coincident enteritis.

Ulcerative colitis (UC) is a chronic inflammatory condition of the large intestine of human beings. It is characterized by the presence of bloody diarrhea and severe abdominal cramp and abnormal immune functions, defect in intestinal epithelial cell barrier function, and gut microbiota (Campieri and Gionchetti 2001, Sasaki et al 2012). UC is also considered to be a pre-condition in colorectal cancer (CC) (Rhodes and Campbell 2002). *Campylobacter* spp., and Entero Haemorrhagic *E. coli* (EHEC) were predominant in tissue sample collected from patients with UC, but not in tissues from healthy individuals (Gradel et al 2009; Ternhag et al 2008).

Sub-therapeutic use of antibiotics in farm animal production and its consequences. Though antibiotics have served significantly to make foods safer, they present certain limitations which have led many people to rethink on their application in animal feed. There are many direct negative impacts as well as indirect effects of these antimicrobials towards life in general. Since the mid-20th century, when the application of antibiotics against bacterial and fungal diseases was first introduced, it has been considered as the single most important medical event in human history. It has reduced morbidity and mortality in human and animal to a dramatic level. Since then, the use of antibiotics accounts for several million tons worldwide both for medication and in farm animal production (Andersson and Hughes, 2010). The intensive use of antibiotics caused a huge influence in the frequency of resistance among bacterial pathogens including *Campylobacter*. Enhanced rate of microbial resistance could be reduced or minimized if we could use the antibiotics effectively and properly in treatment of human diseases and farm animals. As the antibiotic resistant microorganisms are more virulent and aggressive in respect to disease occurrence, this may increase the risk of complicated situations and fatality, escalate the economic burden on public health and may eventually introduce a dreadful post-antibiotic era.

Since the 1950s, large amounts of antibiotics have been used in agricultural animal production in the US as growth promoters because of their effect on animal growth acceleration

and ability to enhance feed conversion efficiency. Flavophospholipol and virginiamycin are generally used in the US for poultry production (Peter and John, 2004). B-lactam antibiotics (e.g., penicillin, lincosamide), macrolids (e.g., erythromycin, tetracycline) are used mainly in pigs (Peter H. and John H., 2004). Some other antimicrobials, such as arsenical compounds, bacitracin, flavophospholipol, pleuromutilins, quinoxalines and virginiamycin, are also used in pig production. In the cattle industry flavophospholipol, monensin, and virginiamycin are generally used as growth promoter because these compounds play important roles in muscle formation and increase milk productivity (Peter H. and John H., 2004). Some antibiotic candidates approved by FDA for pre-harvest use in cattle, swine and poultry are listed in Table 1.1.

Pre-harvest use of antibiotic is common in farm animal production but instead of specific targeted pathogens, antibiotics are used for diverse groups of pathogens. As a result, broad-spectrum antibiotics are used in pre-harvest level. Recent studies suggest that some antibiotic treatment can disrupt the dynamics of gut flora and therefore impair animal health and productivity, and even food safety (Crosswell et al., 2009). Aarestrup et al., (1998) carried out an intensive research on the manifestation of acquired resistance to antimicrobials which were used for animal growth promotion among bacteria that are generally isolated from poultry, cattle and swine in Denmark. They used three groups of bacteria, such as, indicator bacteria (*E. coli*, *Enterococcus faecalis*, *Enterococcus faecium*), 2) zoonotic bacteria (*Campylobacter*, *Salmonella*, *Yersinia enterocolitica*), and 3) animal pathogens (*E. coli*, *Staphylococcus aureus*, coagulase-negative staphylococci (CNS), *Staphylococcus hyicus*, *Actinobacillus pleuropneumoniae*). Antimicrobials that were generally used as growth promoters in Denmark and some structurally related therapeutic agents were included in the study. Those included: avilamycin, avoparcin (vancomycin), bacitracin, carbadox, flavomycin, monensin, olaquindox, salinomycin, spiramycin (erythromycin, lincomycin), tylosin, and virginiamycin (pristinamycin). The research group reported acquired resistance to all of the tested antibiotics. A more frequent incidence of resistance were found against avilamycin, avoparcin, bacitracin, flavomycin, spiramycin, tylosin and virginiamycin, whereas resistance to carbadox, monensin, olaquindox and salinomycin was less frequent. For these consequences, the European Union banned the use of antibiotics for farm animal production as growth promoters (avoparcin in 1997 and bacitracin, spiramycin, tylosin and virginiamycin in 1999). In the US, certain use of cephalosporin in production animals were restricted by the U.S. Food and Drug Administration (FDA). FDA is considering to follow the

footstep of the EU to ban non-therapeutic use of antibiotics in food animal production. As a result, alternative growth promoters and antimicrobial agents are required more than ever.

Alternative approaches for prophylaxis and animal growth promotion. To combat the current situation regarding bacterial contamination, antibiotic resistance and animal growth promotion, natural phenolic compounds can play important roles to reduce pre-harvest colonization of zoonotic bacterial pathogen, specifically *Campylobacter* from animal (poultry) gut. Polyphenols, a widely available groups of compounds naturally occurring in plants, are an integral part of the human and animal diet. Chemically, polyphenols are characterized by hydroxylated phenyl moieties. In fruits and vegetables, they are typically occurred in their glycosylated forms, though alterations such as polymerization or methylation are frequently observed. Polyphenols have gained interest in the last decades for having possible health-promoting effects such as antimicrobial, anti-inflammatory, anti-estrogenic, cardio-protective, chemo-protective and neuro-protective properties (Steinberg et al., 2003; Selma et al., 2009) as well as antioxidant and pro-oxidant activities both *in vitro* and in animal models (Halliwell et al., 2007).

Polyphenols are prevalent in many fruits and vegetables. For example, they are the main components of pomegranate. Clinical research reported that it takes up to 56 hours for the phenolics, mostly punicalagins, to leave the colon, because they are absorbed very slow and not completely (Seeram et al., 2006). During this time, it is proved that pomegranate tannins inhibit a large number of pathogens in that section of the gut without affecting most beneficial bacteria (Bialonska et al., 2009). Pomegranate constituents seem to disturb both Gram-positive and Gram-negative intestinal pathogenic bacteria. According to previously published reports (Bialonska et al., 2009), ellagic acid inhibited the growth *Clostridium clostridioforme, C. perfringens,* and *C. ramosum*. Ellagic acid together with punicalagins was effective against the growth of *Bacteroids fragilis* and *S. aureus*. The effect of pomegranate polyphenols on bifidobacteria was species-specific. The number of *Bifidobacterium. breve* and *B. infantis* increases in the presence of the pomegranate ellagitannins which indicated that pomegranate products may help regulate pathogens without adverse effects on beneficial bacteria (Howell et al., 2013).

In red raspberries the main phenolic compounds are ellagitannins, followed by flavonoid and anthocyanins. Antimicrobial activity of tannins against microorganisms is well documented. Phenolic compounds, including ellagitannins, anthocyanin, and flavonols (Nohynek et al., 2006)

showed similar selective bactericidal effects on both Gram-positive and Gram-negative bacteria. Raspberries are effective inhibitors of *Staphylococcus* and *Salmonella* Typhimurium (Puupponen-Pimia et al., 2005). Other types of bacteria being affected by the phenolic compounds in raspberries include *E. coli* (Nohynek et al., 2006) and *Salmonella enterica* (Puupponen-Pimia et al. 2001). The growth of *Lactobacillus* was not inhibited by any of the raspberry extracts at low concentrations. However, in high concentrations, the growth of the probiotic was clearly disrupted (Puupponen-Pimia et al. 2005).

The composition of the cranberry polyphenols has been widely documented as a combination of flavonoids and phenolic acids like catechin, myricetin, and benzoic acid, the latter of which is the most prominent one in a freshly squeezed sample of juice (Chen et al., 2001). Cranberries are also rich in a phenolic compound called proanthocyanidins. Proanthocyanidins have been cataloged as the ones responsible for the cranberry's great ability to disrupt bacterial adherence to cultured human cells (O'May and Tufenkji, 2011). Howell et al., (2005), concluded that the proanthocyanidins in cranberry juice can prevent the adhesion of *E. coli* to the urinary tract thus preventing urinary tract infections. Furthermore, biofilm formation in uroepithelial cells can also be reduced by ingesting cranberry juice (Reid et al., 2001). The antimicrobial effects of cranberry juice have been recorded several times. Historically, women have been told to drink the juice in order to prevent and even cure urinary tract infections. In the early 1990s, researchers found that the monosaccharide fructose present in cranberry and blueberry juices competitively inhibited the adsorption of pathogenic *E. coli* to urinary tract epithelial cells, acting as an analogue for mannose (Zafriri et al, 1989). Different research studies have proven the antimicrobial properties of cranberries against both Gram-positive and Gram-negative bacteria. Cranberry extract has been demonstrated being effective towards *Bacillus cereus*, *Clostridium perfringens*, and *Staphylococcus epidermis*; lyophilized cranberry had a bacterocidic impact on *Salmonella* Typhymurium, *Staphylococcus aureus* and *Listeria monocytogenes* (Puupponen-Pimia et al. 2005). Also, coumaric acid, another phenolic acid present in cranberry juice, has confirmed efficacy on *Lactobacillus plantarum* (Nualkaekul and Charalampopoulos, 2011). In a separate study, proanthocyanidins were proved to be effective in blocking part of the motile system of *Pseudomonas aeruginosa* (O'May and Tufenkji, 2011). This study showed that the growth of *P. aeruginosa* was not inhibited, however, the characteristic migrating branching pattern of the

bacteria was disrupted, which indicated a disruption in swarming motility in the presence of cranberry phenolics.

Phytochemical additives from plant sources are potent dietary supplement for animals including poultry. Some of these compounds stimulate appetite (e.g. menthol), provide antioxidant protection (e.g. anthocyanin, cinnamaldehyde) or reduce harmful microbial growth. Plant essential oils induce endogenous digestive enzymes in the intestinal tract. Oregano essential oils were shown to act as growth promoter in poultry though the exact mechanism could not be explained. However, carvacrol from oregano extract shows antimicrobial effect, its essential oils can modulate the gut microflora and reduce bacterial load by suppressing the proliferation of bacteria. Phenolic extracts from plant origin stimulate gastric juice secretion which improves digestive power in animals. Some polyphenols are also purported to function by raising blood circulation rate, which leads to faster detoxification in animal body that were generated from metabolic pathways. Flavonoids are important plant polyphenols which maintain the shape of small blood vessels and connective tissue.

Natural bioactive extracts from berry pomace. Bioactive phytochemicals from berries, especially blackberry (*Rubus fruticosus*) and blueberry (*Vaccinium corymbosum*) pomace as feed or water supplement to reduce pre-harvest level of *Campylobacter* colonization in poultry, might be a feasible alternative because extraction of bioactive phytochemicals from fruit juice is too expensive for large scale application in the production animals specifically poultry. Pomaces, the by-products of fruit juice industry, are the solid or semisolid remains of fruits after separating out the juice or oily portion. It consists of skins, stem, seeds and small amount of pulp of the fruit. Berry juice and fruit processing industries produce a significant amount of pomaces. Pomace generally accounts for as much as 20-30% of the weight of the whole fruit. According to National Agricultural Statistics Service (2009), a significant amount of pomaces are produced in the US annually which result in disposal problem as they cannot be used as animal feed for having low protein content and acidic pH. As a result, an alternate use of these byproducts will be highly appreciated. Cultivation of berries is season dependent but they are stored by the juice factories to have a year round supply of fruits confirming a year round supply of pomaces. So these byproducts can be used to extract polyphenolic compounds, which have been demonstrated to have numerous health benefits including anti-carcinogenic, anti-atherosclerotic, anti-adhesive, anti-inflammatory,

anti-allergic, anti-hypertensive, anti-arthritic and anti-microbial properties (Boivin et al., 2007). They also inhibit lipid peroxidation, act as free radical scavengers and metal ion chelators. Moreover, these phenolics are concentrated in the outer layers of fruit (e.g. skin) as their biosynthesis is dependent on UV-light (sunlight). So, cheap extraction of the phenolics from berry pomace would provide a solution to both the fruit industry and the poultry industry.

Composition of berry pomace. The major bioactive components present in pomaces (a list of phytochemicals present in pomaces and essential oils are summarized in Table 1.2) are polyphenols, phytosterols, tocopherols (mostly as α- tocopherol), fibre, protein and biotic (Puupponen- Pimiä et al., 2001; Puupponen- Pimiä et al., 2005; Puupponen- Pimiä et al., 2008). There are different classes of polyphenols and each class has unique characteristics which separate one from other. Phenolic compounds are one of the most diverse secondary metabolites found berry pomace. Major components of the polyphenols are anthocyanins (cyanidine-3-galactoside and cyanidine-3-glucoside), procyanidins and hydroxycinnamic (α-Cyano-4-hydroxycinnamic acid). Procyanidins, present in the pomace, is a class of polymeric polyphenolics and it contains catechin or epicatechin at polymerized form (Djilas et al., 2009). Cyanidin-3-glucoside and cyanidin-3-galactoside are particular types of anthocyanin pigments found in many berries including blackberry and blueberry (Sasaki et al., 2007). The highest concentrations of cyanidin are found in the skin and seed of these fruits because their biosynthesis are stimulated by sunlight (ultraviolet light), so higher concentrations are found in the most outer layer (skin) of the fruit. Different linkages between catechin and epicatechin units are found in procyanidins, the most common being β-type linkages. Oligomeric procyanidins demonstrated greater antimicrobial activity even at lower concentrations than catechin and epicatechin though the degree of polymerization on antimicrobial activity still remains unclear.

Antibacterial properties of berry phenolics. Bioactive compounds extracted from pomaces (especially berry fruits) have been shown to inhibit different foodborne pathogens as well as food-spoiling microorganisms. According to Puupponen-Pimiä et al. (2001), berry extracts inhibited the growth of *Salmonella, Escherichia, Staphylococcus* but not *Lactobacillus* and *Listeria* species. Cavanagh et al. (2003) showed inhibitory effect of several berry extracts on the growth of wide range of Gram-positive and Gram-negative human pathogenic bacteria. Biswas et al. (2012) found

blueberry extract has negative impact on the growth of *Salmonella, Escherichia, Campylobacter* and *Listeria* whereas stimulates the growth of probiotic *Lactobacillus*. In other study, human intestinal bacteria such as *Bacteroids fragilis, Clostridium perfringens, E. coli* and *Salmonella* Typhimurium were found to be inhibited by tannins while *Bifidobacterium infantis* and *Lactobacillus acidophilus* were unaffected by this group of phenolics (Chung et al., 1998). Inhibitory effect of phenolic compounds such as flavonoids, against Multi-Drug Resistant (MDR) bacteria and methicillin-resistant *Staphylococcus aureus* has been documented (Belofsky et al., 2004). Roccaro et al., (2004) found that catechin can increase tetracyclin activity against tetracyclin-resistant staphylococcal isolates. Pomace essential oils also have been documented to have effect against various microorganisms. Muthiyan et al. (2012) found bioactive essential oils can be used as anti-Staphylococcal agent. Essential oils and terpineol were also found to extend the shelf life of milk to more than 56 days when stored at 4˚C. According to Kim et al. (1995), carvacrol, citral and geraniol had strong activity against *Salmonella* Typhimurim and its rifampicin resistant mutant *in vitro* but nerolidol, limonene or β-ionone had no effect on similar kind of bacteria suggesting different compounds have different target microorganism as well as different mechanism of action.

Several mechanism of action of berry pomace extracts have been proposed which include cytoplasmic membrane destabilization, permeabilisation of plasma membrane, extracellular microbial enzyme inactivation, direct effect on microbial metabolism, deprivation of substrates mandatory for microbial growth (Puupponen- Pimiä et al., 2004). These extracts may also play role in alteration of host epithelial cell-pathogenic bacteria interactions which is a prerequisite for colonization and infection of many pathogenic bacteria. Clifford et al. (2004) documented that dietary phenolics are poorly absorbed in the small intestine and 90-95% accumulate in colon which possess the capability to alter host cell-bacterial interactions. Cytoplasmic membrane, a semipermeable membrane in microbes is a phospholipid bilayer which contain embedded proteins (Millard, 2002) that regulate the movement of solutes and different metabolites in and out of the cell. Phenolics penetrate cytoplasmic membrane and interact with cellular proteins. Concentration dependent mechanism is also proposed from several researches. Cellular enzyme activity is affected at lower concentration of phenolics whereas higher concentration may cause protein denaturation. Walsh et al. (2003) reported membrane damage as a mode of action of phenolic essential oil components. They may interfere with electron transport, nutrient uptake, and nucleic

acid synthesis. Carvacrol acts on cytoplasmic membrane, acts as a proton exchanger and hampers the pH gradient across the cytoplasmic membrane, disrupt proton motive force and deplete intracellular ATP resulting in cellular death (Ultee et al., 2002). Hydroxycinnamic acids, due to having less polar side chain, can penetrate inside the cell very easily (Campos et al., 2003). Tannins inhibit oxidative phosphorylation by disrupting extracellular microbial enzymes or eliminating substrates for enzymatic reaction which affect microbial metabolism (Scalbert 1991). Tannins can also cause complexation of metal ions required for bacterial growth. The effect of phenolics on MDR bacteria is due to its ability to impair efflux pumps, which bacteria use to get rid of antibiotics and that cause an increase in the drug retention time inside bacterial cells. Yoda et al. (2004) concluded that green tea polyphenol, EGCG directly bind to cell wall components and causes inhibition whereas Zhao et al. (2001) showed EGCG and β-lactams attach to the same site which is peptidoglycan of bacterial cell wall and confer synergistic relationship to β-lactams. EGCG have also been shown to interfere with the transfer of conjugative R plasmid in *E. coli* which is responsible for the conjugative plasmid mediated antibiotic resistance in bacteria. Most of the acidic components of pomace extracts show similar mode of action, the pKa of most phenolic acids ranges between pH 3 and pH 5, due to the higher pH of cytoplasmic membrane compared to surrounding medium, these compounds dissociates and release proton which acidifies cytoplasm (Cotter and Hill 2003). Anionic portion of these acidic compounds which cannot escape accumulate within bacterial cell and impair metabolic function such as increase osmotic pressure which causes incompatibility to bacterial cell. There are other hypothesized modes of actions which need to be evaluated.

Anti-inflammatory properties of berry phenolics on host and potential as alternative growth promoter in production animals. In vitro and *in vivo* studies in animal model have found polyphenolic compounds to alter the animal immunity and mainly serve by exerting anti-inflammatory effect. Polyphenols can modify and/or alter various biochemical pathways related to signal transduction in eukaryotic cells which elicit their beneficial properties. These alterations include, modulation of pro-inflammatory cytokine gene expression such as interleukin-6, interleukin-8, cyclooxygenase, nitric oxide synthases, lipoxygenase, and several non-specific cytokines. These modulations are generally carried out by several alterations in mitogen-activated protein kinase signaling and nuclear factor-kappa B (Santangelo et al., 2007). Crouvezier et al.

(2001) reported that polyphenols, such as epicatechin gallate, epigallocatechin and epigallocatechin gallate decreased the production of pro-inflammatory cytokine interleukin-1β and enhanced the production of anti-inflammatory cytokine interleukin-10, but had no effect on the production of interleukin-6 or tumor necrosis factor. Deng et al., (2010) carried out a study on the immunomodulatory potential of polyphenolics in piglet model. Their study concluded that polyphenols stimulated the activation and proliferation of T lymphocytes. The ratio of CD4+/CD8+ cells was also raised, which indicated a recovering mechanism against oxidative stress mediated immune damages. The attenuation of pro-inflammatory cytokine interleukin-1 caused by oxidative stress was observed, and the amount of serum interferon-γ was depleted by polyphenolic supplemented feed. However, the serum concentrations of interleukin-4, a prominent anti-inflammatory cytokine, were significantly enhanced suggesting an immune shift from Th1 to Th2. A more recent study showed that phenolic extracts reduced the morbidity and inflammation induced by Avian Pathogenic *E. coli* (APEC) (Zhong et al., 2014). APEC causes colibacillosis which results in inflammation in multiple organs of chickens, and results in serious economic loss to the poultry industry. Quantitative real-time polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA) results showed that the phenolic extracts reversed the induction of the Toll-like receptor (TLR) 2, 4, and 5, genes and down-regulated nuclear factor-kappa B signal transduction pathway activation, and inhibited the production of proinflammatory cytokines.

Anti-inflammatory properties can be attributed with growth promotion in animals, especially in poultry. From the last five decades, sub-therapeutic concentrations of antibiotics have been used to grow production animals for growth promotion. Several mechanisms have been proposed, none of which is beyond controversy. In earlier hypotheses, the growth promoting effects of antibiotic growth promoters (AGP) were linked with only their antimicrobial properties which seems unlikely because sub therapeutic concentrations of AGPs possess limited growth inhibitive effect on microbes, and AGPs are still in effect despite the wide-spread development of resistance among pathogens (Cox and Popken, 2010). Furthermore, down regulation of intestinal mucosal immune responses was observed in a rodent model after sub-therapeutic use of tetracycline (Costa et al., 2011). As a result, anti-inflammatory properties of AGPs are more linked to animal growth promotion. Production animals can grow to their complete hereditary potential in the absence of immunological challenges. Inflammation is associated with lower appetite, and

muscle catabolism and as a result Metabolic Inflammation (MI), i.e., local intestinal inflammation, is more important for production animals. Non-symptomatic infections and MI in production animals can be drastic which lead to reduced weight gain. Supplementation of anti-inflammatory compounds in feed may act as a remedy in such a situation and logically, polyphenol rich berry pomace extracts possess high potential to be an alternative to AGPs.

Bioavailability of berry phenolics in host gut through gut microbial metabolism. The animal colon and gut serves as reservoir for an extremely compound microbial ecosystem, at concentrations of $>10^3$ microbes per gram of gut content, while the microbiome surpasses the human genome by 100-fold, in terms of genetic diversity (Donohoe et al., 2011). The composition and diversity of gut microbiota diverges greatly from species to species, and even within species; so the composition of gut microflora is unique to an individual host and generally modulated by farming practices, diet, environmental conditions, healthy or diseased status, genotype, and so on (Turnbaugh et al., 2008; Zoetendal et al., 2006).

Depending on the degree of polymerization and the glycosylation pattern, a significant portion of dietary polyphenols can remain in the colon. These polyphenols are exposed to the colonic microbial community where microbial co-metabolism of polyphenols are carried out with a common pattern in which the tremendously varied collection of natural polyphenols are funneled to a relatively smaller group of metabolites. The microbial community of the gut possess various mechanisms to carry out O- and C-deglycosilation, amide and ester hydrolysis, and deglucuronidation of large flavonoids and also fermentation of the flavonoid structure. There are ample examples for the bioconversion of active nutritional composites into metabolites of higher bioavailability. For example, isoflavones from soy or hop prenyl-flavonoids are modulated by colonic microbial population for the pseudoestrogenic activity (Possemiers et al., 2006; Rowland et al, 1999). *In vivo* study on rat model with red wine powder detected microbial metabolites excretion through urine. The bioactive metabolites included hydroxybenzoic, hippuric, vanillic, phenylacetic, p-coumaric, phenylpropionic, caffeic, ferulic acids and catechins, though a majority of the above metabolites were absent in the wine powder that was used for feed supplementation in rats (Gonthier et al., 2003). Aura et al., (2005) showed that deglycosylation and breakdown of the anthocyanidin (a major plant phenolic) heterocycle were the major part of microbial degradation. They found that most of the anthocyanins were degraded just after 1 h of treatment

with 5% fecal inoculum. Later studies found that inoculums from human and animals deglycosylated and hydrolyzed anthocyanins into a wide array of metabolites including 4-hydroxybenzoic acid, syringic acid, vanillic acid, protocatechuic acid, and phloroglucinol aldehyde, where initial anthocyanins were completely disappeared from the *in vitro* system (Keppler et al., 2005; Fleschhut et al., 2006). These phenolic acids were derived from the β ring of the respective anthocyanidins, while α rings were broken down to aldehyde derivatives (Morand et al., 2004). In addition to the deglycosylation by microbial enzymes, the microflora are capable of mild transformation such as dihydroxylation, demethylation, and catabolism of polyphenols into simpler derivatives (Cermak et al., 2006). Specialists suggest that these derivatives, rather than their original precursors, may be responsible for the beneficial effects of polyphenols, since these derivatives are more easily absorbed and resilient to additional metabolism.

So far, a very limited number of bacterial species have been identified in the laboratory to be capable of metabolizing polyphenols. The majority of the bacteria belong to the Clostridia group, lactic acid bacteria especially *Lactobacillus*, *Bifidobacterium* and *Enterococcus* spp. The enzymes of *Lactobacillus plantarum* involved in polyphenol metabolism include benzyl alcohol dehydrogenase, reductase, decarboxylase, tanase, β-glucosidase, phenolic acid decarboxylase and esterase (Rodríguez et al. 2009). Probiotic fermentation of phenolic extracts with *Lactobacillus helveticus* was used to mobilize phenolics and improve biological functionality by maintaining a constant phytochemical profile and as a result α-glucosidase inhibitory activity and *Helicobacter pylori* inhibitory potentials were increased (Ankolekar, 2013). Glycosylated flavanone, hesperidin derived from microbial metabolism of polyohenols showed antibacterial activity against *Aeromonas hydrophila*; a study on murin model revealed that hesperidin inhibited bacterial colonization and significantly increased anti-LPS IgM levels and reduced anti-LPS and anti-ECP IgA levels (Abuelsaad et al., 2013). Hidalgo et al., (2012) reported growth stimulation in *Bifidobacterium*, *Lactobacillus* and *Enterococcus* spp in presence of anthocyanin while forming gallic, syringic and p-coumaric acids. These findings indicate that polyphenols and their bioactive metabolites exert a positive modulation of the colonic microbial population.

Synergistic relationship between traditional antibiotics to natural phenolics and possibility of resistance development in Campylobacter against phenolic extracts. In vitro synergistic interaction of crude phenolic extracts from *Acorus calamus* (rhizome) *Hemidesmus indicus,*

Holarrhena antidysenterica, *Plumbago zeylanica*, *Camellia sinensis*, *Lawsonia inermis*, *Punica granatum*, *Terminalia chebula* and *Terminalia belerica* were detected with tetracycline, chloramphenicol, ciprofloxacin, cefuroxime and ceftidizime (Aqil et al., 2005; Aqil et al., 2006). Antibiotic-phenolics combination studies have demonstrated synergistic relationship between streptomycin and phenolics including ferulic acid, gallic acid, allylisothiocyanate chlorogenic acid, and 2-phenylethylisothiocyanate against the Gram-negative bacteria (Saavedra et al., 2010). Bakar et al., (2012) reported synergistic relationship between flavanone hesperidin aglycon extracted from citrus fruits with vancomycin and oxacillin to inhibit vancomycin-intermediate *Staphylococcus aureus* (VISA) and *Helicobacter pylori*. Liu et al., (2009) found that kaempferol glycosides isolated from *Laurus nobilis*, kaempferol-3-O-alpha-L-(2",4"-di-E-p-coumaroyl)-rhamnoside and kaempferol-3-O-alpha-L-(2"-E-p-coumaroyl-4"-Z-p-coumaroyl)-rhamnoside, greatly potentiated anti-Methicillin Resistant *Staphylococcus aureus* (MRSA) activity of fluoroquinolones. They also found that the synergism was more prominent with hydrophilic fluoroquinolones, for example, norfloxacin and ciprofloxacin, but not with hydrophobic quinolones. These findings suggest potential role of polyphenols in rescuing traditional antibiotics against which bacterial resistance already have been developed.

However, here comes the burning question, "what if bacteria becomes resistant to natural phenolics." There are no reports, so far, on the resistance of bacterial pathogens including *Campylobacter* against natural phenolics. Crude or purified plant phenolic extracts and their microbial metabolites make up an enormous array of diversified bioactive compounds, which come in contact with bacterial pathogens. These compounds attack the pathogens with varied mechanistic approach from different directions. Moreover, plant polyphenols have been a part of human and animal diet from pre-historic era and so, pathogenic bacterial resistance would have been reported earlier if the phenomenon was supported by the Mother Nature. Yet, experiments need to be carried out to investigate the hypothesis.

Overall Hypothesis and Specific Aims

Natural bioactive components extracted from blackberry and blueberry pomaces can persistently reduce the colonization of *Campylobacter* in poultry gut while promoting gut health and poultry productivity.

To investigate the hypothesis, the following aims need to be fulfilled,

Aim 1: Extraction, composite analysis, and roles of berry pomace extracts on the growth and pathogenicity of *Campylobacter jejuni*.

Aim 2: Determine the effects of berry pomace extracts on poultry pathogens of commercial importance and probiotics *in vitro* and cultured host cells *ex vivo*.

Aim 3: Role of berry pomace extracts on *Campylobacter jejuni* colonization in poultry gut using day-old chick model.

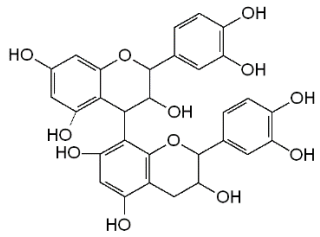
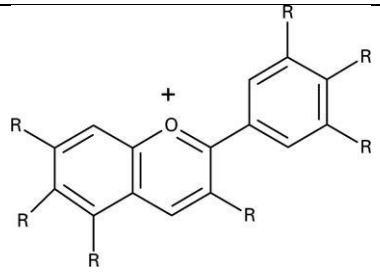
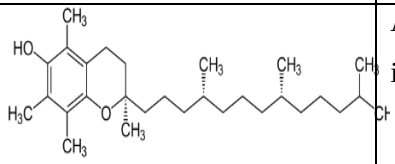
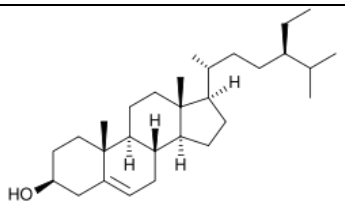
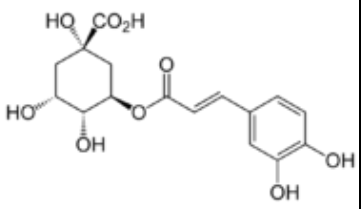
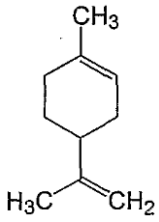
Aim 4: Berry pomace extracts on poultry growth promotion through modulation of gut microbiome and long term consequences.

List of Figures and Tables

Table 1.1. FDA-approved antibiotics for pre-harvest subtherapeutic use in cattle, swine and poultry.

Antibiotics	Cattle	Swine	Poultry
Apramycin		+	
Arsanilic acid		+	+
Avilamycin			+
Avoparcin			+
Bacitracin zinc	+	+	+
Bambermycins	+	+	+
Carbadox		+	
Chlortetracycline	+	+	+
Laidlomycin	+		
Lasalocid	+		
Lincomycin		+	+
Monensin	+	+	
Oxytetracycline	+	+	+
Penicillin		+	+
Roxarsone		+	+
Spiramycin			+
Tilmicosin		+	
Tylosin	+	+	+
Virginiamycin	+	+	+

Table 1.2: Bioactive phytochemicals present in pomaces and their biological roles

Compound	Generalized chemical Structure	Targeted microorganism and mechanism of action	Ref.
Procyanidin		Gram-positive and Gram-negative bacteria	Mayer et al., 2008
Anthocyanin		Gram-positive bacteria and few Gram-negative bacteria Membrane damage and interaction with intracellular materials	Cisowska et al., 2011
A-Tocopherol		Antioxidant, anti-inflammatory	Singh et al., 2004
Phytosterol		*Staphylococcus, Streptococcus, E. coli, Pseudomonas, Klebsiella*	Sharma 1992
Cholinergenic acid		*Staphylococcus, Streptococcus, Bacillus, E. coli, Shigella, Salmonella* Alteration of Plasma membrane permeability	Lou et al., 2011
Terpens (monoterpenes)		Gram-positive and Gram-negative bacteria Biomembrane damage	Trombetta et al., 2005

Chapter 2: Extraction, composite analysis, and roles of berry pomace extracts on the growth and pathogenicity of *Campylobacter jejuni*

Introduction

Campylobacter jejuni is one of the major enteric pathogens and is the source of much of the foodborne bacterial illnesses in the US. In 2012, Foodborne Diseases Active Surveillance Network (FoodNet) identified 14.3 *Campylobacter*iosis cases for every 100,000 inhabitants of the twelve states of USA and this rate was 14% higher than the rates found in 2006-2008. This high prevalence rate may be attributed to the increased consumption of white meat specifically chicken and turkey. In recent years, poultry has been recognized to be the major source of *Campylobacter*iosis in humans (Lin, 2009). *Campylobacter* colonizes the poultry gut as part of the normal flora. So far, the most widely used control measure against the colonization of *Campylobacter* in poultry gut is the use of antibiotics in feed and water, but resistant strains of *C. jejuni* to several antibiotics, including ciprofloxacin, nalidixic acid, erythromycin, tetracycline, and streptomycin, have been reported (Wieczorek, Szewczyk, and Osek, 2012). Consequently, the search for alternative natural and organic antimicrobials is now more essential than ever. Bioactive phytochemicals from berries, especially blackberry (*Rubus fruticosus*) and blueberry (*Vaccinium corymbosum*) pomace as feed or water supplement to reduce pre-harvest level of *C. jejuni* contamination in poultry, might be a feasible alternative.

Recently, the antimicrobial activity of phenolic compounds present in berry fruits and their pomaces against *Campylobacter* species and other intestinal pathogens was demonstrated (Biswas et al., 2012; Puupponen-Pimiä, Nohynek, Alakomi, and Oksman-Caldentey, 2005). Extraction of bioactive phytochemicals from fruit juice is too expensive for large scale application in the poultry industry. However, fruit pomace, the (semi) solid remains of crushed fruit after juice extraction, may provide a cheaper alternative. Fruit pomaces may make up as much of 30% of the original fruit weight and are produced in significant amounts in the US, where they are considered a waste product. Pomaces cannot be fed to animals directly since they are low in protein content and too acidic. Cheap extraction of the phenolics that are mainly responsible for the antibacterial action would provide a solution to both the fruit industry and the poultry industry. These phenolics are

concentrated in the outer layers of fruit (e.g. skin) as their biosynthesis is dependent on UV-light (sunlight).

Several modes of actions of berry phenolics have been suggested for pathogen inhibition. These mechanisms include damaging the bacterial cell membrane (Lacombe, Tadepalli, Hwang, and Wu, 2013), inhibition of extracellular microbial enzymes (Scalbert, 1991), direct effect on microbial metabolism, and deprivation of substrates mandatory for microbial growth (Puupponen-Pimiä et al., 2004). These mechanisms would act indiscriminately against benign and pathogenic bacteria. Nevertheless, we have provided evidence that berry juice inhibited pathogenic bacteria while stimulating the growth of probiotics (Yang, Hewes, Salaheen, Federman, and Biswas, 2014). The aim of this work was two-fold: (1) Development of a cheap extraction method to concentrate the antibacterial ingredients of berry pomace; and (2) Investigation of the effects of these active ingredients on the expression of virulence genes of *C. jejuni*, in order to explain the noted sensitivity of this pathogen for the antibacterial activity of berry pomace. The findings from this research will provide significant insight into the effects of blackberry and blueberry pomace extracts on *C. jejuni* growth, alteration of physicochemical properties, virulence gene expression and its interactions with host cells.

Material and Methods

Bacterial strain and growth condition. C. jejuni RM1221 (ATCC BAA-1062TM) was used in the current study. The bacterium was grown in Blood agar (Himedia, India) with 5% defibrinated sheep blood (Ward's Science) at 37˚C under microaerophilic (10% CO2, 5% O2, and 85% N2) condition.

Preparation of pomace extracts. Blackberry and blueberry whole fruits were purchased from a local market (College park, MD) and brought into the lab. A kilogram of blackberry or blueberry was treated with 1 liter boiling water for 3 minutes and the water was discarded. After cooling, a mixture of 400 mL sterile water and 82 µL (a rate of 0.0827 mL/kg of berry) pectinase enzyme (Novozyme Corp., Bagsvaerd, Denmark) was added to the treated berry and incubated at room temperature for 1 hour followed by vigorous blending. The pomace was concentrated by filtration to remove juice (which was stored at 4˚C for further use), dried at 40˚C in an oven and stored in

the dark at 4˚C. For further analysis of pomaces that are produced commercially as by-products from the berry juice industry, we obtained blackberry and blueberry pomaces from Milne Fruit Inc™.

Extraction was tested with the following solvents: water, 10% v/v ethanol and 10% v/v methanol. 2.5 g pomace was suspended in 50 mL extraction solution and incubated for 24 hours at 37 and 60˚C. After 24 hours, the solid portion was separated using centrifugation (3000 x g for 20 min) and the supernatant was sterilized with 0.45 µm filter (AcroVac Filter Unit, Pall Life Sciences, NY). The solvent was evaporated by vacuum freeze drying and resuspended in deionized water.

Determination of total phenolic contents in pomace extracts. Total phenolic content in each extract was determined using the spectrophotometric method described previously (Singleton, Orthofer, and Lamuela-Raventos, 1999). Briefly, 20 µL extract was dissolved to 1.58 mL water and 100 µL Folin-Ciocalteu reagents (MP; CAT NO.195186) was added. 300 µL 7.5 % Na¬2CO3 was added to the mixture and allowed to leave for another 2 hours at room temperature. The absorbance was determined with spectrophotometer (PerkinElmar, Lambda Bio) at λmax = 765 nm. The similar procedure was applied using standard solutions of Gallic acid and the standard calibration curve was constructed. To construct the calibration curve, 50 mL 10 mg/mL Gallic acid stock solution was prepared and 0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/mL Gallic acid solutions were formulated from the stock solution for individual points in the curve. The total phenolic content was expressed as Gallic Acid Equivalent (GAE).

Determination of minimum inhibitory concentration of pomace extracts against *C. jejuni*. Minimum Inhibitory Concentration (MIC) was determined using broth micro-dilution method described previously (Nkanwen, Gatsing, Ngamga, Fodouop, and Tane, 2009). The concentration ranging from 0.0 mg/mL to 1.0 mg/mL GAE for both blackberry and blueberry pomace extracts were added to Bolton broth with 10% defibrinated blood to a final volume of 990 µL. 10 µL bacterial suspensions containing approximately 5×105 CFU/mL was added to each of the well of 24-wells plates. Plates were incubated for 24 and 48 hours at 37˚C under microaerophilic condition. MIC was recorded as the lowest concentration of blackberry and blueberry pomace extracts that prevented visible growth of *C. jejuni* compared to the control.

Minimum Bactericidal Concentration (MBC) was determined by culturing the last well showing visible growth and the wells with no visible growth on a Karmali *Campylobacter* Agar (HIMEDIA). The lowest concentration that caused a significant reduction compared to the control (>3-logs, 99.9%) was considered as MBC. MBC:MIC ratio was also determined which illustrates a relationship between in vitro minimum bactericidal concentration and MIC of any drug against specific pathogen. If the value is less than 2, the drug is considered to be bactericidal against that pathogen; if this ratio exceeds 16 the drug is considered bacteriostatic and the ratio is more than 32, the pathogen is regarded to be tolerant to that drug (May, Shannon, King, and French, 1998; Konaté et al., 2013).

Time dependent growth patterns of *C. jejuni* in the presence of various concentrations of blackberry or blueberry pomace extracts were also determined. Bacterial inoculum was prepared as described above and inoculated into broth containing various concentrations blackberry or blueberry pomace extracts. Deionized water was used in the control instead of berry pomace extracts. Bacterial solutions were diluted in PBS, plated on Karmali Agar plates and CFU was counted at various time points upto 72 hours.

HPLC-tandem mass spectrometry (LC-MS/MS) analysis. Extracts were was prepared from the dried phenolic powder in 10% v/v ethanol and the total phenolic contents were measured. The concentrations of the stock solutions were adjusted to 6-8 g GAE/L. Berry pomace extract (BPE) was comprised of blackberry and blueberry pomace extracts at 1:1 v/v ratio. Total phenolic content in each extract was determined using spectrophotometric method (Singleton et al., 1999) and expressed as Gallic Acid Equivalent (GAE). The pH of the crude extracts were 4.5-5 and pH varied depending on the treatment concentration. A phenolic screen was accomplished using HPLC-MS (Peng et al., 2014). Sample injections were 5 µL and separations were performed on an Agilent 1100 system, coupled to an Agilent MSD-TOF (time-of-flight) mass spectrometer. Reversed-phase liquid chromatography was used to separate the samples. A Waters Atlantis T3 column (3 µm, 150 x 2.1 mm i.d.) was used. A binary mobile phase consisting of solvent systems A and B was used in gradient elution where A was 0.1% formic acid (v/v) in ddH2O and B was 0.1% formic acid (v/v) in acetonitrile. Mobile phase flow rate was 0.3 mL/min. The linear gradient was as follows: time 0 – 1 minute, 0% B; time 40 minutes, 90% B; time 41 minutes, 90% B; time 42 minutes, 0% B; time 52 minutes, 0% B. Following the separation, the column effluent was

introduced by electrospray ionization (ESI) into the MSD-TOF. Samples were assayed, using both negative and positive mode ESI. Source parameters were: gas temperature 350˚C, gas flow 9 L/min, nebulizer 35 psi, fragmentor 125 V, capillary voltage 3500 V. Data were acquired with a mass range of 75 - 1000 m/z. Accurate mass accuracy was guaranteed by the continuous infusion of Agilent Reference Mass Solution (G1969-85001). Individual chromatographic peaks were identified using Agilent's Mass Hunter Qualitative Analysis software (v. B.06). Compounds were identified using Agilent's Mass Profiler Professional software (v. 13.1). Peaks in duplicate injections were aligned to account for instrumental drifts in retention time and mass. Compounds were retained only if they appeared in both duplicate samples. Compounds were annotated by querying Agilent's METLIN human metabolite database, with a mass error criteria of < 5 ppm.

Evaluation of physicochemical properties C. jejuni treated with pomace extracts

Hydrophobicity: The bacterial cells were grown in the absence (no treatment) and predetermined sublethal (concentration at which reduced but not complete growth inhibition is observed) concentrations of blackberry/blueberry pomace extracts at 37˚C for 18 h. Cells were suspended in 2 mL of phosphate buffer saline (PBS, pH 7.2) and optical density (OD) was adjusted (OD570) to 0.5 (Ht0). The solution was mixed with 1 mL of n-hexadecane and incubated for 5 min at room temperature. The aqueous phase was measured at 570 nm (Ht5) using microplate reader (Multiskan FC, Thermo Scientific, MA). The hydrophobicity was calculated using the equation: Hydrophobicity (%) = (1 – Ht5/Ht0) × 100.

Auto-aggregation: The bacterial cells were incubated at 37˚C for 24h either in only broth (control) or broth supplemented with blackberry or blueberry pomace extracts. The cells were centrifuged at 3,000 × g for 20 minutes, the supernatant was decanted, and the cells were resuspended in 3 mL of PBS (pH 7.2) and adjusted the OD (OD570) to 0.5 (At0). The cell suspensions were incubated at 37 ˚C for 2h. The supernatants were separated and absorbencies were measured using a Multiskan microplate reader at 570 nm (At2). Aggregation was expressed as Auto-aggregation (%) = (1 – At2/At0) × 100.

Swimming and swarming motility: Motility assay was performed on semi-solid medium as described previously (Golden, 2002). In brief, OD600 of *C. jejuni* suspension was adjusted to 0.10. Two µL of the bacterial suspension was stabbed onto 0.45% (swarming motility) or 0.25% (swimming motility) Muller Hinton (MH) agar containing 0.3 mg/mL GAE of berry pomace

extracts. For negative control, equal amount of water was added to the wells. The plates were incubated at 37˚C for 48 hours under microaerophilic condition and diameter of zone for control (ZDC) and treatments (ZDT) were measured and calculated following the equation:
Motility rate (%) = ZDT/ ZDC × 100.

Adhesion and invasiveness assay. Adherence and invasiveness assays were performed according to the method described previously (Biswas et al., 2000). Briefly, 100 µL bacterial suspension, containing CFU approximately 100 times higher than host (INT-407 or DF-1) cell number, was inoculated into triplicate wells of a 24-well tissue culture plate. Each well contained semi-confluent monolayers of INT- 407 or DF1 cells covered with DMEM with 10% Fetal Bovine Serum (FBS) and 0.10 mg/mL and 0.08 mg/mL GAE of blackberry and blueberry pomace extracts respectively to a final volume of 900 µL. Infected monolayers were incubated for 3 h at 37˚C under a 5% CO2 humidified atmosphere. The infected monolayers were washed five times with DMEM containing 1% FBS and then re-incubated for another 1 h in fresh DMEM containing 10% FBS and 250 µg/mL of gentamicin. The monolayers were washed three times with DMEM containing 1% FBS and lysed with 0.1% Triton X-100 for 15 min. The suspensions were diluted and the numbers of viable bacterial cells were determined on Karmali agar plates.

RNA extraction and cDNA synthesis. The cells were grown in the absence or presence of sub-lethal concentrations of blackberry and blueberry pomace extracts and the extraction of RNA was carried out according to the protocol of ZR Bacterial RNA MiniPrep kit (Zymo Research Corp., Irvine, CA). RNA quantification was carried out using a NanoDrop spectrophotometer (Thermo Scientific Inc., West Palm Beach, FL). The cDNA synthesis was performed according to the protocol of qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). The eluted RNA (1 µg) was mixed with 4 µL of 5X qScript cDNA SuperMix containing optimized concentration of $MgCl_2$, dNTPs, RNase inhibitor protein, qScript reverse transcriptase, random primers, oligo(dT) primer, and stabilizers and then incubated at 25˚C for 5 min, 42˚C for 30 min, and 85oC for 5 min.

Quantitative RT-PCR assay. The mixture containing 10 µL of PerfeCTa SYBR Green FastMix, 2 µL of each primer (100 nM), 2 µL of cDNA (10 ng), and 4 µL of RNase-free water was amplified using an Eco RT PCR system (Illumine, San Diego, CA), which was denatured at 95°C for 30 sec,

followed by 40 cycles of 95°C for 5 sec, 55°C for 15 sec, and 72°C for 10 sec. The custom-synthesized oligonucleotides (Erofins MWG Operon; Huntsville, AL) used as primers to target conserved regions of *C. jejuni* are listed in Table 2.2. The relative expression levels of genes were calculated by the comparative method (Livak, and Schmittgen, 2001). The CT values of target genes in treated *C. jejuni* cells were compared to the CT values obtained from the control. The housekeeping gene, 16S ribosomal RNA, was used as the reference gene for normalization of target gene expression.

Statistical Analysis. All data were analyzed using the Statistical Analysis System software (SAS, Institute Inc., Cary, NC, USA). One-way analysis of variance (ANOVA) was used, followed by Tukey's test to determine significant differences among treatments at $P < 0.05$.

Results

Role of solvents on phenolic content extraction from pomaces. We have extracted the pomace phenolics using three different solvents (10% ethanol, 10% methanol in water, and water alone) and incubated at two different temperatures, 37˚C or 60˚C, (Table 2.3). We found that as a solvent, 10% ethanol in water was the most effective in extracting phenolic compounds from both blackberry (1.66 mg/mL GAE) and blueberry (1.43 mg/mL GAE) pomaces compared to other two solvents though the difference between ethanol and methanol in terms of extracting phenolic compounds was not significant. Due to toxicity issue, we further used ethanol as solvent. We also observed that 95% ethanol in water failed to extract the phenolic compounds in pomaces. In the presence of 95% ethanol in water, the mixture became too hazy (data not shown). Water alone was found to be the least effective in extracting the phenolic contents of pomaces. We observed that 10% methanol in water could extract the phenolic compounds in pomaces (1.55 mg/mL and 1.35 mg/mL GAE from blackberry and blueberry pomaces respectively) better than water alone (0.75 mg/mL and 0.51 mg/mL GAE from blackberry and blueberry pomaces respectively) but lower than 10% ethanol in water extraction (1.66 mg/mL and 1.43 mg/mL GAE from blackberry and blueberry pomaces respectively). We also found that temperature had a significant impact on total phenolic content. A higher temperature (60˚C) had significant impact in extracting phenolic compounds from both blueberry and blackberry pomaces compared to a lower temperature (37˚C). Among all the solvents and temperatures, we found that 10% ethanol in water extracted the highest amount of phenolic compounds in berry pomaces at 60˚C.

Composition of BPE. BPE solution remained stable (no significant change in total phenolic content) at room temperature upto 1 month in room temperature and 6 months under refrigerated condition. HPLC-MS analysis of these crude extracts with negative and positive ionization mode showed the presence of a wide array of components (Supplementary file 1). In the negative ionization mode, 5108 (2320 unique) and 4445 (2221 unique) compounds were detected in blackberry and blueberry pomace extracts, respectively (Table 2.1). Major compounds in these extracts included, but not limited to, apigenin, acetoxyeugenol, chlorogenic acid, cinnamic acid, coumarin, ellagic acid, flavan, flavanone, gallic acid, gingerol, glucuronides, hibiscetin,

hydroxydaidzein, myricetin, phenols, quercetin, quinones, rhamnosides, stilbenol, triamcinolone, and xanthine. Wide structural variability in the phenolic derivatives were observed.

Inhibition of C. jejuni growth with pomace extracts. The effect of blackberry and blueberry pomace extracts on *C. jejuni* growth is shown in Table 2.4. We found that MIC and MBC of blackberry pomace extract were 0.6 mg/mL and 0.8 mg/mL GAE respectively, whereas MIC and MBC of blueberry pomace extract were 0.4 mg/mL and 0.5 mg/mL GAE. MBC:MIC ratio shows values < 2 for both of the extracts which indicates both of them are bactericidal (Table 2.4) but the bactericidal activity of blueberry pomace extract was stronger than that of blackberry pomace extract.

Time required for inhibition of growth of C. jejuni with pomace phenolic extracts. Based on the previous results showing growth inhibition and bactericidal effect of blueberry and blackberry pomace extracts on *C. jejuni* growth, we monitored the growth inhibition at various time points. The CFU/mL of the *C. jejuni* strain incubated in different concentration of blackberry and blueberry pomace extracts were reduced in a time-dependent manner. In Fig. 2.1, we showed the time dependent inhibition of *C. jejuni* in the presence of various concentrations of berry pomace extracts. We observed that the same concentration (0.6 mg/mL GAE) of blackberry and blueberry pomace extract showed difference in growth inhibition. 0.6 mg/mL GAE of blackberry pomace extract reduced the CFU/mL of *C. jejuni* approximately 2 logs compared to the control at 24 h time point. In the same experiment, 0.6 mg/mL GAE of blueberry pomace extract reduced the CFU/mL of *C. jejuni* approximately 6.5 logs after 24 hours of incubation compared to the control. Though inhibition of *C. jejuni* growth with 0.8 mg/mL GAE blackberry pomace extract was > 4 logs after 24 hours compared to the control (recorded as MBC), but growth inhibition was not observed during the first six hours at the ranges of concentrations from 0.6 to 0.7 mg/mL GAE. On the other hand, higher concentrations of blueberry pomace extract (0.5 mg/mL GAE) showed antimicrobial effect right from the beginning of the growth cycle of *C. jejuni* (Fig. 2.1).

This fact was further supported when inhibition of growth pattern of *C. jejuni* was observed for longer periods of time in the presence of sub-lethal concentrations (0.3 mg/mL GAE) of both of the blackberry and blueberry pomace extracts. Blackberry pomace extract showed mild inhibitory effect compared to its blueberry counterpart over a shorter time period (Fig. 2.1c) but blueberry

started to lose effectiveness after 24 hours while blackberry retained effective even after 72 hours providing > 2 log reduction compared to the control.

Alteration of virulence properties of C. jejuni in the presence of pomace extracts. In this study, in the presence of blueberry or blackberry pomace extracts, autoaggregation capability of *C. jejuni* strain decreased significantly (Table 2.5). Untreated bacterial cells showed higher autoaggregation, nearly 66% whereas in presence of blackberry and blueberry pomace extracts the values were reduced to 62% and 42%, respectively. But cell surface hydrophobicity of *C. jejuni* was increased by treating with both berry pomace extracts. The motility phenotypes of *C. jejuni* treated with berry pomace extracts were examined through solid-based movement (swarming motility) and liquid-based movement (swimming motility) on semi-solid agar plates containing berry pomace extracts (Table 2.5). Both blackberry and blueberry pomace extracts reduced bacterial migration, while strong migration was observed in the absence of pomace extracts (Fig. 2.2). We found that blackberry pomaces extract reduced the motility of *C. jejuni* more effectively (>85%) than blueberry pomace extract (<40%).

Role of berry pomace extracts on host cell-C. jejuni interactions. Invasion of *C. jejuni* into INT407 cells and DF1 cell was reduced significantly (Fig. 2.3) in the presence of low concentrations of berry pomace extracts (0.10 mg/mL and 0.08 mg/mL GAE of blackberry and blueberry pomace extract respectively). The concentrations of pomace extracts were selected depending on their cytotoxic effect on cultured (INT-407 and DF1) cells (data not shown). In the presence of blackberry and blueberry pomace extracts, reduction of invasion abilities of *C. jejuni* into DF1 cells were >75% and >30%, respectively. In INT407 cells, invasion abilities of *C. jejuni* were also reduced similarly, approximately 79% in the presence of blackberry and approximately 52% in the presence of blueberry pomace extracts, respectively. We also observed that both blueberry and blackberry pomace extracts also reduced the adhesion abilities of *C. jejuni* to DF1 cells significantly, but not to INT407 cells. Adhesion abilities of *C. jejuni* to DF1 were reduced by 42% and 29% in the presence of blackberry and blueberry pomace extract, respectively whereas adhesion abilities of *C. jejuni* to INT407 cells were reduced by 19% and 27% in the presence of blackberry and blueberry pomace extracts, respectively.

We also carried out an experiment where bacterial cells were pretreated with pomace extracts before infection phase. We needed to collect the cells with centrifugation for infecting host cell. We found that centrifugation at 3000×g for 20 min reduced the adhesion or invasiveness of *C. jejuni* RM1221 (data not shown).

Role on virulence gene expression. The common and known genes responsible for *C. jejuni* adherence and invasion ability and bacterial cell motility were examined for their expression in the absence or presence of two different concentrations (0.6 and 0.4 mg/mL GAE blackberry and 0.4 and 0.2 mg/mL GAE blueberry) of blackberry and blueberry pomace extracts (Fig. 2.4). In both concentrations, blackberry pomace extract increased the expression of flaA by 3 to 5 fold. Expression level of the other genes, including flaB, cadF, ciaB and cdtB, did not alter significantly. But we observed the numerical decrease in cadF in the presence of blueberry pomace extracts at both concentration levels (0.4 mg/mL GAE or 0.2 mg/mL GAE).

Discussion

There are many natural components that are able to limit and inhibit the spread and virulence of foodborne bacterial pathogens. In our previous study, we found that both blueberry (Biswas et al., 2012) and blackberry (Yang, Hewes, Salaheen, Federman, and Biswas, 2014) juice inhibited the growth of various foodborne bacterial pathogens including *C. jejuni*. Several literatures also indicated that phenolic components of berry and berry products have antioxidant and offer many other health benefits (Biswas et al., 2012; Puupponen-Pimiä, Nohynek, Alakomi, and Oksman-Caldentey, 2005). Considering the economical and practical approach of using bioactive components of berries in agricultural animal production, in this study we initiated an innovative method to extract the bioactive components from the byproduct of the blueberry and blackberry juice industry. We also tested the role of these bioactive extracts of berry pomaces in the cellular and molecular properties and dynamics of interaction with host cells of *C. jejuni*. Our results suggested that berry pomace extracts used as feed additives or water supplements may reduce the colonization level of *C. jejuni* in poultry. Though the nontoxic concentration of phenolic extracts of berry pomace could not stop complete growth of *C. jejuni*, it reduced the growth significantly. A risk assessment study found that *C. jejuni* infection in humans could be reduced by 30-fold if the number of this pathogens in poultry was reduced by 2 logs (Rosenquist, Nielsen, Sommer, Nørrung, and Christensen, 2003).

Berry pomaces are rich in phenolic compounds making them a plausible source for extraction. Proper application of these cheap and vastly abundant byproducts could help reduce the waste management problems in the juice industries and increase their profits. In this study, we focused on the extraction of bioactive components from the pomace of locally grown blueberry and blackberry and investigated the role of these the bioactive extracts against the most common foodborne pathogenic enteric bacteria, *C. jejuni*.

We extracted the pomace phenolics using different concentrations of three solvents and two incubation temperatures. We found that as a solvent, 10% ethanol in water and 60˚C incubation temperature was the most effective in extracting phenolic compounds from both blackberry and blueberry pomaces. In our study, 10% ethanol solution served more efficiently in extracting phenolics from berry pomaces compared to 10% methanol in water or water alone. This finding is in agreement with previous studies (Franco et al., 2008) who reported ethanol to be more

efficient than methanol in extracting polyphenols. Moreover, the toxicity issue favors ethanol over methanol as solvent. Temperature is another important variable that affects polyphenol extraction and higher extraction temperature (60˚C) was found to increase the amount of total phenolics regardless the types of solvent (Pinelo, Rubilar, Jerez, Sineiro, and Núnez, 2005; Spigno and De Faveri, 2007). We also found extraction temperature of 60˚C was more efficient compared to 37˚C, which is in complete agreement with previous findings.

Previous studies focused on the ability of berry phenolics to inhibit pathogens (Nohynek et al., 2006) or the MIC of different phenolics against *C. jejuni* (Klancˇnik, Mozˇina, and Zhang, 2012). In addition to that, we focused on the growth pattern of *C. jejuni* in the presence of sub-lethal concentrations of blackberry and blueberry pomace phenolics. Both blackberry and blueberry pomace extracts were proved to be bactericidal considering the MBC:MIC ratio. Higher concentration of blackberry pomace extract was required to get similar bactericidal effect to its blueberry counterpart. But a sub-lethal concentration of blackberry pomace extract exerted long term bactericidal effect, even after 72 hours of bacterial growth, whereas a sub-lethal concentration of blueberry pomace extract started to lose effect after 24 hours. There might be difference in polyphenol composition between these two pomaces extracts. Berries contain four major classes of phenolic compounds: flavonoids, phenolic acids, lignans, and polymeric tannins (Puupponen-Pimiä, Nohynek, Alakomi, and Oksman-Caldentey, 2005) and we hypothesize compositional differences might be the reason for the different effects of blackberry and blueberry pomace extracts on *C. jejuni*.

This study also found that blackberry and blueberry pomace extracts altered several virulence properties of *C. jejuni*. Two important physicochemical surface properties of pathogenic bacteria are autoaggregation and hydrophobicity. Autoaggregation is considered to be a marker of virulence in several Gram-negative bacteria (Chiang, Taylor, Koomey, and Mekalanos, 1995; Menozzi, Boucher, Riveau, Gantiez, and Locht, 1994). Though role of autoaggregation in *C. jejuni* pathogenesis is not well documented, it has been suggested that autoaggregation might play role in *C. jejuni* invasiveness into intestinal epithelial cells (Golden, 2002). We found that due to treatment with sublethal concentration of blueberry pomace extracts, *C. jejuni* autoaggregation decreased significantly whereas blackberry pomace extract caused numerical decrease (but not significant) in autoaggregation. Hydrophobicity is another important surface physicochemical property. Cell surface hydrophobicity and autoaggregation were found to be positively correlated

to bacterial adhesion (Saran, Bisht, and Singh, 2012). Some studies have suggested that there is no correlation between hydrophobicity and autoaggregation or bacterial attachment (Vinderola, Medici, and Perdigón, 2004). We observed that treatment with berry pomace extracts caused an increase in *C. jejuni* surface hydrophobicity unlike autoaggregation, which decreased significantly.

Both in vitro and in vivo study indicated that motility also plays a critical role in pathogenicity of *C. jejuni* (Yao et al., 1994). In this study, we investigated the motility phenotypes of *C. jejuni* in presence of sublethal concentration of blackberry and blueberry pomce extracts in liquid-based movement (swimming motility) and solid-based movement (swarming motility) on semi-solid agar plates. Both kinds of motility decreased significantly in the presence of both pomace extracts, though *C. jejuni* seemed less motile in the presence of blackberry rather than blueberry pomace extract. It has also been found that natural carvacrol reduced *C. jejuni* motility by disrupting the function of flagella (Van Alphen, Burt, Veenendaal, Bleumink-Pluym, and van Putten, 2012), but this treatment did not alter flagellar biosynthesis, which is in complete agreement with our findings. The flagella-mediated swimming motility is mainly responsible for bacterial translocation to evade the host immune system (Chow, Gu, Jiang, and Nassour, 2011). The surface-associated swarming motility plays critical role in bacterial colonization (O'May and Tufenkji, 2011). The decrease in swimming and swarming motility indicates that blackberry and blueberry pomace extracts can effectively control bacterial interactions with host cells.

Attachment is the major prerequisite for colonizing *C. jejuni* on intestinal epithelial cells and invasiveness is considered to be an important virulence property. Adhesion and invasion abilities of *C. jejuni* to the cultured human intestinal epithelial (INT407) cells and chicken fibroblast (DF1) cells were compared in the presence and absence of blueberry and blackberry pomace extracts. Significant decrease in invasiveness into both cell lines were observed and numerical decrease in adhesion were found. The above mentioned paralyzed physicochemical properties (decreased autoaggregation and mitility) might have impact on the reduction of adherence and invasion abilities of *C. jejuni*. The findings agrees with the previous report (Golden, 2002) which also showed that decreased motility caused reduction in invasiveness in *C. jejuni*.

We assessed the expression level of several virulence genes of *C. jejuni*. The genes include flaA (Flagellin A subunit synthesis), flaB (Flagellin B subunit synthesis), cadF (*Campylobacter* adhesion fibronectin-binding protein), cdtB (Cytolethal distending toxin) and ciaB (*Campylobacter* invasion antigen). A significant increase in relative expression of flaA was

observed, but the expression of other genes under study remained unaltered. The mechanism behind the increase in flaA expression is still unknown, but it plays an important role in pathogenesis, specifically the motility and colonization of *C. jejuni*. We hypothesize, disruption of flagellar integrity due to BPE treatment may be compensated with induction of flaA. To our knowledge, this study is the first report exploring the expression level of virulence genes of *Campylobacter* in presence of natural and organic berry phenolics.

Conclusions

1. Major compounds in BPE included, but not limited to, apigenin, catechol, chlorogenic acid, cinnamic acid, coumarin, ellagic acid, eugenols, flavan, gallic acid, gingerol, glucosides, glucuronides, myricetin, phenols, quercetin, quinones, rhamnosides, stilbenol, tannins, triamcinolone, and xanthine.
2. BPE inhibited the growth of *C. jejuni in vitro* and altered physicochemical properties of bacterial cell membrane.
3. BPE reduced *C. jejuni* growth and motility, *in vitro*, resulting in lower adherence and invasiveness to chicken fibroblast cells.

List of Figures and Tables

Table 2.1. Composition of berry pomace extracts

Source	Ionization mode	No. of compounds*	No. of unique compounds
Blueberry Pomace Extract	(+) ve	1103	605
Blueberry Pomace Extract	(-) ve	4445	2221
Blackberry Pomace Extract	(+) ve	1638	985
Blackberry Pomace Extract	(-) ve	5108	2320

*****Complete list of compounds are included in Supplementary file 1.

Table 2.2. Molecular functions and primer sequences of target genes used in qRT-PCR analysis for *C. jejuni*

Function/protein	Gene	Primer	Sequences (5'-3')	References
Flagellin A	*flaA*	Forward	GCAGCAGATGATGCTTCAGGGAT	Klančnik et al., 2006
		Reverse	GCTTGAAGCATGGTTCTTGT	
Flagellin B	*flaB*	Forward	CCGTTTCCATCACCATCTTC	Tu et al.,2008
		Reverse	ACACGCTTTGAAACAGGAGG	
Fibronectin binding protein, Cadherin	*cadF*	Forward	TATGGTGTAGAAAAAAGTCGCATCA	Fouts et al., 2005
		Reverse	ATCCGCTCTACCTTCTTTAGTGTCA	
Cytolethal Distending toxin	*cdtB*	Forward	AATGCAAGCTGAAGAAGTGATTGT	Lara-Tejero, and Galan, 2001
		Reverse	AGCATCATTTCCATTGCGAAT	
Campylobacter invasion antigen	*ciaB*	Forward	CAACTTTATATTTGCACTCCGATG	Poly, and Guerry, 2008
		Reverse	GGAACGACTTGAGCTGAGAATAAAC	
	16S rRNA	Forward	AGAGTTTGATCCTGGCTCAG	Klančnik et al., 2006
		Reverse	TGTCTCAGTTCCAGTGTGACT	

Table 2.3. Total phenolic content in blackberry and blueberry pomace extracts in presence of different solvents

Source	Extraction Solvent	Extraction Temperature (˚C)	Total Phenolic Content (mg/mL GAE)
Blackberry	10% Ethanol	60	1.66 ± 0.30
		37	0.75 ± 0.25
	10% Methanol	60	1.55 ±0.42
		37	0.79 ± 0.19
	Water	60	0.75 ± 0.23
Blueberry	10% Ethanol	60	1.43 ± 0.18
		37	1.09 ± 0.21
	10% Methanol	60	1.35 ± 0.31
		37	0.98 ± 0.19
	Water	60	0.51 ± 0.24

Table 2.4. Antibacterial effect of blackberry and blueberry pomace extracts on *C. jejuni*

Treatment	MIC (mg/mL GAE)	MBC (mg/mL GAE)	MBC/MIC	Bactericidal/Bacteriostatic
Blackberry pomace extract	0.6	0.8	1.33	Bactericidal
Bluebery pomace extract	0.4	0.5	1.25	Bactericidal

Abbreviations: MIC, Minimum Inhibitory Concentration; MBC, Minimum Bactericidal Concentration; GAE, Gallic Acid Equivalent.

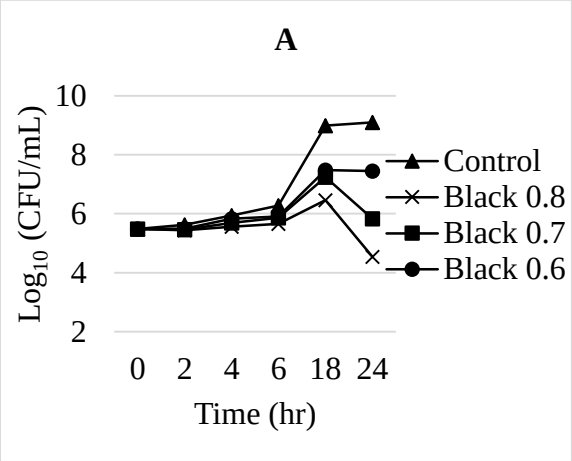
Table 2.5. Physicochemical properties and mechanical behaviours of *C. jejuni* treated with blackberry and blueberry pomace extracts.

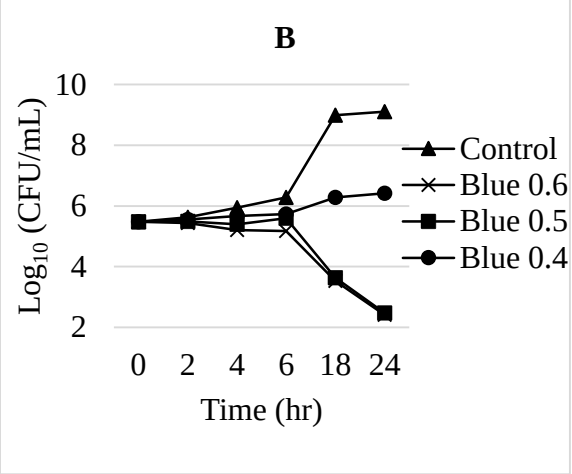
Treatments	Auto-aggregation (%)	Hydrophobicity (%)	Swimming motility (%)	Swarming motility (%)
Control	66.45±3.08 ǂ, a	1.27±0.74 a	100 ~~A~~, a	100 a
Blackberry pomace extract	62.36±0.37 a	4.16±2.81 a	10.02±0.50 b	14.04±1.46 b
Blueberry pomace extract	42.64±5.42 b	7.17±1.40 b	71.45±7.21 c	60.51±9.53 c

ǂ Means with different letters within an individual column (a-c) are significantly different at $P < 0.05$.

~~A~~ Motility values are normalized to untreated control (100%).

Fig. 2.1. Time dependent growth inhibition pattern of *C. jejuni* in presence of various concentrations (in mg/mL GAE units) of blackberry (A) or blueberry (B) pomace extracts. Comparative growth inhibition of blackberry and blueberry pomace extracts at particular concentration (0.3 mg/mL GAE) is also shown (C). Each data point represents mean of three different experiments.





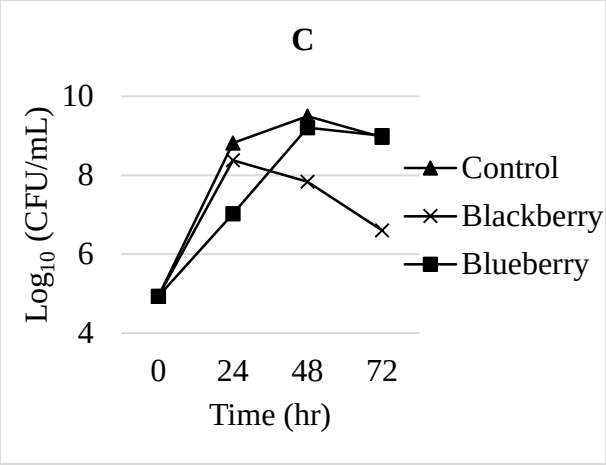

C
10
8
6
4
Log10 (CFU/mL)
0
24
48
72
Time (hr)
Control
Blackberry
Blueberry

Fig. 2.2. Swimming and swarming motility of *C. jejuni* in presence of pomace extracts. Photographs were taken using Canon digital camera from the same distance for each well.

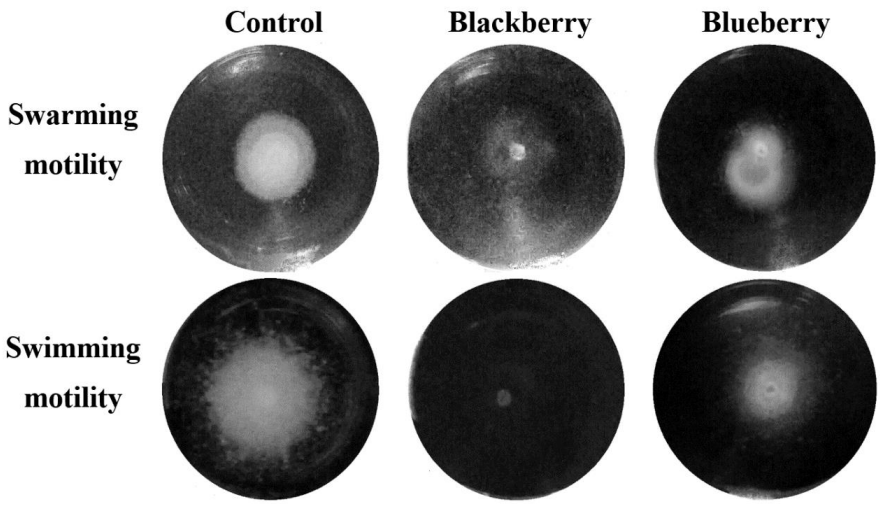
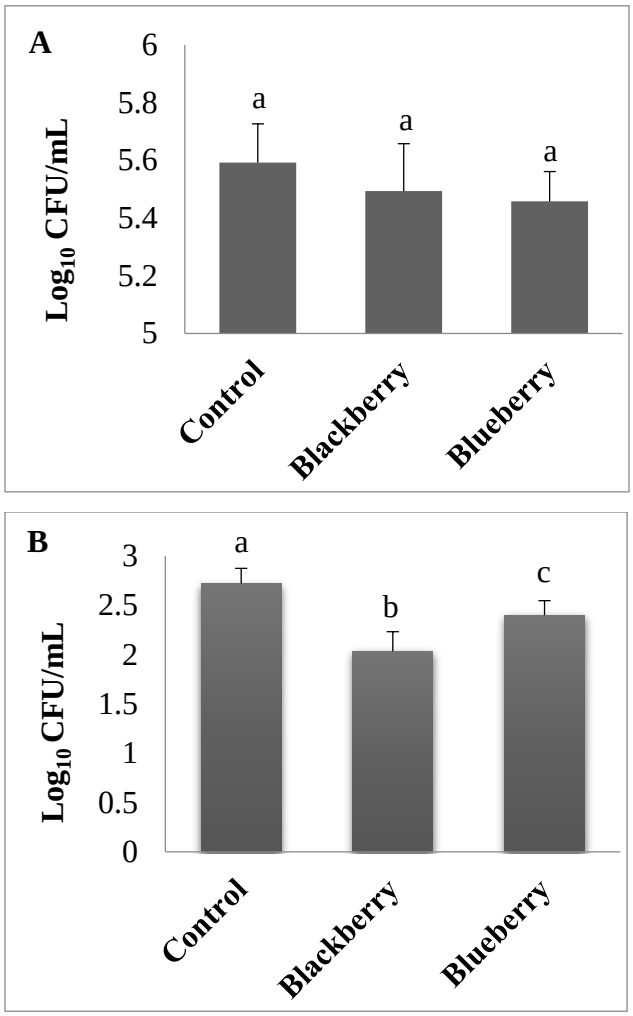
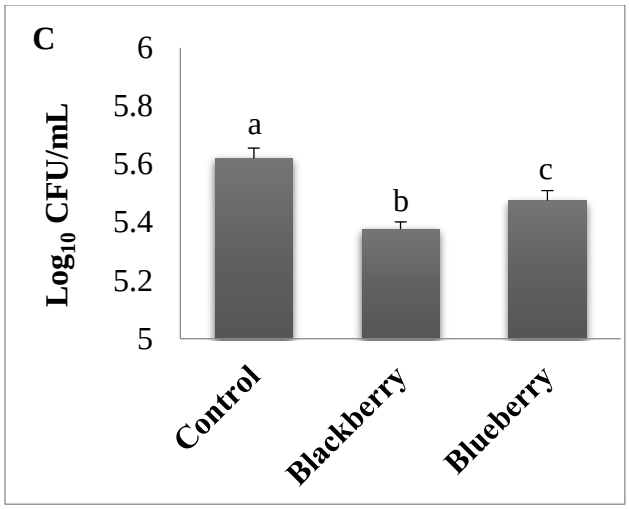


Fig. 2.3. Adhesion (A and C) and invasiveness (B and D) of *C. jejuni* to INT407 cells and DF1 respectively in presence of blackberry and blueberry pomace extracts. Means with different letters (a-c) are significantly different ($P<0.05$)




C
6
5.8
5.6
5.4
5.2
5
Log10 CFU/mL
a
b
c
Control
Blackberry
Blueberry

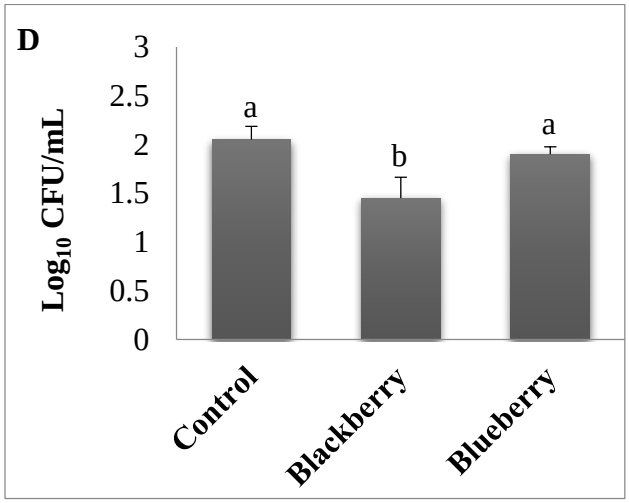
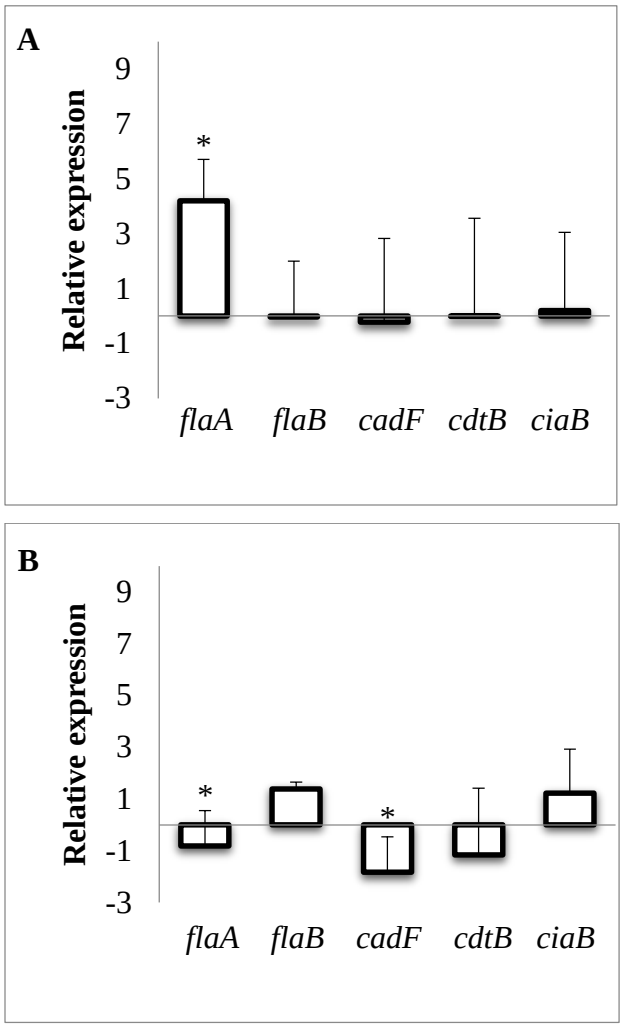
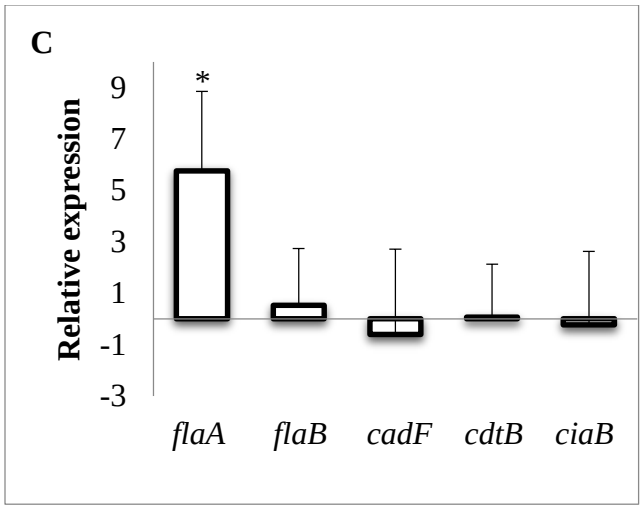
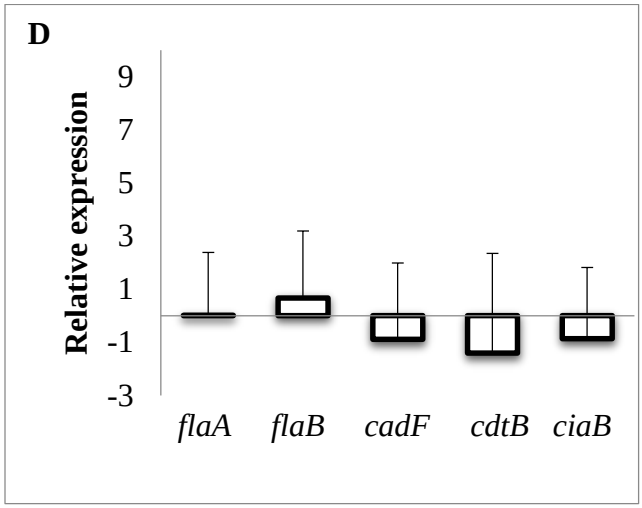

D
3
2.5
2
1.5
1
0.5
0
Log10 CFU/mL
a
b
a
Control
Blackberry
Blueberry

Fig. 2.4. Relative expression of virulence genes of *C. jejuni* treated with (A) 0.4 mg/mL GAE blackberry, (B) 0.2 mg/mL GAE blueberry, (C) 0.6 mg/mL GAE blackberry, and (D) 0.4 mg/mL GAE blueberry pomace extracts. * indicates significantly increased or decreased relative expression of genes at $P < 0.05$.




C
Relative expression
9
7
5
3
1
-1
-3
*
flaA
flaB
cadF
cdtB
ciaB


D
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ciaB

Chapter 3: The effects of berry pomace extracts on poultry pathogens of commercial importance and probiotics *in vitro* and cultured host cells *ex vivo*.

Introduction

Maintaining the health of food-animals health is important from an animal welfare, animal production and public health standpoint. The health status of the poultry is always critical to the producer whether he maintains a backyard or commercial poultry flock. Identification of the cause of disease and appropriate treatment or adequate feeding can prevent a condition from becoming an unprofitable enterprise due to poor production and quality of the birds. Several bacterial pathogens are involved in poultry diseases and economic losses caused by them are currently 10 to 20 percent of the gross value of production in developed poultry industries and are likely to be higher in organic/pasture farming (Fanatico 2008; Hanning et al 2010). Major bacterial diseases such as fowl typhoid, caused by *Salmonella* enterica serovars Gallinarum and Pullorum, and fowl cholera, caused by *Pasteurella multocida* remain a large threat to the poultry industry specifically pastures poultry (Porter 1998; Christensen and Bisgaard 2000; Herath et al 2010). Recently, it has been found that most of the gastrointestinal diseases and animal health are strongly influenced by gut flora and gut floral composition can be modulated with feeds (Backhed et al 2012).

Pasteurella multocida is one of the most common poultry pathogen which causes highly fatal disease known as fowl cholera (Herath et al 2010). Fowl cholera has a great economic impact in large and small scale poultry productions including both organic and conventional back-yard poultry production. The disease has global occurrence and is capable of infecting all avian species (Christensen et al 2000, Herath et al 2010). In addition with fowl cholera, *P. multocida* also cause lameness and neurological disorder in poultry and several other diseases in cattle (bovine mastitis) and pig (diarrhea) (Wilkie et al., 2012). In early age mortality of poultry is one of the biggest hurdles of poultry industries and this pathogen play a key role.

Currently, free-range and pasture-flock poultry is a sustainable agricultural production system and up on the rise on the top of the organic food lists (Adl et al., 2011). It is becoming more popular due in part to a negative perception of conventionally raised chicken. Free-range and pasture-flock poultry spend more than half of their life in outdoor open areas and come in contact with birds, rodents, pest and other animals (Hanning et al 2011). Therefore, biosecurity and higher

risk of various infectious diseases are major concerns for free-range and pasture-flock poultry. According to national organic regulations, organic livestock farming is not allowed to use antibiotics and other synthetic chemicals. Instead of routinely giving antibiotics to their animals, organic farmers try to keep animals healthy through proper nutrition and sanitation, by reducing animal stress and with preventative and holistic animal health practices. But without chemical and antibiotics, organic farm animals specifically poultry tend to become more susceptible to various bacterial infectious diseases. Fowl cholera is recognized as a big player in pasture and free-range poultry and the disease pose a serious threat to gastrointestinal health and overall flock livability and mortality (Porter 1998).

A Danish survey of large organic layer flocks reported high mortality rates (15-20%) of two to three times higher than layers in conventional battery cages (Kritensen 2012). In a manuscript entitled "Causes of mortality in commercial organic layers in Denmark" Stckholm et al (2010) reported that the mean of flock mortality for organic layers was 20.8% compared with 7% for confined flocks on deep litter (Hanning et al 2011). In the US, broiler mortality rates vary from 5 to 10 percent in organic production and this numbers for organic layer flocks are from 3 to 5 percent (Fanatico 2008). That indicates that due to higher mortality, organic farmers lose millions of dollars every year in the US.

The most common and available current option for controlling poultry diseases in poultry flocks is the addition of synthetic antimicrobials to poultry feeds and water and/or vaccination. Due to poor efficacy and inconvenience in use in poultry flocks, most of the farmers prefer to use antibiotics for disease control as well as growth promotion. But, according to the USDA recommended substances removal from the NOP's National List, organic poultry farmers are not allowed to use those antimicrobial chemicals for their flocks for either disease control or growth promotion. Moreover, pasture flocks and free-range chickens are raised with access to the outdoors for at least one-third of their entire life cycle (Hanning et al 2010). The lack of proper biosecurity measures potentially increases the possibility of their coming into close contact with sources of foodborne pathogens including birds, pests and other wild animals (Hanning et al 2010). Organic farmers are in need of natural antimicrobial products to employ in disease control and to improve the gut flora of the birds in order to maintaining the flocks' good health and sustain their business long term.

Berries, native North American fruits and their byproducts (pomaces) are of interest to researchers because of their potential market value and contribution to human health as well as their strong antimicrobial effect. Berries are rich in several phytochemicals such as phenolic acids, proanthocyanidins, anthocyanins and other flavonoids (Bomser et al 1996; Mantley and Buslig 1998). Many of these compounds exhibit a wide range of biological effects including antioxidant, antimicrobial, anti-inflammatory and vasodilators (Mantley and Bushlig 1998). The antimicrobial activity of these compounds has been intensively studied as a means to control invasion by and growth of plant pathogens (Boivin et al 2007; Jepson and Craig 2007). There has been growing interest in a variety of potential chemo preventive activities of edible berries, specifically blueberry, blackberry and cranberry. Pomace (byproducts) consists primarily of seeds and skins of fruits used for juice and wine production and shows antimicrobial activity against various bacterial pathogens. There is evidence that flavanols that reach the large intestine may provide prebiotic-like benefits by promoting the growth of beneficial bacteria while inhibiting the growth of harmful bacteria (Tzounis et al 2011; Salaheen et al 2014). Since uses of fruit pomaces are limited, fruit processing industries are becoming increasingly interested in exploring alternate and potentially economically advantageous ways to utilize their waste materials. Given the emphasis on the potential application of the bioactive extracts of the blueberry and blackberry pomaces in poultry disease control and on improved gut flora, it is critical to provide in depth research-based information that will aid in a demonstrating clear evidence of the functional roles of these extracts as allowable alternatives to substances recommended for removal from the NOP's National List of Prohibited Substances specifically antibiotics. The aim of this study is to develop an alternative bioactive intervention strategy for common poultry diseases and improve the gut flora using functional extracts of berry pomace (byproducts). We hypothesize that bioactive components from the byproducts of blueberry and blackberry will be an alternative functional agent for gut flora modulation and control of bacterial poultry diseases such as fowl cholera and fowl typhoid. In addition, we will determine if the use of this bioactive component through the feed can alter viral colonization.

Material and Methods

Bacterial strains and growth conditions. Pasteurella multocida (ATCC 15742) was used in this study. Prior to each experiment, bacterial strain was cultured on blood agar plates containing 5% sheep blood (SBA) at 37°C for 18 h. As liquid medium, tryptone soy broth (Oxoid) and for counting Luria Bertani (HIMEDIA) were used for experimental purpose. *Salmonella* enterica serovars Gallinarum (CAT375, Presque Isle Cultures, Erie, PA) and Pullorum (CAT3751, Presque Isle Cultures, Erie, PA) were used as poultry bacterial pathogens and *Lactobacillus* plantarum (ATCC 8014TM) was used as a probiotic. Prior to each experiment, *S.* Gallinarum and *S.* Pullorum were grown on Luria Bertani (LB) (Himedia, India) agar plate and *L. plantarum* was grown on deMan, Rogosa, and Sharpe (MRS) agar (EMD, MA) plates at 37°C for 18-24 h from -80°C glycerol stock. Afterwards, for growth inhibition assay and colony counting LB or MRS agar were used.

Preparation of pomace extracts. Blackberry and Blueberry whole fruit were bought from local market and brought in lab. One liter water was heated up to 95˚C. A kilogram of blackberry or blueberry was treated with this water for 3 min and water was discarded. Mixture of 400 ml sterile water and 82 µl (a rate of 0.0827 ml/kg of berry) pectinase enzyme (Novozyme Corp., Bagsvaerd, Denmark) was added to treated berry and incubated at room temperature for 1 hour followed by vigorous blending. Juice portion was filtered out, stored at 4˚C and pomace was separated, dried at 40˚C in a HERATherm oven (Thermo Scientific) and stored in dark container at 4˚C.

Ethanol (10%) in water was used as solvent for extraction. 2.5 g pomace was suspended in 50 ml extraction solution and incubated overnight at 60˚C. After 24 hours, solid portion was separated using centrifugation and the supernatant was sterilized with 0.45 µm filter (AcroVac Filter Unit, Pall Life Sciences). The solvent was evaporated by vacuum freeze drying and it was resuspended in demonized water and adjusted the concentration. Total phenolic content was calculated as milligrams of gallic acid equivalent per milliliter of solution.

Determination of total phenolic contents in pomace extracts. Total phenolic content in each extract was determined using the spectrophotometric method described by Singleton et al., 1999. Briefly, the reaction mixture was prepared by mixing 20 µL extract to 1.58 ml water and 100 µL Folin-

Ciocalteu reagents (MP; Cat #195186). After 5 min incubation, 300 µL 7.5 % Na¬2CO3 was added to the mixture and allowed to leave for another 2 hours at room temperature. The absorbance was determined with spectrophotometer (PerkinElmar, Lambda Bio) at λmax = 765 nm. All the samples were prepared in triplicate for each analysis and the mean value of absorbance was determined using calculation method. The similar procedure was applied using standard solutions of gallic acid and the standard calibration curve was constructed. To construct the calibration curve, 50 ml 10 mg/ml gallic acid stock solution was prepared and 0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/ml gallic acid solutions were formulated from the stock solution for individual points in the curve. The total phenolic content was expressed as gallic acid equivalent (GAE).

Determination of minimum bactericidal concentration of pomace extracts against P. multocida. Minimum Inhibitory Concentration (MIC) was determined using broth micro-dilution method previously described by Nkanwen et al., (2009). The concentrations ranging from 0.0 mg/ml to 1.0 mg/ml berry pomace extracts or 0% to 1% citrus oil in TSB were used. 10 µL bacterial suspensions containing approximately 5×105 cfu/ml was added to each of the well of 24-wells plate. Plates were incubated for 24 hours at 37˚C with or without shaking. MIC was recorded as the lowest concentration of blackberry and blueberry pomace extracts or citrus oil that prevented visible growth of *P. multocida*. Minimum Bactericidal Concentration (MBC) was determined by culturing the last well showing visible growth and the wells with no visible growth on a blood agar plate. The lowest concentration that showing a significant reduction (>3 logs, 99.9%) was considered as MBC.

Determination of cell surface Hydrophobicity. The bacterial cells were grown in the absence (no treatment) and predetermined elevated concentrations (concentration which did not show growth inhibition in 1 hour) of blackberry/blueberry pomace extracts and citrus oil at 37˚C for 1 h. The cells were then centrifuged at 3,000 × g for 20 min. The supernatants were poured out and the cells were resuspended in 2 ml of phosphate buffer saline (PBS, pH 7.2) and optical density (OD) was adjusted (OD570) to 0.5 (Ht0). The bacterial suspensions were mixed with 1 ml of n-hexadecane and incubated for 5 min at room temperature. The aqueous phase was measured at 570 nm (Ht5) using a Multiskan microplate reader (Multiskan FC, Thermo Scientific). The hydrophobicity was calculate using the equation: Hydrophobicity (%) = $(1 - H_{t5}/H_{t0}) \times 100$.

Adhesion and invasiveness assay. Adherence and invasiveness assays were performed according to the method described previously (Biswas et al. 2000). Chicken fibroblast cell (DF1) and bovine mammary gland cell line (MacT) were used. Briefly, 100 µl bacterial suspension, containing CFU approximately 100 times higher than host (MacT or DF-1) cell number, was inoculated into triplicate wells of a 24-well tissue culture plate containing semi-confluent monolayers of MacT or DF1 cells. Infected monolayers were incubated for 1 h at 37³C under a 5% CO2 humidified atmosphere. The infected monolayers were washed five times with DMEM containing 1% Fetal Bovine Serum (FBS) and then reincubated for another 1 h in fresh DMEM containing 10% FBS and 250 µg/ml of gentamicin. The monolayers were washed three times with DMEM containing 1% FBS and lysed with 0.1% Triton X-100 for 15 min. The suspensions were diluted and the numbers of viable bacterial cells were determined on LB agar plates.

Growth Performance of S. Gallinarum, S. Pullorum and L. plantarum in the Presence of Pomace Extracts in Broth, Fecal Medium or Water. For growth inhibition assay in broth, *S.* Gallinarum and *S.* Pullorum were grown in LB broth and *L. plantarum* was grown in MRS broth, respectively, at 37 °C for 18 h. Then the cells were pelleted by centrifugation at 4000×g for 5 min and resuspended in phosphate buffered saline (PBS, pH 7.4). The optical density of the bacterial cell suspensions were adjusted to 0.1 at OD600 using spectrophotometer (PerkinElmer, MA). In the wells of 24-well culture plate, LB or MRS broth with 1.0-2.0 mg AGE/L blackberry or blueberry pomace extracts were prepared by adding required volume of extracts from the stock solutions. Autoclaved deionized water was added instead of the extracts in the control wells. Ten microliter bacterial suspension containing approximately 2×10^6 cfu was added to each of the well of 24-wells plates. Plates were incubated for various time points (24, 48 and 72 h) at 37˚C with shaking at 120 rpm. After incubation, bacterial suspensions were made serial dilution in PBS and plated on LB agar for *S.* Gallinarum and *S.* Pullorum or MRS agar for *L. plantarum* to determine the colony forming units (cfu).

For growth inhibition assay in fecal medium, chick feces was collected from poultry facility at the University of Maryland, College Park on campus farm. One gm feces was mixed with 5 mL deionized water to make a semisolid medium and sterilized by autoclaving. Various concentrations of blackberry or blueberry pomace extracts were added to semisolid fecal medium

in 24-well culture plate. *S.* Gallinarum, *S.* Pullorum and *L. plantarum* inoculums were prepared as described above and inoculated into the fecal medium. Plates were incubated for 24, 48, and 72 h at 37˚C with shaking at 120 rpm followed by dilution in PBS and plating on agar medium for bacterial counts.

For growth inhibition assay in water, similar protocol was applied. Various concentrations of blackberry or blueberry pomace extracts were added to autoclaved tap water and bacterial suspensions were added to each well. Plates were incubated for 15, 30, 45 and 60 min at 37˚C with shaking at 120 rpm followed by dilution in PBS and plating on agar medium for bacterial counts.

Co-culture of pathogen and probiotic in fecal medium. Co-culture of *S.* Gallinarum and *L. plantarum* or *S.* Pullorum and *L. plantarum* was carried out in semisolid fecal medium in the presence or absence of blackberry or blueberry pomace extracts and compared to the growth of pathogens alone in the same medium. Pathogen and probiotic were attempted to mix equal volume of bacterial suspension (1:1) containing approximately 106 cfu/mL. Inocula were prepared in PBS as described above. Culture tube containing 980 µL semisolid fecal medium with various concentrations of blackberry or blueberry pomace extracts and 10 µL of each bacterial suspension was incubated for various time points (0, 24, 48, and 72 h) at 37 °C with shaking at 120 rpm. Viable cell counts were determined by the serial-dilution method using LB agar for *S.* Gallinarum and *S.* Pullorum, and MRS agar for *L. plantarum*, respectively.

Determination of the effects of BPE on cultured host cells. Viability of cultured INT407, DF1, and HEK001 cells in the presence of various concentration of BPE was measured following the method described previously (Strober, 2001) with some modifications. In brief, 2×10^5 cells were inoculated into triplicate wells of a 24-well tissue culture plate containing serum-free Keratinocyte-SFM medium containing 5 ng/mL EGF (DMEM with 10% FBS for INT407 and DF1) incubated for 24 hr at 37˚C under 5% CO2. After incubation, cell monolayers were washed with Keratinocyte-SFM medium for three times and 1 mL of fresh Keratinocyte-SFM medium containing 5 ng/mL EGF and various concentrations of BPE (0, 50, 100, 200, 400, and 800 µg GAE/mL) were placed in triplicate wells. The plate was incubated for 2 hr at 37˚C under 5% CO2, the medium was decanted and viability assay was carried out with 0.4% Trypan Blue solution (Sigma, MO). Treated HEK001 cell monolayers were also imaged with Nexus 6 (Google Play

Store) and the images were processed in Microsoft Office Picture Manager (Version 2010). Treated HEK001 cell monolayers were also used to extract RNA with Quick-RNA MiniPrep Kit (Zymo Research) for quantitative RT-PCR assay.

Statistical Analysis. All data were analyzed using the Statistical Analysis System software (SAS, Institute Inc., Cary, NC, USA). One-way analysis of variance (ANOVA) was used, followed by Tukey's test to determine significant differences among treatments at $P < 0.05$.

Results

Inhibition of P. multocida growth with berry pomace extracts. We found that pomace extracts from both blackberry and blueberry and citrus oil inhibited growth of *P. multocida* (Fig. 3.1). Paper disks containing 30 µg blackberry pomace phenolics and 22.5 µg blueberry pomace phenolics created clear zone of diameter 11 mm and 9.5 mm respectively (Fig. 3.1). These clear zones visibly indicated the growth inhibitory effect of blackberry and blueberry pomace extracts on *P. multocida*. In the same study, we also found that citrus essential oil showed inhibitory effect.

Time dependent inhibitory effect of pomace extracts on P. multocida growth. The Minimum Bactericidal Concentration (MBC) of blackberry pomace extract was 0.3 mg/ml; that was slightly lower than blueberry pomace extract. MBC for blueberry pomace extract was 0.4 mg/ml (Table 3.1). In the same study, we observed that 0.05% of citrus oil inhibited the growth of *P. multocida*. We also found that both blueberry and blackberry pomace extracts, and citrus oil showed higher antimicrobial activity during incubation with continuous agitating condition. At that condition, MBC values for blackberry and blueberry pomace extracts, and citrus oil decreased to 0.05 mg/ml, 0.25 mg/ml and 0.025% respectively. Based on these results, we decided the concentration for both pomace extracts for *P. multocida* growth inhibition and bactericidal effect assays at various time points. It was found that growth of *P. multocida* was inhibited with various concentrations of blackberry and blueberry pomace extracts in a time dependent manner. All three concentrations (0.3, 0.2 and 0.1 mg/ml for blackberry and 0.4, 0.3 and 0.2 mg/ml for blueberry) for both blueberry and blackberry pomace extracts showed inhibitory effect on *P. multocida*. We observed that all three concentrations (0.3, 0.2, 0.1 mg/ml) of blackberry pomace extract inhibited the growth of *P. multocida* completely after 24 hour time period whereas two higher concentrations of blueberry pomace extract (0.4 and 0.3 mg/ml) caused complete inhibition of *P. multocida* growth. The lowest concentration (0.2 mg/ml) of blueberry pomace extract used in this study inhibited the growth partially. This growth inhibition pattern suggested that an equilibrium condition where the rate of bacterial death and proliferation were almost equal (Fig. 3.2 B). We found that blackberry pomace extract showed delayed inhibitory effect on growth of *P. multocida* that became effective after 12 h of incubation whereas blueberry pomace extract showed its inhibitory effect within few hours of incubations. Compared to blackberry or blueberry pomace extracts, citrus oil showed more rapid

growth inhibition. Higher concentrations (0.05 and 0.025%) reduced CFU/ml of *P. multocida* more than 7 logs in 24 hours (Fig. 3.2 C). Whereas, 0.0125% citrus oil showed inhibitory effect upto 4 hours but did not affect *P. multocida* growth after 4 hours.

Effect of berry pomace extracts on cell surface hydrophobicity of P. multocida. Cell surface hydrophobicity of *P. multocida* treated with berry pomace extracts and citrus oil were monitored. Bacterial cells were treated with 0.6 mg/ml blackberry and blueberry pomace extracts or 0.05% citrus oil for 1 hour. For control, deionized water was used instead of extracts. Treatments with both blackberry and blueberry pomace extracts caused significant increase in cell surface hydrophobicity but citrus oil did not show any effect. The control groups had a hydrophobicity value of around 7% and group treated with citrus oil had similar hydrophobicity that of control group. But blackberry treated group had 13% and blueberry treated group had approximately 21%. So, it is evident that, both of the treatments caused two to three folds increasing in cell surface hydrophobicity (Fig 3.3).

Role of berry pomace extracts in Adhesion to and invasion into host cells by P. multocida. Both blueberry and blackberry pomace extracts reduced adhesion ability of *P. multocida* to both chicken fibroblast (DF1) and bovine mammary gland (MacT) cells. In DF1 cells, number of adhered bacteria decreased more than 50% in the presence of both blackberry and blueberry pomace extracts (Fig 3.4). Similarly, the reduction of adherence ability of *P. multocida* to MacT cells was observed. We found that both pomace extracts reduced the adherence ability of *P. multocida* to MacT cells more than 80% (Fig 3.4). Invasiveness of *P. multocida* in the presence of both blackberry and blueberry pomace extracts were also monitored. We found that both blackberry and blueberry berry pomace extracts reduced invasion ability of *P. multocida* into MacT cells by approximately 80%. In the same study, blueberry pomace extract reduced *P. multocida* invasion into DF1 cells by more than 30% whereas phenolics extract from blackberry pomace extract significantly increased (approximately 70%) the rate of invasion of DF1 cells by *P. multocida*. For adherence and invasion assay, the role of citrus oil on P *multocida* was not carried out due to high lytic ability of the citrus oil. *P. multocida* cells treated with citrus oil were lysed with very trace amount of oil within few minutes of incubation.

Growth performance of probiotic and pathogens in broth. We found that bioactive extracts from both blackberry and blueberry pomace inhibited the growth of *S.* Gallinarum and *S.* Pullorum in LB broth significantly (Fig 3.5). Blackberry pomace extract, at a concentration of 2.0 mg AGE/L, reduced the growth of *S.* Gallinarum by approximately 5, 6 and 8 logs at 24, 48 and 72 h, respectively. The same concentration of blackberry pomace extract reduced the growth of *S.* Pullorum by 5 logs at 24 h and eliminated completely by 48 h of incubation. Blueberry pomace extract showed similar growth inhibition pattern. We observed that 2.0 mg AGE/L of blueberry pomace extract reduced the growth of both *S.* Gallinarum and *S.* Pullorum by more than 5 logs at 24 h and completely eliminated by 48 h (Fig 3.5). However, 1.0 mg AGE/L of blackberry or blueberry pomace extract did not show any inhibitory effect on the growth of any of these pathogens in this study. On the other hand, 1.0 or 2.0 mg AGE/L blackberry or blueberry pomace extracts did not affect the growth of probiotic *L. plantarum* at 24, 48 and 72 h time points in broth (Fig 3.5). We also observed that up to 4.0 mg AGE/L of blackberry or blueberry pomace bioactive extracts did not show any significant effect on the growth of *L. plantarum* in broth (data not shown).

Growth performance of probiotic and pathogens in fecal medium in the presence of BPE. In Fig 3.6, we showed the effect of blackberry or blueberry pomace bioactive extract on the growth of avian pathogenic *Salmonella* or probiotic *L. plantarum* in a simulated gut environmental conditional medium where poultry feces served as nutrient source. In this simulated gut conditional medium, blackberry pomace extract at a concentration of 2.0 mg AGE/L reduced the growth of *S.* Gallinarum and *S.* Pullorum by more than 4, 5 and 5 logs at 24, 48 and 72 h time points, respectively. We also found that the lower concentration of blackberry pomace extract (1 mg AGE/L) showed the inhibitory effect against these pathogens significantly. One mg AGE/L of blackberry pomace extract caused growth inhibition by more than 2 logs at each time points (24, 48 and 72 h) for both *S.* Gallinarum and *S.* Pullorum. Blueberry pomace bioactive extract showed slightly different growth inhibition pattern compared to blackberry pomace extract in fecal medium. Two mg AGE/L blueberry pomace extract completely inhibited *S.* Pullorum within 24 h but 1 mg AGE/L concentration caused complete inhibition of this pathogen at 72 h (Fig 3.6 B). The growth of *S.* Gallinarum was reduced significantly by both 1.0 and 2.0 mg AGE/L blueberry pomace extract (Fig 3.6 A). One mg AGE/L reduced *S.* Gallinarum growth by 3-5 logs at each

time points tested whereas *S.* Gallinarum growth went to undetectable level in the presence of 2 mg AGE/L of blueberry pomace extract at 24 h. However, *S.* Gallinarum cells revived at 48 and 72 h in the presence of 2 mg AGE/L blueberry pomace extract. On the other hand, the growth of probiotic *L. plantarum* did not differ significantly from the control in the presence of various concentration of blackberry or blueberry pomace extracts at different time points (Fig 3.6 C). Yet, numerical but not significant growth stimulation of *L. plantarum* was observed at 72 h in the presence of both blackberry or blueberry pomace extracts, where 2.0 mg AGE/L showed slightly higher growth stimulation compared to 1.0 mg AGE/L for both extracts (Fig 3.6 C).

Growth inhibition of S. Gallinarum and S. Pullorum in water. We observed that blackberry or blueberry pomace extract were able to completely inhibit avian pathogenic *Salmonella* within 1 h in autoclaved tap water (Fig 3.7). The log cfu of both *S.* Gallinarum and *S.* Pullorum remained stable in tap water over the period of 60 min in absence of berry pomace extracts. Blackberry and blueberry pomace extracts showed different effects in terms of amount of time required to inhibit the pathogens. *S.* Gallinarum was completely inhibited by 1.0 mg AGE/L of blueberry and blackberry pomace extracts at 45 and 60 min respectively in tap water. Both bioactive extracts of berry pomaces inhibited these poultry pathogens at a concentration of 2 mg AGE/L by 30 min completely, but blueberry pomace extract showed quick activity compared to blackberry pomace extracts. Blueberry pomace extract inhibited the growth of *S.* Gallinarum by more than 4 logs within 15 min, whereas blackberry pomace extract caused a reduction of just 0.5 logs at that time period (Fig 3.7 A). Growth of *S.* Pullorum was inhibited completely by 2.0 mg AGE/L blueberry and blackberry pomace extracts at 30 and 45 min, respectively. At the lower concentration (1 mg AGE/L), both extracts required 60 min to inhibit completely but inhibition pattern suggested that blueberry pomace extract inhibited *S.* Pullorum at a faster rate compared to blackberry pomace extract over the period of 60 min (Fig 3.7 B).

Co-culture of L. plantarum and S. Gallinarum in the Presence of Berry Pomace Extracts. In Fig 3.8, we showed the growth performance of *S.* Gallinarum and *L. plantarum* when co-cultured in semisolid fecal medium. *L. plantarum* reduced the growth of *S.* Gallinarum by approximately 1, 2 and 2 logs at 24, 48 and 72 h, respectively in co-culture condition compared to *S.* Gallinarum grown alone in fecal medium. But in the presence of 1 mg AGE/L blackberry or blueberry pomace

extracts in co-culture, no detectible growth of *S.* Gallinarum was observed after 24 h (Fig 3.8 A). On the other hand, growth and survivability of *L. plantarum* remained unchanged regardless the growth condition, grown alone or co-cultured with *S.* Gallinarum in fecal medium. We observed that in the presence of 1 mg AGE/L blackberry pomace extract, the growth of *L. plantarum* in co-culture with S. Gallinarurm increased significantly by ~0.5 log at 48 h. Both blackberry and blueberry pomace extracts numerically stimulated *L. plantarum* growth at 72 h compared to the control at the same time point (Fig 3.8 B).

Co-culture of L. plantarum and S. Pullorum in the Presence of Berry Pomace Extracts. Growth performance of *S.* Pullorum in co-cultured with *L. plantarum* in fecal medium is shown in Fig 3.9. When co-cultured with *L. plantarum* in fecal medium, the growth of *S.* Pullorum was reduced by 1, 2 and 3 logs at 24, 48 and 72 h, respectively compared to control at these time points. However, in the presence of 1 mg AGE/L blackberry or blueberry pomace extracts in co-culture of *L. plantarum* with *S.* Pullorum reduced to non-detectable level of *S.* Pullorum after 24 h (Fig 3.9 A). Growth pattern of *L. plantarum* was unaltered whether grown alone or in co-culture with *S.* Pullorum. But when 1 mg AGE/L blackberry or blueberry pomace extracts were present in the co-culture medium, *L. plantarum* was stimulated significantly by 0.5-1.5 logs at 48 and 72 h compared to the control (Fig 3.9 B).

Effect of BPE on host cell viability. Trypan blue exclusion assay indicated a dose dependent reduction in the viability of adherent host cells to the plastic surfaces (Fig 3.10). The percentages of 96.06, 92.72, 92.42, 91.51, 90.61, and 87.88 of adherent HEK001 cells to the plastic surfaces were remained viable after 2 hr treatment with 0, 50, 100, 200, 400, and 800 µg GAE/mL of BPE, respectively (Fig. 3.10). Usually, HEK001 human cultured keratinocytes are large, epithelial, round, adherent cells growing as a confluent monolayer and after 2 hr exposure to the tested concentrations of BPE, neither substantial changes in cell morphology, nor any detachment from the surface were noticed microscopically. However, a slight dehydration was observed with the increasing concentrations of BPE. Investigation into the expression of HEK001 genes playing important roles in necrosis, apoptosis, and autophagy pathways indicated that treatment with BPE induced the expression of Bcl-2 (inhibitor to apoptosis and autophagy pathways) by ~ 2 while down-regulating TRADD and Bax genes (inducers of apoptosis pathway) by 2-3 folds (Fig. 3.10).

No significantly differential expression of host (HEK001) cells was observed in the other tested genes (TRAF2, Ripk1, Map1Lc3, Casp3, APAF1, BECN1, MLKL, Atg12, Cacp8, and Ripk3) tested.

Anti-inflammatory effect of BPE on chicken macrophage cells (HD11). Infection of HD11 cell with *C. jejuni* did not cause any detectable alteration in the expression of inflammatory genes. We also infection the HD11 cell with Avian Pathogenic *E. coli* (APEC) which resulted in higher level of expression of inflammatory genes. However, in the presence of BPE, the expression of pro-inflammatory cytokine genes, e.g., IL-1B, IL-6, and NOS were significantly downregulated (Fig. 3.11).

Discussion

Uses of antimicrobial compounds as feed supplement and/or veterinary drugs in animal infectious diseases treatment are important for safer and economical production of farm animals. Growing antimicrobial bacterial resistance pattern is also an emerging issue and there is an unsolved debate about the role of antimicrobial use in farm animal production. Currently, organic products are getting popularity and growing faster than ever but microbial safety of the products and production lost due to various animal diseases in the absence of antimicrobial are making them fragile. Considering these emerging issues, we aimed to find an alternative and natural organic antimicrobial compounds for production of both conventional and organic farm animals.

For alternative antimicrobials from natural sources for organic animal production and replacing synthetic antimicrobial for conventional farming, in this study we extracted the bioactive compounds from the byproducts of berries using ethanol-water and tested its inhibitory effect on the common poultry and cattle pathogen, *P. multocida*. We also tested the citrus oil, a byproduct of orange juice company, in inhibition of *P. multocida* growth. All the antimicrobials used in this study, blackberry and blueberry pomaces extracts and citrus oil, inhibited the growth of *P. multocida* effectively. In previous studies, we found that blueberry and blackberry juice inhibited the growth of human enteric bacterial pathogens including *Campylobacter jejuni*, *Salmonella* enterica serovar Typhimurium, enterohemorrhagic *E. coli* and *Listeria monocytogenes* (Biswas et al 2012, Yung et al 2013). In this study, we showed that extracts from the byproducts of both blueberry and blackberry inhibited the poultry pathogen. Therefore, berry juices or its extracts could be potential antimicrobial for human food preservation or supplement whereas the cheap organic and consumer friendly byproducts of berries could be an alternative feed supplement for farm animal specifically poultry. We also observed that susceptibility to these bioactive compounds increased due to agitation, which indicates increased contact between phytochemicals and bacterial cell is important for inhibition. Lacombe et al., (2013) found that blueberry phytochemicals inhibit bacteria by disrupting bacterial cell wall. We hypothesize, citrus oil and berry pomace phytochemicals have affinity towards outer membrane component of *P. multocida*, hence agitation caused increased interaction of these phytochemicals to outer membrane components and ultimately increased inhibition was found. Further experiments are underway to justify this hypotheses.

In respect of required time for effectiveness, blueberry pomace extract inhibited *P. multocida* growth faster compared to blackberry. Though, a higher concentration of blueberry pomace extract was needed to inhibit similar degree of *P. multocida* growth compared to blackberry pomace extract. Compositional differences in bioactive phytochemicals present in these two extracts might be the main reason of different rate of *P. multocida* growth inhibition. Compared to both blackberry and blueberry pomace extracts, citrus oil showed faster growth inhibition, even 0.0125% reduced *P. multocida* growth upto 4 hours but growth inhibition waned after that time period, which indicates exhaustion of responsible bioactive compounds in citrus oil. Limonene, a volatile monoterpene, is the major component of citrus essential oil and shows bactericidal effect against pathogenic bacteria (Fisher et al., 2008; Espina et al., 2013). So our results indicated that evaporation of limonene caused reduced inhibitory effect over longer period time.

We also checked physicochemical property of *P. multocida* after treatment with natural bioactive extracts. When the bacterial cells were treated with blackberry and blueberry pomace extracts, the physicochemical properties of *P. multocida* specifically cell surface hydrophobicity was increased significantly. Previously Arif et al., (1998) showed that inability to synthesize capsular material by *P. multocida* enhanced cell surface hydrophobicity which indicated a direct correlation between presence of capsule and cell surface hydrophobicity. As capsule is an important virulence factor of *P. multocida* (Harper et al., 2006) and play an important role in bacterial adherence to host cells (Al-haj et al., 2004). Increased hydrophobicity in the presence of berry pomace extracts indicated the capsule disruption. On the other hand, treatment with citrus oil did not alter cell surface hydrophobicity of *P. multocida*.

In addition, we also observed that berry pomace extracts altered host cell-*P. multocida* interactions. Adherence to DF1 and MacT cells decreased significantly due to the pre-treatment of bacterial cells with pomace extracts. This decreased adherence provided further support in capsular disruption in the presence of pomace extracts. In another study, Esslinger et al., (1994) have found decreased adhesion of *P. multocida* serotype A to HeLa cells due to treatment with hyaluronidase which acted against hyaluronic acid in capsule. Borrathybay et al., (2003) found the opposite, an increased adherence to chicken embryo fibroblast (CEF) due to treatment with hyaluronidase but decreased adherence after treatment with trypsin and hyaluronidase combined, indicating the importance of surface protein on *P. multocida* adherence. Dubreuil et al., (1992) suggested capsule

as well as outer membrane proteins are important for *P. multocida* virulence. Decreased adherence in our study suggests, berry pomace extracts not only disrupts bacterial capsule but affects surface proteins also. *P. multocida* invasion into MacT cells also decreased due to treatment with berry pomace extract phenolics. Similar decrease in invasion was found in DF1 cells when treated with blueberry pomace extract but interestingly an increased invasion occurred due to treatment with blackberry pomace extract. Further study is necessary to explain this phenomenon.

Maintaining the well-being of poultry health is always crucial to the producers in terms of productivity and product safety, irrespective of the rearing system adopted: MCLF or backyard, free-range pasture organic or conventional poultry farming. In addition, prognosis of disease and implementation of appropriate treatment strategies are important to ensure safer and cost effective production of poultry. Further, increased antimicrobial resistance among zoonotic pathogens have raised a multifaceted debate on whether nontherapeutic use of antibiotics in farm animal/livestock production should be endured any longer. Due to vast popularity of organic products among consumers, sustainable organic agricultural systems are now gaining increased attention among farmers. However, microbiological safety of organic products and production loss followed by economic instability due to various animal diseases in the absence of antibiotics are making organic farming practice an unprofitable enterprise. Considering these emerging issues, we focused on finding alternative and natural bioactive compounds to apply in both conventional and organic farming systems that will potentially replace antibiotics and chemical antimicrobials.

In this study, we used bioactive antimicrobial components extracted from byproducts of blackberry or blueberry called pomace and tested their inhibitory effect on avian pathogenic *Salmonella* strains such as *S.* Gallinarum and *S.* Pullorum. We also evaluated the efficacy of these pomace extracts to alter the growth dynamics of probiotic or modulate the growth of these poultry pathogens. We found that blackberry and blueberry pomace extracts inhibited the growth of *S.* Gallinarum and *S.* Pullorum in broth. In our previous studies, we also found that these extracts inhibited the growth of human enteric bacterial pathogens including *Campylobacter jejuni*, *Salmonella* enterica serovar Typhimurium, enterohemorrhagic *E. coli* and Listeria monocytogenes and poultry pathogen *Pasteurella multocida* (Biswas et al., 2012; Yang et al., 2014; Salaheen et al., 2014a; Salaheen et al., 2014b). Therefore, these bioactive compounds extracted from cheap, organic and consumer friendly byproducts of berry juice industry could act as natural feed additives for livestock production, especially for poultry.

Fecal materials make up a major portion of cecum content in poultry and most of the gut bacteria come in close contact to feces during their life cycle. As a result, fecal medium can be considered as a simulated poultry gut composite and bacterial behavior in fecal medium should partly correlate with in vivo situation. So we were interested to investigate the effects of bioactive components extracted from blackberry and blueberry pomace extracts on the growth of avian pathogenic *Salmonella* and probiotic *L. plantarum* in fecal medium. However, the quality and composition of fecal medium varies depending on the poultry diet, hence it might provide variable results. In this experiment, we found that fecal medium served as a good source of nutrient for the growth of both the pathogens and probiotic. In the presence of 1.0 mg AGE/L blackberry or blueberry pomace extracts, the growth of *S.* Gallinarum or *S.* Pullorum was reduced significantly in fecal medium whereas no such reduction was found in broth which suggested that these pathogens were more vulnerable in the semisolid fecal medium compared to broth. On the other hand, numerical increase on the growth of *L. plantarum* at 72 h time point was observed in the presence of blackberry or blueberry pomace extract in fecal medium but not in broth. This result suggested that, *L. plantarum* was able to switch its preference for source of nutrient and utilized some portions of the berry extract as nutrient in fecal medium but due to high nutritious condition in broth, this phenomenon was not observed while grown in broth.

S. Gallinarum and *S.* Pullorum are able to transmit and colonize in poultry gut via both vertical and horizontal route (Berchieri et al., 2011; Olasupo et al., 2003; Prakash, 2006). Fecal-oral route plays important role in horizontal transfer because pathogens remain viable in feces and water for many days. We found that, in the presence of blackberry or blueberry pomace extracts in water both *S.* Gallinarum and *S.* Pullorum were completely inhibited within 1 h. This supports the hypothesis that bioactive berry extracts will be able to reduce horizontal transfer of these pathogens when used as water supplement. Blueberry pomace extract inhibited *S.* Gallinarum and *S.* Pullorum growth quicker compared to blackberry in water. Difference in bioactive phytochemical profile of these two extracts might be the main reason of dissimilar rates of growth inhibition.

Previous studies have reported that *Lactobacillus* can inhibit the growth of pathogens in co-culture condition (Williams, 1981; Soria et al., 2013; Servin, 2004). We also found that *L. plantarum* reduced the growth of *S.* Gallinarum and *S.* Pullorum when co-cultured in fecal medium. In the presence of even 1.0 mg AGE/L blackberry or blueberry pomace extracts,

inhibition of pathogens was more drastic when co-cultured with *L. plantarum* which suggested a synergistic relationship between the probiotic and berry extract. Berry extracts are good source of anthocyanin, hydroxycinnamic acid, hydroxybenzoic acid, polymeric tannins and other polyphenols (Puupponen-Pimiä et al., 2005; Hütt et al., 2006). Bioconversion of phenolic acids and anthocyanin by *Lactobacillus* have been reported (Salaheen et al., 2014c; Herrmann 1989). Though, metabolic pathways of *Lactobacillus* to utilize phenolic compounds have not been unearthed yet, it has been shown that bioconversion of phenolic compounds of some food caused an increase in their antioxidant activity (Rodríguez et al., 2008). We hypothesize that, partial degradation of berry pomace phenolics by *L. plantarum* increased bioavailability and antimicrobial properties of these derivatives, hence reduced avian pathogenic *Salmonella* while in co-culture.

Conclusions

1. BPE inhibited the growth of *P. multocida*, *S.* Gallinarum, and *S.* Pullorum *in vitro*.
2. BPE stimulated the growth of probiotic *Lactobacillus* in fecal medium and provided selective bias towards probiotics when co-cultured with pathogenic *Salmonella*.
3. Anti-inflammatory property of BPE reduced the expression of pro-inflammatory cytokines in chicken macrophage HD11 after infection with Avian Pathogenic *E. coli*.
4. BPE showed no visible cytotoxicity or morphological alterations on cultured host cells.

List of Figures and Tables

Table 3.1. Antibacterial effect of berry pomace extracts on *P. multocida.*

Treatments	MBC (without agitation)	MBC (with agitation)
Blackberry	0.3 mg/ml	0.05 mg/ml
Blueberry	0.4 mg/ml	0.25 mg/ml

Abbreviation: Minimum Bactericidal concentration, MBC.

Fig. 3.1. Growth inhibition of *P. multocida* with blackberry (B) and blueberry (C) pomace extracts, compared to PBS (A) using disk diffusion method.

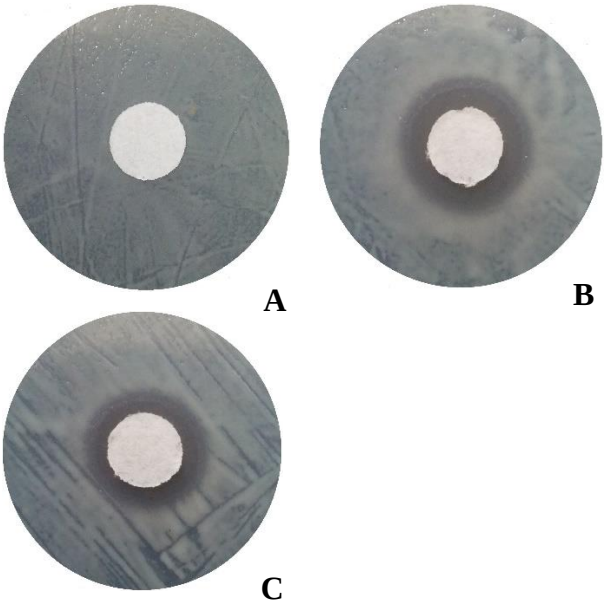


Fig. 3.2. Time dependent growth pattern of *P. multocida* in presence of various concentrations of blackberry (A) and blueberry (B) pomace extracts. Means of three different experiments were used.

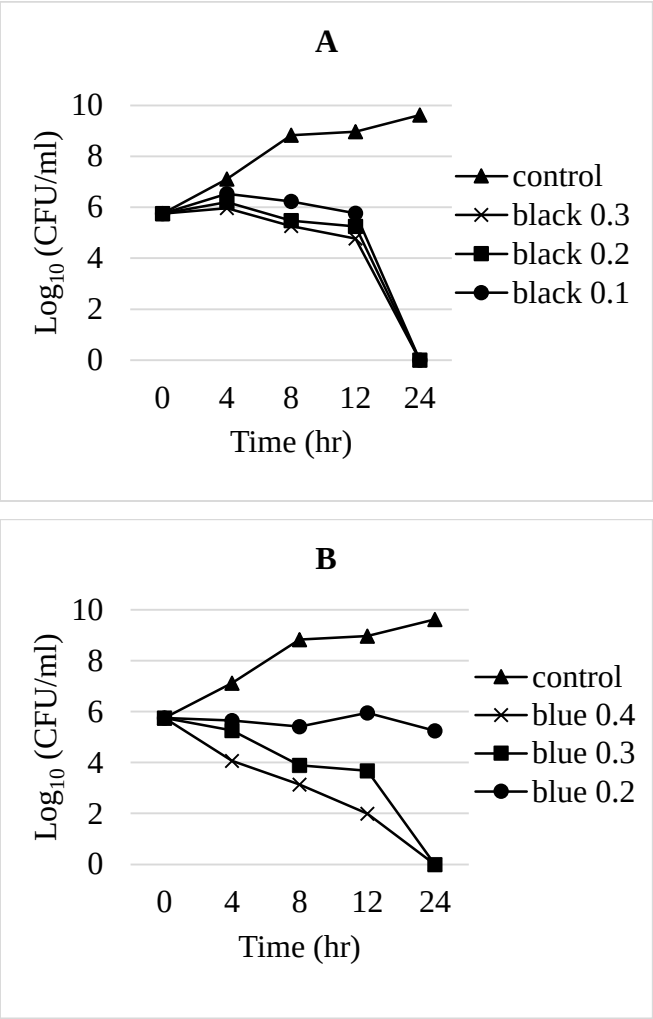


Fig. 3.3. Cell surface hydrophobicity of *P. multocida* treated with blackberry or blueberry pomace extracts. Bars containing * are significantly different from the control (P<0.05).

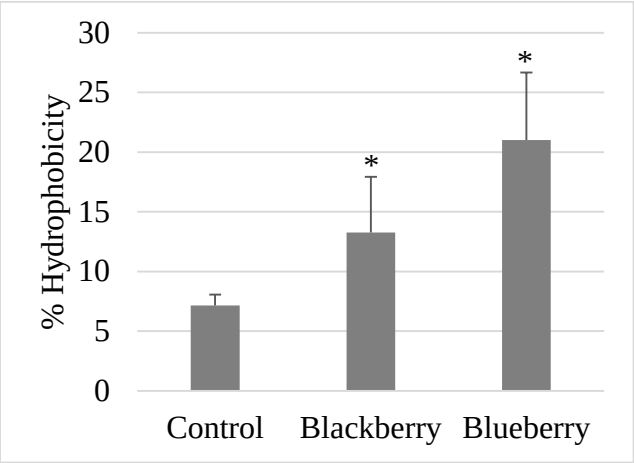
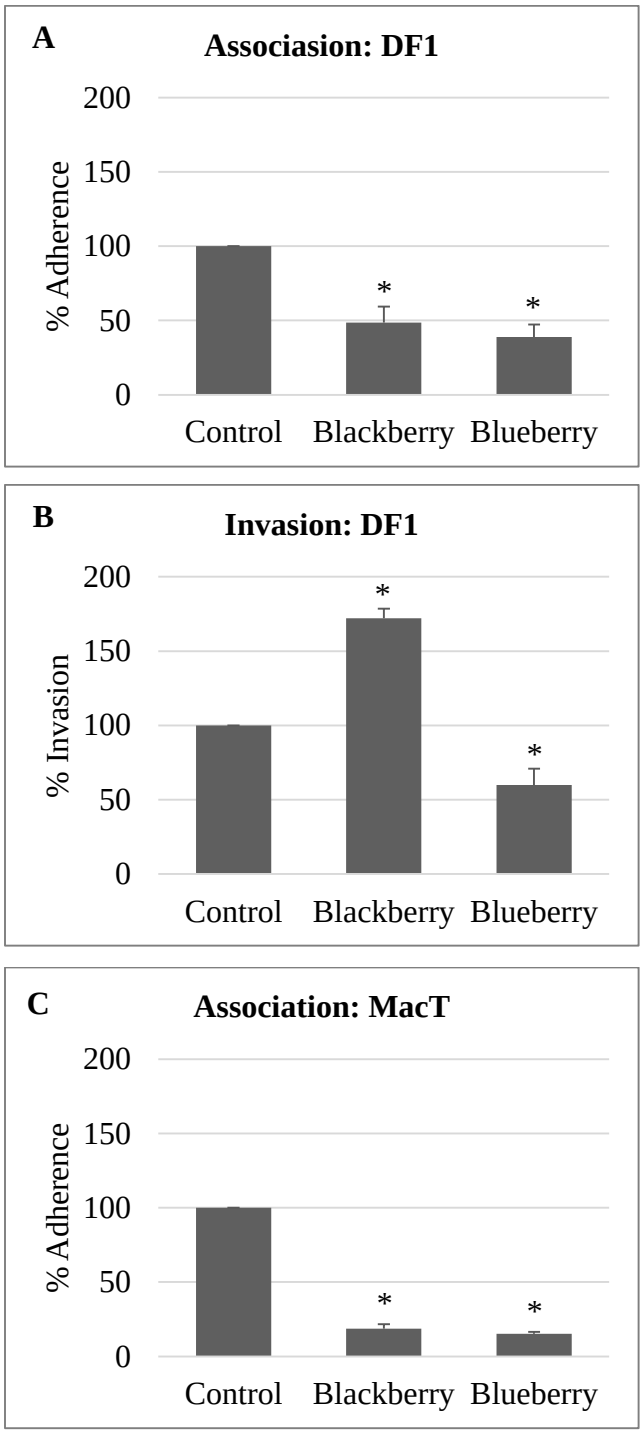


Fig. 3.4. Adhesion (A and C) and invasiveness (B and D) of *P. multocida* to DF1 and MacT cells respectively in presence of blackberry and blueberry pomace extracts. Bars containing * are significantly different from the control (P<0.05).



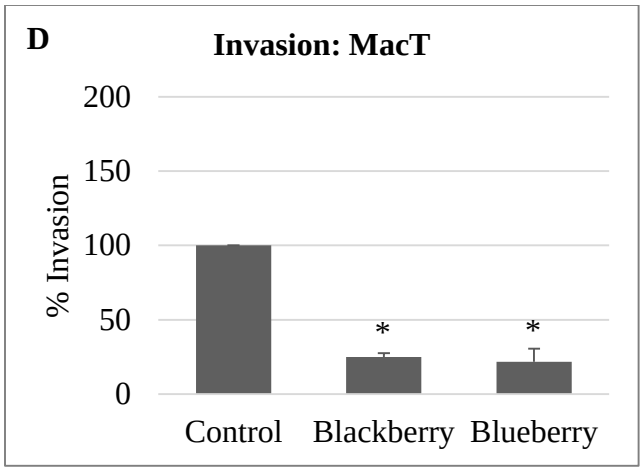

D
Invasion: MacT
200
150
100
50
0
% Invasion
*
*
Control
Blackberry
Blueberry

Fig 3.5. Growth performance of *S.* Gallinarum (A), *S.* Pullorum (B) and *L. plantarum* (C) in broth supplemented with 1.0 or 2.0 mg AGE/L of blackberry (Blk) and blueberry (Blb) pomace extracts. Bars containing * are significantly different from the control ($P < 0.05$).

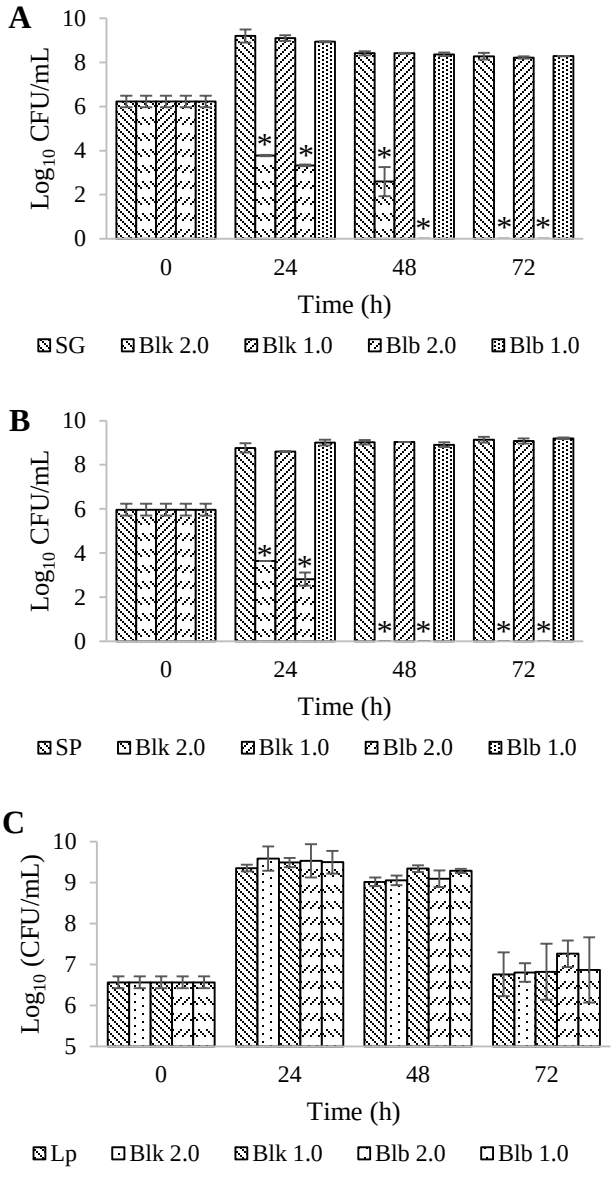


Fig 3.6 Growth performance of *S.* Gallinarum (A), *S.* Pullorum (B) and *L. plantarum* (C) in fecal medium supplemented with 1.0 or 2.0 mg AGE/L of blackberry (Blk) and blueberry (Blb) pomace extracts. Bars containing * are significantly different from the control ($P < 0.05$).

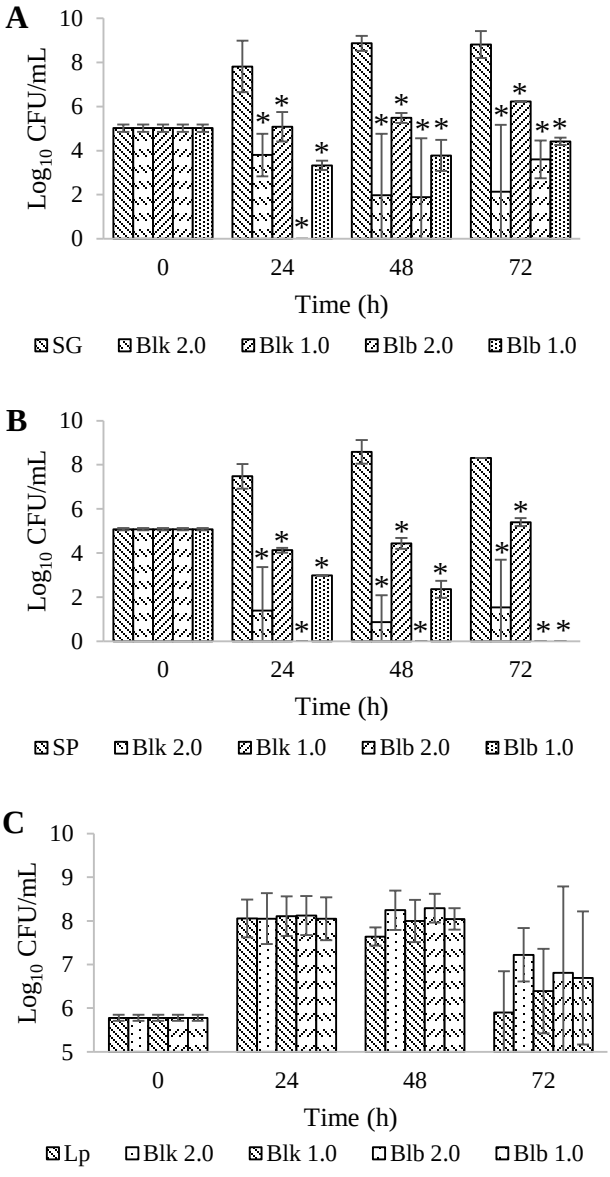


Fig 3.7. Inhibition of *S.* Gallinarum (A) and *S.* Pullorum (B) in deionized water supplemented with 1.0 or 2.0 mg AGE/L of blackberry (Blk) and blueberry (Blb) pomace extracts. Means from three independent experiments were used for each data point.

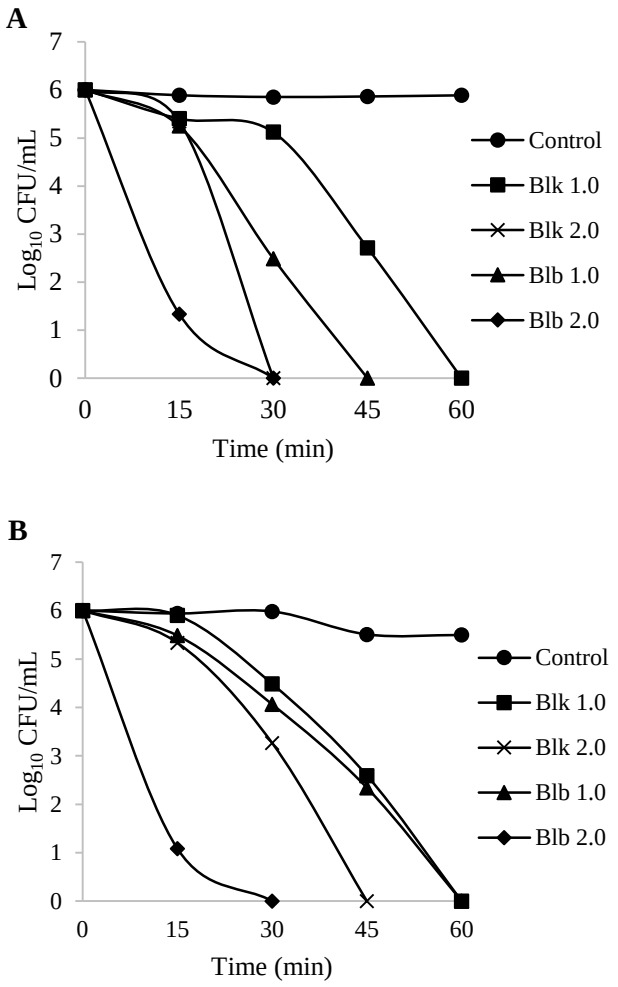


Fig 3.8. Growth performance of *S.* Gallinarum (A), and *L. plantarum* (B), when co-cultured in fecal medium supplemented with 1.0 mg AGE/L of blackberry (Blk) or blueberry (Blb) pomace extracts. Bars containing * are significantly different from the control ($P < 0.05$).

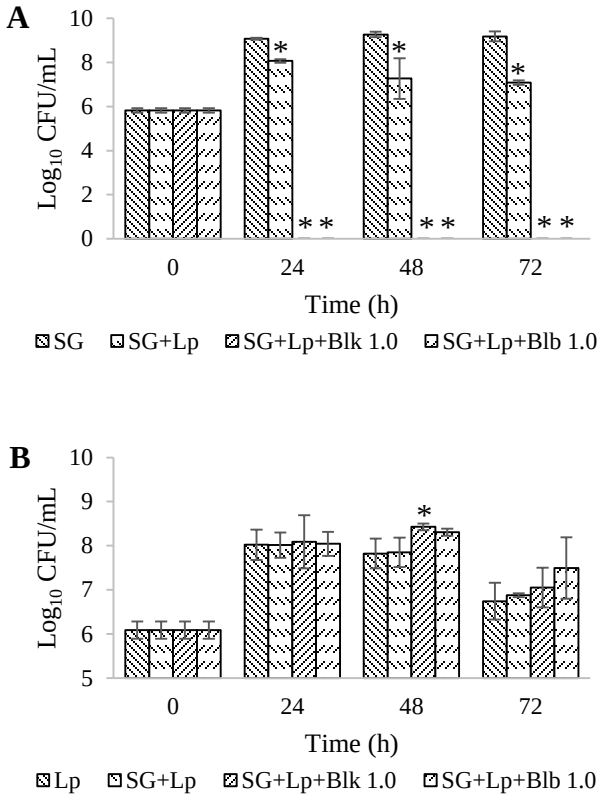


Fig 3.9. Growth performance of *S.* Pullorum (A), and *L. plantarum* (B), when co-cultured in fecal medium supplemented with 1.0 mg AGE/L of blackberry (Blk) or blueberry (Blb) pomace extracts. Bars containing * are significantly different from the control ($P < 0.05$).

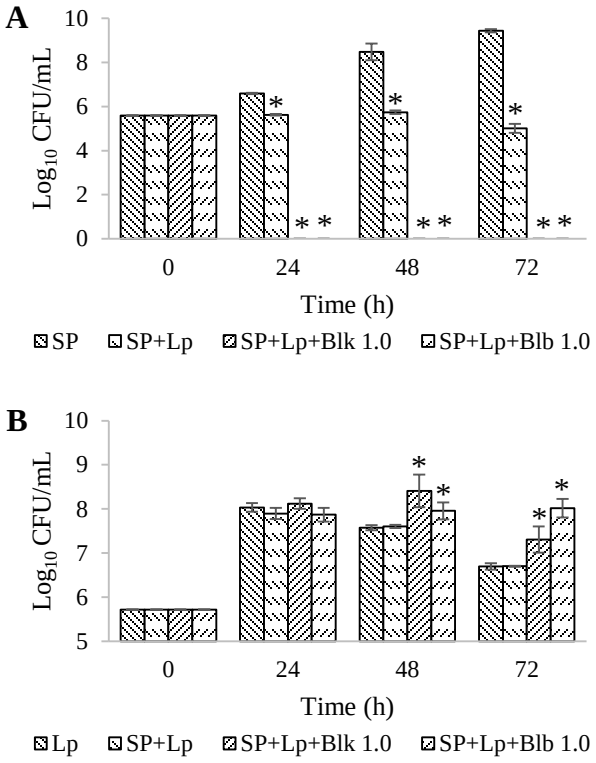
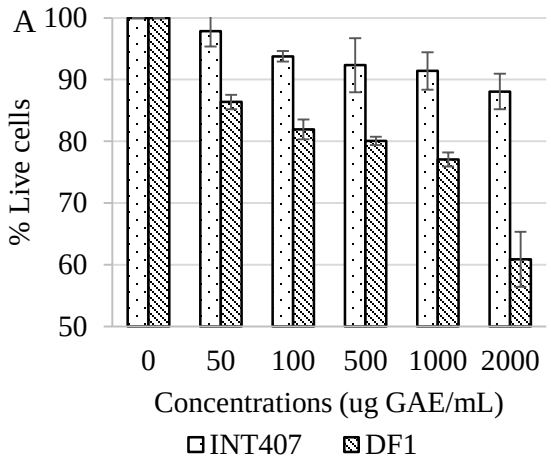
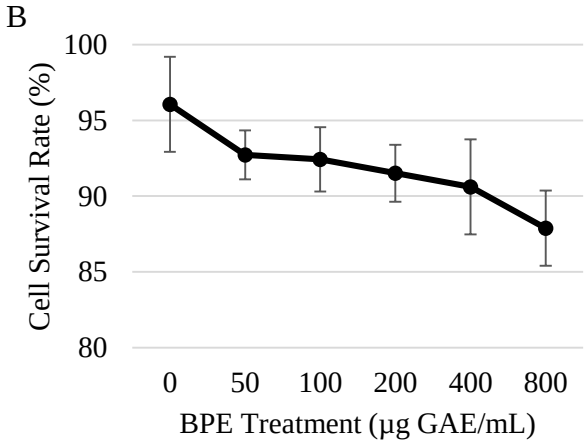
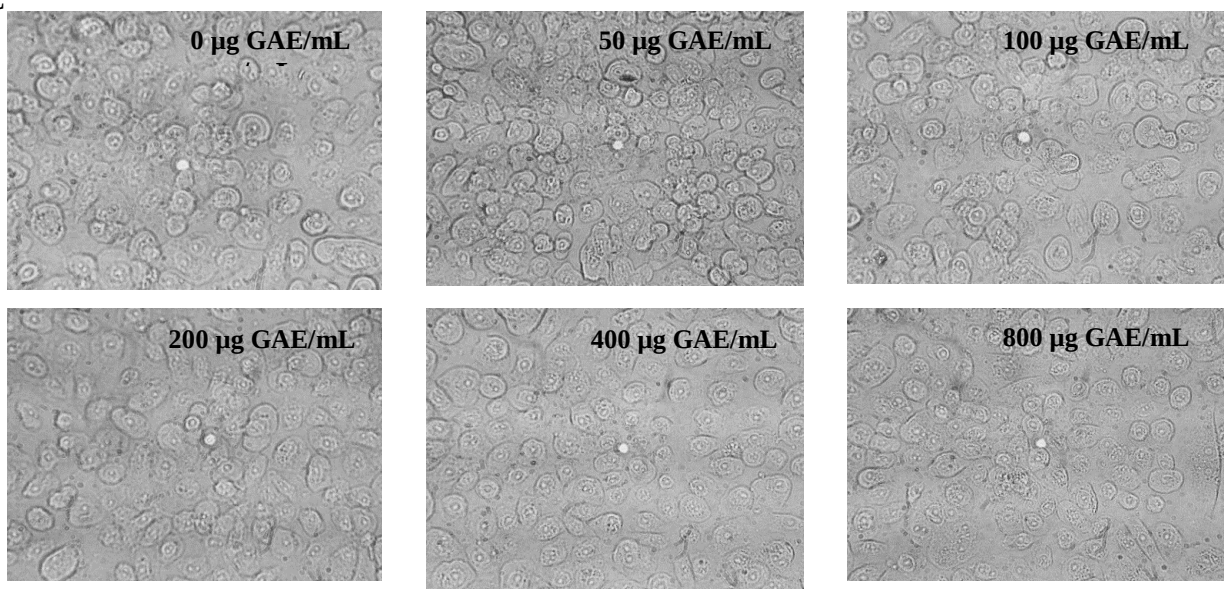


Fig 3.10. Cytotoxicity of BPE on host cell-lines: INT407, DF1, and HEK001using microscopic and Trypan Blue methods. A, survival rate of INT407 and DF1 cells; B, HEK001 cell survival rate at various concentrations of BPE; C, visualization of cell morphology in the presence of BPE; D, expression of the genes related to cell growth and apoptosis after treatment with BPE.





C


0 μg GAE/mL
50 μg GAE/mL
100 μg GAE/mL
200 μg GAE/mL
400 μg GAE/mL
800 μg GAE/mL

D

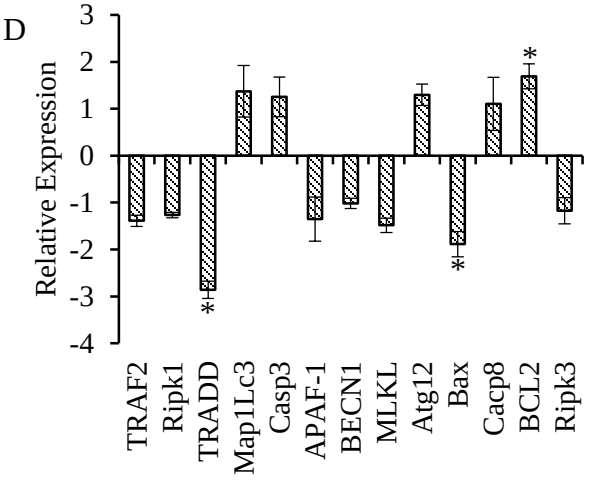
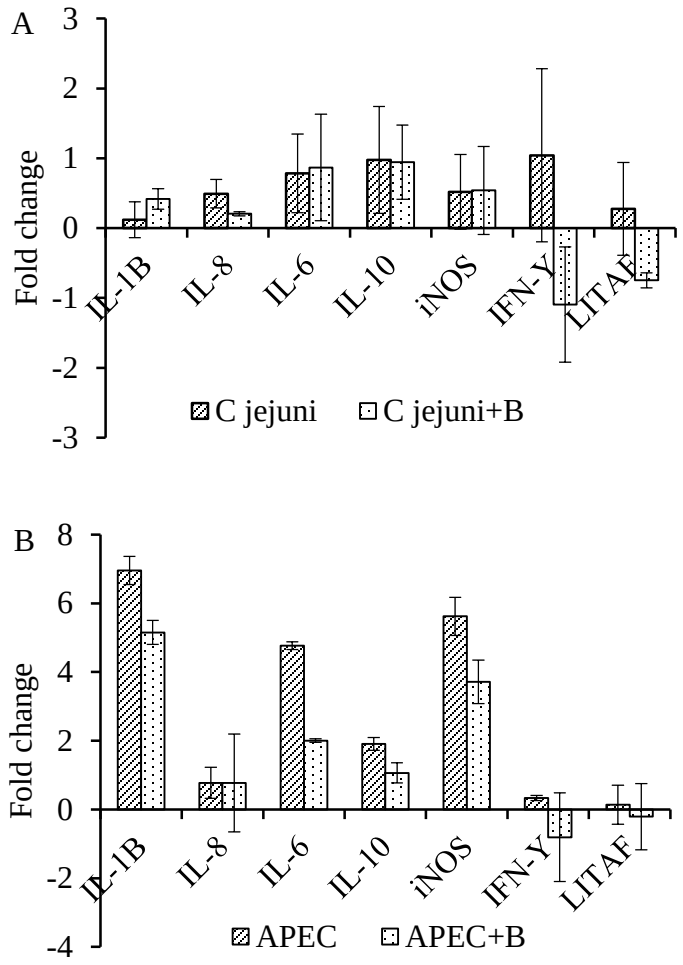

Relative Expression
3
2
1
0
-1
-2
-3
-4
*
*
*
TRAF2
Ripk1
TRADD
Map1Lc3
Casp3
APAF-1
BECN1
MLKL
Atg12
Bax
Cacp8
BCL2
Ripk3

Fig 3.11. Anti-inflammatory effect of BPE on chicken macrophage cells (HD11) using qRT-PCR. A, infection with *C. jejuni* in presence or absence of BPE; B, infection with Avian Pathogenic *E. coli* in presence or absence of BPE.



Chapter 4: Role of berry pomace extracts on *Campylobacter jejuni* colonization in poultry gut.

Introduction

Campylobacter jejuni, a major foodborne enteric pathogen, is among the leading causative agents of acute gastroenteritis in the world. According to the Centers for Disease Control and Prevention (CDC), *C. jejuni* causes one million illnesses, 19,000 hospitalizations and 76 deaths per annum in the US (Scallan et al., 2011). Raw and undercooked poultry and poultry products are considered one of the major sources of campylobacteriosis (Mughini-Gras and van Pelt, 2014). *C. jejuni* along with other species of *Campylobacter* colonize in the poultry gut as normal flora. The ability of *C. jejuni* to adhere to host intestinal epithelial cells plays a primary role in the enteropathogenesis, multiplication and colonization. Therefore, the adherence phase can be considered as a critical control point in early intervention strategies to prevent the colonization of *C. jejuni* in host gut. Important physicochemical and mechanical properties, i.e., auto-aggregation, hydrophobicity, cellular motility are associated with the adhesion ability of bacterial pathogens to the host epithelial cells (Parker et al., 2001; Saran et al., 2012), and these properties eventually lead to bacterial colonization followed by invasion. These phases of activities should be considered in the quest of intervention strategies to reduce the colonization of *C. jejuni* in poultry gut with the ultimate goal to prevent *C. jejuni* cross-contamination in poultry products and reduce the *C. jejuni* associated foodborne infections in humans.

Commonly used control measures against the colonization of *C. jejuni* in poultry gut include the use of antibiotics, synbiotics, and bacteriophages in feed and water. However, development of antibiotic resistance, low efficacy of synbiotics, and high strain specificity of bacteriophages render these control measures tricky. In response to increased public health concern on antibiotic resistance, the U.S. Food and Drug Administration has announced to gradually withdraw non-therapeutic use of antibiotics from farm animal production (Kuehn, 2014). Consequently, the search for alternative natural and green antimicrobials is now more essential than ever. Bioactive phenolics from berries, especially blackberry (*Rubus fruticosus*) and blueberry (*Vaccinium Corymbosum*) pomace as feed or water supplement to reduce pre-harvest level of *C. jejuni* contamination in farm animals, specifically poultry, might be a feasible alternative. Antimicrobial effects of phenolics present in berry fruits and their pomaces against enteric

bacterial pathogens have been extensively studied (Biswas et al., 2012; Puupponen-Pimiä et al., 2005; Salaheen et al., 2014a; Yang et al., 2014). In our previous studies, we showed the bactericidal effect of phenolic extracts from berry fruits on *Campylobacter jejuni, Salmonella* Gallinarum, *Salmonella* Pullorum, and *Pasteurella multocida* (Salaheen et al., 2014a, 2014b; Salaheen et al., 2015). Proposed mechanism of pathogen inhibition of these phenolics include damage of bacterial cell membrane (Lacombe et al., 2013), inhibition of extracellular microbial enzymes (Scalbert, 1991), distortion of microbial metabolism, and deprivation of substrates mandatory for microbial cell proliferation and pathogenicity (Puupponen-Pimiä et al., 2004). Importantly, synergisms among various phenolic derivatives act indiscriminately against benign and pathogenic bacteria.

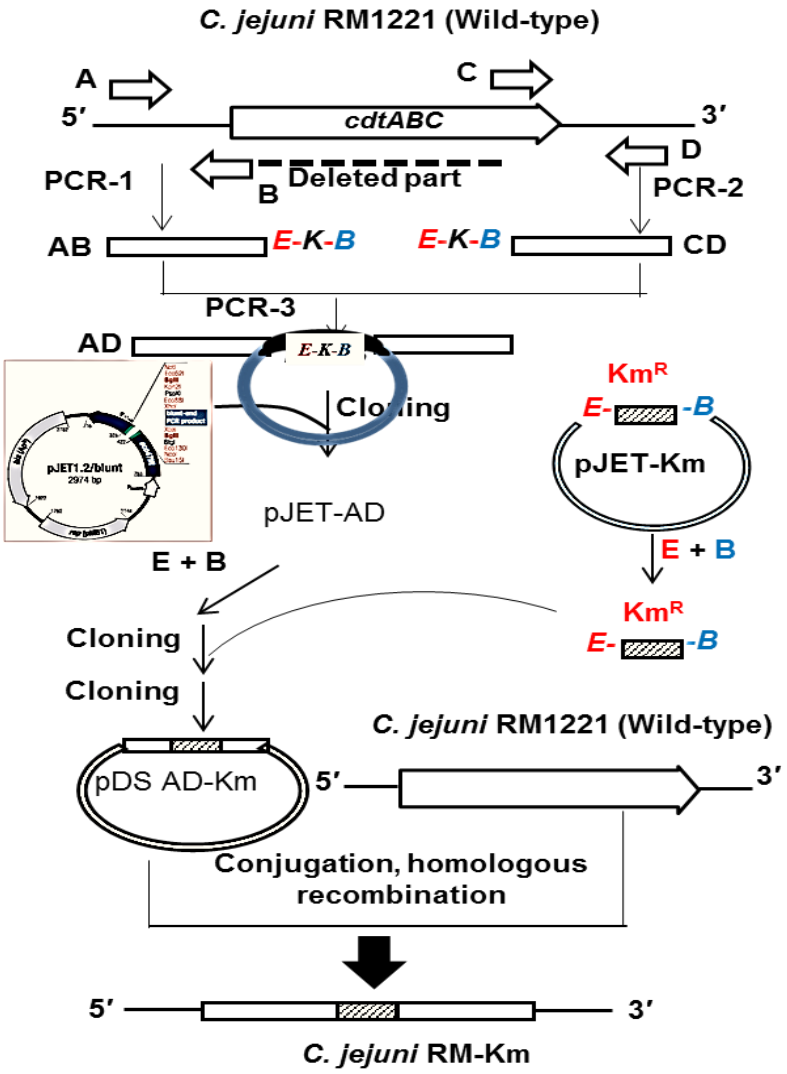
Therefore, in this study we aimed to evaluate the effect of these extracts on the colonization of *C. jejuni* in chick cecum. Findings from this study will provide significant insight into the alternative preventive and therapeutic antimicrobial regime to reduce *C. jejuni* infection by developing a new, effective, and green antimicrobial against bacterial infections.

Material and Methods

Preparation of pomace extracts and HPLC-tandem mass spectrometry (LC-MS/MS) analysis. Extracts were prepared according to the protocol previously described (Salaheen et al., 2014a). Total phenolic content in each extract was determined using spectrophotometric method (Singleton et al., 1999). Total phenolic content was expressed as Gallic Acid Equivalent (GAE). The pH of the crude extracts were 4.5-5 and pH varied depending on the treatment concentration. A phenolic screen was accomplished using HPLC-MS (Peng et al., 2014). Sample injections were 5 µL and separations were performed on an Agilent 1100 system, coupled to an Agilent MSD-TOF (time-of-flight) mass spectrometer. Reversed-phase liquid chromatography was used to separate the samples. A Waters Atlantis T3 column (3 µm, 150 x 2.1 mm i.d.) was used. A binary mobile phase consisting of solvent systems A and B was used in gradient elution where A was 0.1% formic acid (v/v) in ddH_2O and B was 0.1% formic acid (v/v) in acetonitrile. Mobile phase flow rate was 0.3 mL/min. The linear gradient was as follows: time 0 – 1 minute, 0% B; time 40 minutes, 90% B; time 41 minutes, 90% B; time 42 minutes, 0% B; time 52 minutes, 0% B. Following the separation, the column effluent was introduced by electrospray ionization (ESI) into the MSD-TOF. Samples were assayed, using positive mode ESI. Source parameters were: gas temperature 350˚C, gas flow 9 L/min, nebulizer 35 psi, fragmentor 125 V, capillary voltage 3500 V. Data were

acquired with a mass range of 75 - 1000 m/z. Accurate mass accuracy was guaranteed by the continuous infusion of Agilent Reference Mass Solution (G1969-85001). Individual chromatographic peaks were identified using Agilent's Mass Hunter Qualitative Analysis software (v. B.06). Compounds were identified using Agilent's Mass Profiler Professional software (v. 13.1). Peaks in duplicate injections were aligned to account for instrumental drifts in retention time and mass. Compounds were retained only if they appeared in both duplicate samples. Compounds were annotated by querying Agilent's METLIN human metabolite database, with a mass error criteria of < 5 ppm.

Insertion of genetic marker Kanamycin (Km) gene in C. jejuni RM1221. Kanamycin resistance cassette was infused to *C. jejuni* RM 1221 genome to be used as marked strain according to the protocol depicted in the figure. In brief, the AB and CD PCR products were fused together to generate AD product deleting the internal segment (dotted line) by a third PCR using A and D primers. By sequential cloning steps, *Km* gene was inserted in the middle of AD followed by final cloning into a suicide vector pDS132 and the resulting recombinant suicide plasmid was introduced into the wild-type *C. jejuni* RM1221 strain. After homologous recombination, desired mutant having *Km* insertion in place of *cdtABC* gene, was screened. Here, E-K-B represents EcoRI-KpnI-BamHI



recognition sequences. The new strain was named as *C. jejuni* RM-Km.

Natural colonization of chicks with Campylobacter. Natural colonization of *Campylobacter* in chick model provided with various concentrations of BPE was determined in triplicate trials. In each trial, 100 1-day-old Cobb-500 broiler chicks were obtained from Longenecker's Hatchery Inc, PA. Guidelines recommended in the Institutional Animal Care and Use Committee (IACUC, protocol number R-16-33) were followed for chick husbandry and were provided with commercially available crumbles (Purina Animal Nutrition, MO) with no antibiotic supplementation. In trial 1, chicks were assigned to 4 groups provided with only tap water or tap water with 0.1, 0.5, and 1.0 g AGE/L of BPE. In the following 2 trials, chicks were assigned into 4 groups of 25 chicks each in floor pans using a Completely Randomized Design consisting a negative control, a positive control, and two treatment groups. Negative control group A was provided with only tap water, positive control group B: tap water with AGP (a combination of Oxytetracycline 1 µg/mL, Erythromycin 2 µg/mL, Tylosin 2 µg/mL, Bacitracin 4 µg/mL and Neomycin sulfate 32 µg/mL), treatment group C: tap water with 0.1 g GAE/L of BPE. Treatment group D was provided with tap water with 0.1 g GAE/L of BPE and the treatment concentration was increased to 1 g GAE/L during last 72 h before euthanasia. This way the chicks were reared for 6 weeks. After three weeks, five chicks from each group were euthanized to check the natural colonization level of *Campylobacter* in chick cecum. After six weeks, all the birds were euthanized and ceca were separated. To check the *Campylobacter* colonization, approximately 200 g of cecum content was homogenized in one mL PBS, serially diluted and plated on Karmali *Campylobacter* agar for enumeration. Three representative presumptive isolates from each group were tested with *Campylobacter* specific PCR according to the protocol described by Salaheen et al., (2016). Cecum from each bird was considered an experimental unit for statistical analysis. The number of birds colonized by *Campylobacter* was compared using Fisher's exact test. Differences in the level of colonization (CFUs/g cecum content) were compared by first ranking the data and performing one-way analysis of variance (ANOVA) on the ranked data. Comparison of mean ranks was performed using Tukey's test.

Infection of chicks with C. jejuni RM-KM. A total of 120 chicks were used for 2 trials. Chicks were reared in Avian 12-Cage Isolator Unit. At first, total 60 chicks were used for the initial trial with

berry pomace extracts. The chicks were randomly assigned to control and test groups (20 chicks per group). Four cages of the unit were assigned to each group, so a total of 5 chicks per cage. Stocking density has been calculated and conforms the IACUC guideline. A Completely Randomized Design (CRD) were employed for this purpose. The reason behind using similar number of chicks in the control group was that, in this experiment the control itself (regular water) served as a treatment.

C. jejuni RM-Km was cultured on Blood Agar plates for 24 h under microaerophilic condition and transferred to saline to a concentration of 10^9 cfu/mL.100 uL of this suspension was fed to day-old chicks with oral gavage. For this purpose, chicks were held gently (one at a time). Animal feeding needle attached to plastic syringe was used. 100 uL *C. jejuni* RM-KM suspension was taken into the syringes, and this suspension was fed to one chick using the feeding needle. Chicks were checked for any injury related to the gavage procedure and transferred back to their respective cages. Chicks were kept in close inspection for atleast two days for any abnormalities in their eating or drinking behaviors. The chicks were provided tap water to group A, 1.0 mg AGE/L BPE in tap water to group B. For group C, chicks were provided with 0.1 mg AGE/L BPE throughout, except last 72 h before euthanization, the treatment concentration were increased to 1.0 mg AGE/L BPE in tap water. This way the chicks were reared for 3 weeks, fed with chicken starter diet (mesh) without growth promoters or synthetic chemicals. Water were provided in gravity feed water containers. After week 1, 8 chicks from each group were euthanized. After week 2, 8 chicks from each group were euthanized, and similarly, after week 3, rest of the chicks were euthanized.

Statistical Analysis. Quantitative RT-PCR data will be analyzed using the Statistical Analysis System software (SAS, Institute Inc., Cary, NC, USA). One-way analysis of variance (ANOVA) were used, followed by Tukey's test to determine significant differences among treatments at $p < 0.05$.

Results

Development of kanamycin cassette marked C. jejuni. We developmed a *C. jejuni* mutant possessing kanamycin resistance cassette incorporated in the genome for easy detection of the marked C. jejuni strain in chicken ceca. From the PCR band sizes indicated in Fig 4.1, it was clear that the clone contained right size of band representing kanamycin resistance cassette. We found three clones and used one for the further studies. The product size of the sequence of interest in wild type *C. jejuni* RM1221 was 3204 base paires, whereas the mutants should have been 2649 base paires and this phenomenon is evident in Fig. 1.

Phenotypic characteristics of the newly developed mutant indicated a similar growth pattern of the mutant compared to the wild type *C. jejuni* strain. We prepared the glycerol stocks of the mutants and stored at -80˚C. The efficacy of the mutants was tested by multiple subculturing in kanamycin free medium to check whether the mutants are loosing the kanamycin resistance cassette in the absence of selective pressure of certain concentrations of kanamycin. We observed that the mutants showed resistance to 100 µL/mL of kanamycin in broth even after 14 subcures in kanamycin-free broth medium.

Natural colonization of chicks with Campylobacter. We tested the natural colonization of chicks with *Campylobacter* when provided with 0-1.0 g GAE/L of BPE as water supplement. We checked the natural colonization level by euthanizing five chicks from each of the four groups after three weeks. 100% of the euthanized chicks were naturally colonized with *Campylobacter* after three weeks in groups A, B, and C but 20% chicks in group D, given 0-1.0 g GAE/L of BPE as water supplement (Fig. 4.2). After six weeks, all the chicks were euthanized. The observed median level of colonization of the cecum contents by *Campylobacter* was five logs lower ($p < 0.001$) in presence of 1.0 g AGE/L of BPE compared to the chicks provided with only tap water. However, this high concentration caused an approximately 4% reduction in the chicken weight, though this value was not statistically significant.

Due to this issue, we redesigned our experiment in the second and third trials. Instead of using 1 g GAE/L of BPE for whole 6 weeks period, we suppmenented the water with 0.1 g AGE/L of BPE for 39 days and for the last 3 days before euthanasia, we increased the treatment concentration to 1.0 g GAE/L of BPE for reduction of *Campylobacter* colonization in chicken

ceca. We observed, addition of 1 g GAE/L of BPE in water resulted a reduction of 2 logs in the number of *Campylobacter* colonized in the ceca. But no difference was observed when chickens were provided with 0.1 g GAE/L of BPE in the context of *Campylobacter* colonization in chicken ceca (Fig 4.3).

Colonization of chicks with C. jejuni RM-KM. We infected day-old chicks with *C. jejuni* RM-Km and tested the colonization of chicks with mutant when provided with 0-1.0 g GAE/L of BPE as water supplement. Important to note that, in the experiment, chicks were reared in ventilated cages and gorwed upto 3 weeks. 100% of the euthanized chicks were naturally colonized with *Campylobacter* after one, two and three weeks in groups all the chicks (Fig. 4.4). After one week, 8 chicks per group, after two weeks, 8 chicks per group, and after three weeks, 4 chicks per group were euthanized by cervical dislocation followed by decapitation. The observed median level of colonization of the cecum contents by *Campylobacter* was one log lower ($p < 0.001$) in presence of 1.0 g GAE/ L of BPE compared to the chicks provided with only tap water. Whereas, supplementation of 1.0 g GAE/L of BPE during the last 3 days before euthanasia resulted in 0.5 log reduction in the colonization of marked *C. jejuni* RM-Km in chick gut compared to only tap water.

Discussion

As berries specifically blackberry and blueberry as well as their pomaces are rich sources of phenolic compounds (Nohynek et al., 2006; Puupponen-Pimiä et al., 2004), and berry pomaces are abundant from the fruit juice industry in the US, the berry pomaces are a plausible and economic raw material for extraction of phenolic extracts and can be used in biomedical sector as well as farm animal production. HPLC/high mass accuracy TOF mass spectrometry analysis indicated that major phenolic compounds in both Blk and Blb pomaces included, flavan, flavanone, flavones, glucuronides, glucosides, quinolones, catechol, coumarin, phenols, luteolines, tannins, quercetin, chlorogenic acid, ellagic acid, gallic acid, and xanthoxic acid. This finding remain constant with previous literatures which also reported the presence of these compounds in berries (Mertz et al., 2007; Nohynek et al., 2006; Puupponen-Pimiä et al., 2004) though structural and categorical diversity can be noticed among the phenolic compounds. Factors influencing this diversity include, but are not limited to, species and genetic makeup of berries, agricultural practices, season of harvest, irrigation, soil constituent, types of fertilizers used, processing during juice extraction, and storage of the pomaces. Literature survey demonstrates that crude extracts show better antimicrobial efficacy compared to individual compounds (Bajpai et al., 2012) and we also found that combined mixture of commercially available quercetin, gallic acid, teichoic acid, catechol, and coumaric acid had lower MIC value on Salmonella and Campylobacter compared to each of the individual compounds (data not shown). Due to the reported synergism among various types of phenolic compounds, the use of crude extract instead of purified compounds is justifiable.

After a series of studies on the effect of phenolic extracts from berry pomaces on pathogenic bacteria and probiotics (Salaheen et al., 2014a; Salaheen et al., 2014b; Yang et al., 2014; Salaheen et al., 2015), in this study, we present the bactericidal effect of these extracts against pathogenesis and colonization of *Campylobacter* in chicken gut. However, this does do not deny the bacteriostatic nature that was noticed from the use of sublethal concentrations of these phenolic extracts that showed growth inhibition after 24 h but revealed reduced or no effect after longer period of exposure. This finding agrees with Puupponen-Pimiä et al., (2004) who also reported that raspberry and cloudberry phenolic extracts inhibited bacterial growth at the beginning of the incubation but regrowth occurred after prolonged incubation. The mechanism behind the

inhibition of *Campylobacter* did not solely depend on pH. Depending on the concentration of berry pomace extracts, pH of the solution ranged from 4.5 to 6.5.

Attachment is the prerequisite for *Campylobacter* colonization on intestinal epithelial cells followed by invasiveness, which are considered to be important virulence properties. Association of *Campylobacter* to cultured host cells, e.g., intestinal epithelial INT407, chick macrophage HD11, and chick fibroblast DF1 were altered; decreased association to INT407, increased association to HD11, and association remained unchanged in DF1 cells, after treatment with sublethal concentration of berry pomace extracts. Altered OM protein profile in *Campylobacter* can be a probable cause of increased associated bacterial number to HD11 cells. Hydrophobicity and surface charge of bacterial cells play an important role in the adhesion process as demonstrated previously (Oliveira et al., 2007). In the present case, the extent of adhesion seems to be directly related with cell surface hydrophobicity of *Campylobacter*. Unlike association, treatment with sublethal concentration of berry pomace extract significantly reduced *Campylobacter* invasion into all the host cell types. Alteration of mechanical and physicochemical properties (decreased auto-aggregation and motility) may have impact on the reduction of invasiveness in *Campylobacter* which is supported by previous studies who showed positive correlation between bacterial motility and invasiveness into host cells (Golden, 2002; Salaheen et al., 2014a).

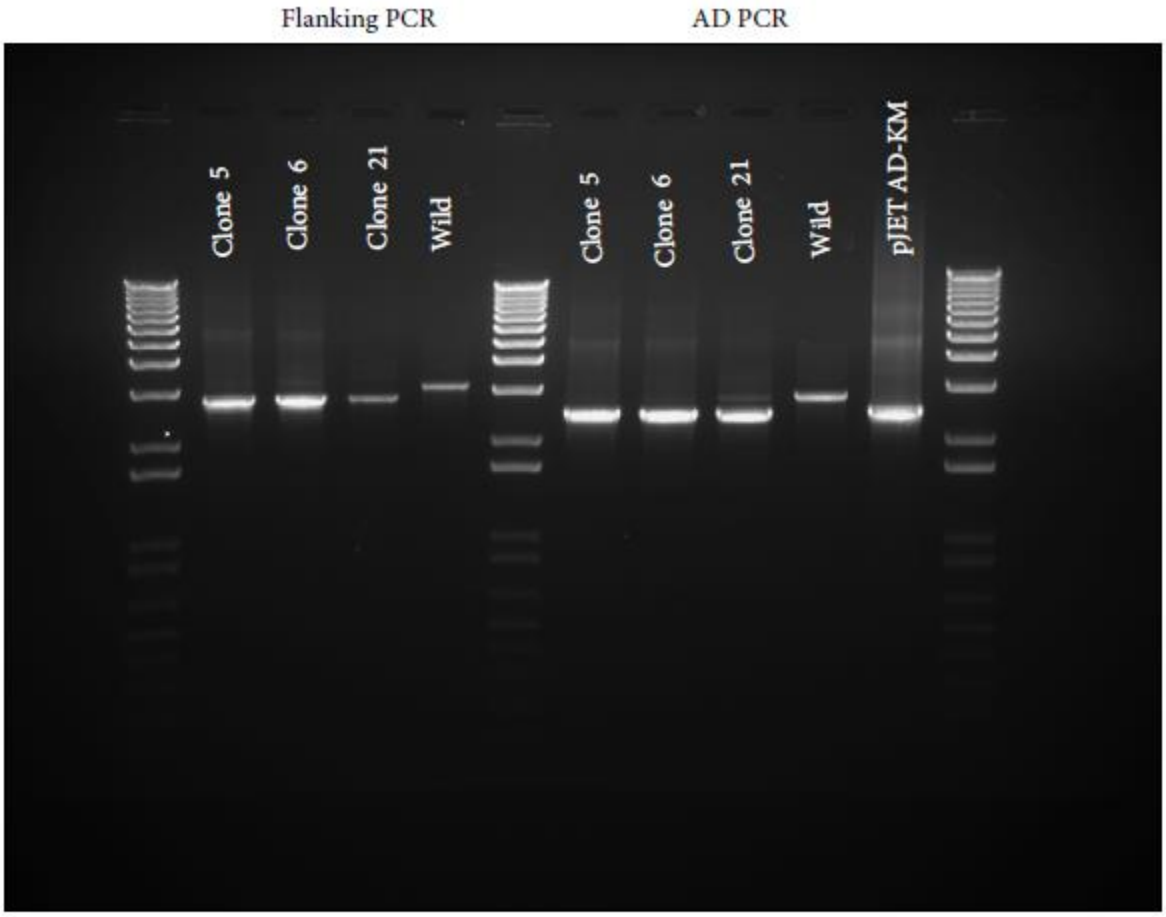
In this study, we found that 0.5-1.0 g GAE/L of berry pomace extract significantly reduced the natural colonization of *Campylobacter* in chick cecum. This phenomenon can be explained by previous report from Clifford (2004), who documented that dietary phenolics are poorly absorbed in the small intestine and 90-95% accumulated in colon resulting high abundance of bioactive phenolic compounds to be interacted with resident bacteria. However, the inhibition of *Campylobacter* colonization depended largly upon how the chicks were grown.

Conclusions

1. 1.0 g GAE/L of BPE reduced natural colonization of *Campylobacter* in chicken ceca by 5 logs at 6 weeks of age in floor pans.
2. 1.0 g GAE/L of BPE reduced *Campylobacter* colonization by 1 log upto 3 weeks of age while grown in isolated cageing system.
3. Treatment with 1.0 g GAE/L of BPE for only 72 h before euthanasia resulted in 2 logs reduction in *Campylobacter* natural colonization in chickens grown in floor pens at 6 weeks of age.

List of Figures and Tables

Fig. 4.1. Development of *C. jejuni* strain marked with kanamycin resistance cassette.



AD PCR: AD-KM for cloned- 2275 bp
AD for wild type-2842 bp

Flanking PCR: Cloned-2649 bp
Wild type-3204 bp

Fig. 4.2. Effect of various concentrations of berry pomace extracts on *Campylobacter* colonization in poultry ceca at 6 weeks of age.

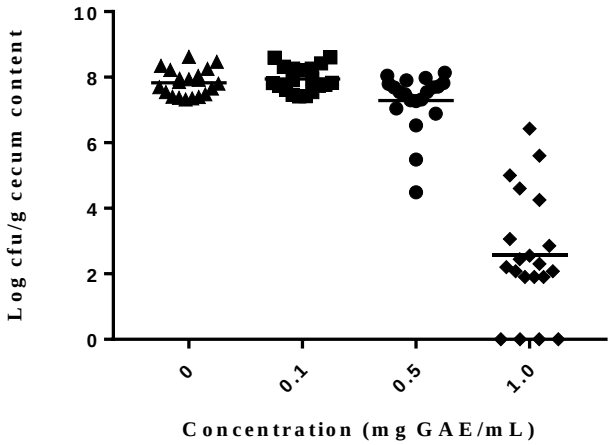


Fig. 4.3. Effect of berry pomace extracts compared to AGPs on the colonization of *C. jejuni* in broiler gut. Groups were assigned in the following manner; broilers from group A (negative control): only tap water, group B (positive control): tap water with AGP, group C: tap water with 0.1 g GAE/L of BPE, and group D: tap water with 0.1 g GAE/L of BPE for 39 days and 1.0 g GAE/L of BPE for last 3 days before euthanasia.

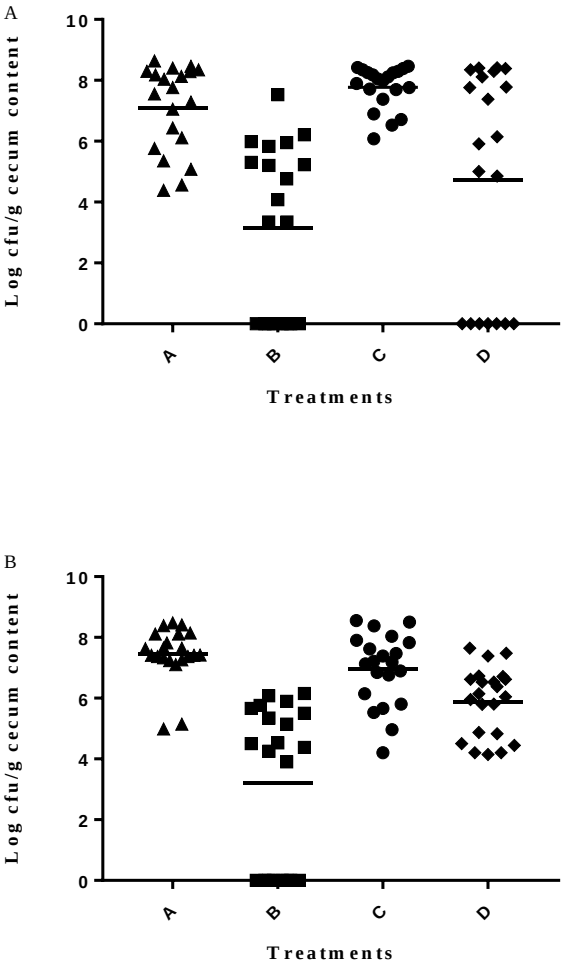
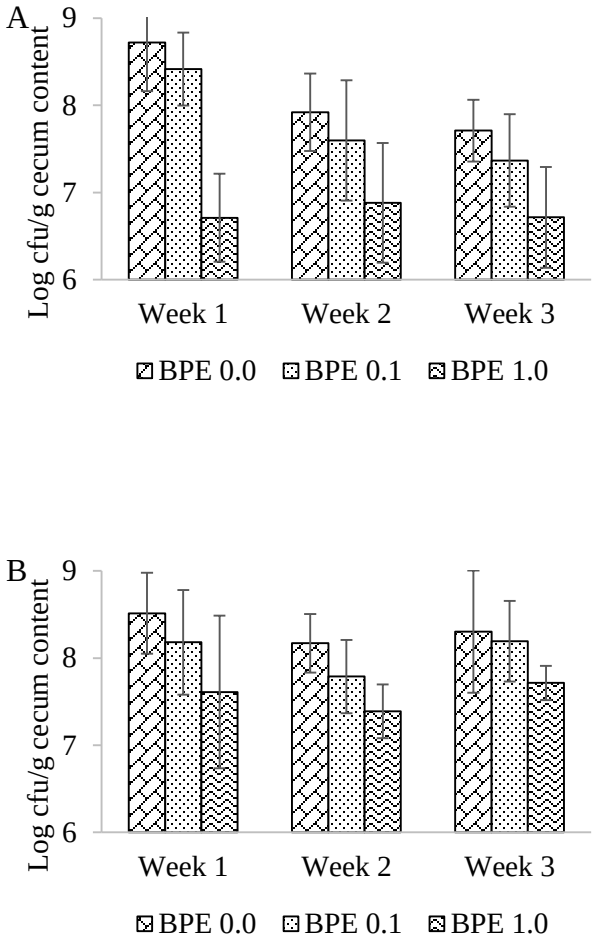


Fig. 4.4. Effect of BPE on the colonization of *C. jejuni* RM-KM in chicken ceca. Panels A and B stand for trials 1 and 2, respectively.



Chapter 5: Berry pomace extracts on poultry growth promotion through modulation of gut microbiome and long term consequences.

Introduction

The discovery of antibiotics in the early 20th century followed by their economization due to large scale production during World War II for controlling human infections, have revolutionized agricultural animal, specifically poultry production in the post war era. Animal agricultural use accounts for more than half of the antibiotics produced in the United States (Lipsitch et al., 2002). However, environmental and public health risks associated with the emergence of antibiotic resistance in zoonotic bacterial pathogens through natural selection, due to therapeutic and/or non-therapeutic use of antibiotics i.e., Antibiotic Growth Promoters (AGPs) for farm animal production, have led the policymakers from worldwide to adopt stringent precautionary measurements. This included the ban of AGP in Sweden starting 1986 followed by a series of events to an EU-wide ban in effect on January 1, 2006 (http://europa.eu/rapid/press-release_IP-05-1687_en.htm). Recently, the US FDA Center for Veterinary Medicine recommended for judicious use of medically important antibiotics in feed based on key reports and scientific literature describing the impacts of AGP on development and transfer of antibiotic resistance traits among intestinal microbiota to the environment and humans through cross-contamination (FDA, 2012, Kleun, 2014). In addition, USDA established the National Organic Program which include organic poultry production that focuses on poultry health, good environmental practices, and production quality without the use of antibiotics as mandated by the USDA. All these precautions as well as organic poultry production system, might help to mitigate the emergence of antibiotic resistance in pathogens in the long run but reduced growth rate in poultry will definitely jeopardize food security. These emerging issues to both conventional and organic poultry industries warrant the need for alternative approaches to render the consequences following AGP removal from feed.

By-products (pomaces) from berry fruits including blueberry (Vaccinium corymbosum) and blackberry (Rubus fruticosus), are major sources of phenolics and have roles in host health improvement through anti-inflammatory, antimicrobial, anti-carcinogenic, anti-oxidant, vasodilatory and other beneficial effects (Boivin et al., 2007; Jepson and Craig, 2007; Tzounis et

al., 2011; Salaheen et al., 2014; Joseph et al., 2014). Dietary supplementation of plant phenolic extracts have been demonstrated to enhance growth performance in broilers but their mechanism of action is far from being elucidated (Herna´ndez et al., 2004). Recent observational and epidemiological studies indicated differential microbial diversity in conventional broilers to their organic counterparts (Torok et al., 2011; Singh et al., 2013; Mancabelli et al., 2016). Neutral effect of AGP on germ free animals indicated the importance of AGP-dependent gut microbiota modulation on growth promotion in animals (Coates et al., 1963). Turnbaugh et al., (2006) revealed the association of two dominant bacterial divisions, the Bacteroidetes and the Firmicutes to weight gain which was supported by other research groups (Gong et al., 2008; Singh et al., 2013; Mancabelli et al., 2016). Gong et al., (2008) reported an increased abundance of *Lactobacillus* spp., Clostridiales and Enterobacteriaceae and later, correlation among Firmicutes to Bacteroidetes (F/B) ratio with increased weight gain and antibiotic treatment was reported (Singh et al., 2013). These findings inspired a comparative view on the effect of AGP versus phenolics on the modulation of broiler gut microbiota, resistome profile, functional enzymes involved in digestion, phage induction and overall potential mechanism behind the improved growth performance.

In this study, we determined the composition of phenolic extracts from blueberry and blackberry and evaluated their roles as an alternative intervention to promote the growth of poultry by modulating gut microbiota. We also aimed to study the resistome profile in poultry ceca which will provide important insights into the applicability of these extracts to replace AGP.

Material and Methods

Preparation of pomace extracts and HPLC-tandem mass spectrometry (LC-MS/MS) analysis. Blueberry and blackberry pomace extracts were prepared according to the protocol previously described using commercial pomaces (powder form) that were kindly donated by Milne Fruit Products Inc., WA and stored at 4°C (Salaheen et al., 2014; Salaheen et al., 2016). Berry pomace extract (BPE) was comprised of blackberry and blueberry pomace extracts at 1:1 v/v ratio. Total phenolic content in each extract was determined spectrophotometricly (Singleton et al., 1999) and expressed as Gallic Acid Equivalent (GAE). The pH of the crude extracts varied from 3.5 to 4.5. Screening of phenolic compounds was performed using the HPLC-MS method described previously by Peng et al. (2015). Briefly, sample injections were 5 µL and separations were performed on an Agilent 1100 system (Agilent Technologies, CA) coupled to an Agilent MSD-

TOF (time-of-flight) mass spectrometer. Reversed-phase liquid chromatography was used to separate the samples with a Waters Atlantis T3 column (3 µm, 150 x 2.1 mm i.d.) (Waters, MA). A binary mobile phase consisting of solvent systems A and B was used in gradient elution where A was 0.1% formic acid (v/v) in ddH_2O and B was 0.1% formic acid (v/v) in acetonitrile. The mobile phase flow rate was 0.3 mL/min. The linear gradient was as follows: time 0 – 1 minute, 0% B; time 40 minutes, 90% B; time 41 minutes, 90% B; time 42 minutes, 0% B; time 52 minutes, 0% B. Following the separation, the column effluent was introduced by electrospray ionization (ESI) into the MSD-TOF. In this study, samples were assayed using negative mode ESI. Source parameters were: gas temperature = 350˚C, gas flow = 9 L/min, nebulizer = 35 psi, fragmentor = 125 V, and capillary voltage = 3500 V. Data were acquired with a mass range of 75 - 1000 m/z. Mass accuracy was guaranteed by the continuous infusion of Agilent Reference Mass Solution (G1969-85001). Individual chromatographic peaks were identified using Agilent's Mass Hunter Qualitative Analysis software (v. B.06). Compounds were identified using Agilent's Mass Profiler Professional software (v. 13.1). Peaks in duplicate injections were aligned to account for instrumental drifts in retention time and mass. Compounds were retained only if they appeared in both duplicate samples. Compounds were annotated by querying Agilent's METLIN human metabolite database, with a mass error criteria of < 5 ppm.

Diet regimens and weight gain in chickens. Diet supplement experiments in chickens were carried out in duplicate trials. In each trial, 100 one-day-old Cobb-500 broiler chicks were obtained from Longenecker's Hatchery Inc. (Elizabethtown, PA). Chick husbandry guidelines recommended by the Institutional Animal Care and Use Committee (IACUC, protocol number R-16-33) were followed. The chicks were provided with commercially available crumbles (Purina Animal Nutrition, MO) with no antibiotic supplementation. The chicks were assigned into 4 groups of 25 chicks each in floor pens using a Completely Randomized Design consisting of a negative control, a positive control, and two treatment groups. The negative control group A was provided non-supplemented tap water; the positive control group B was provided tap water supplemented with AGP (a combination of Oxytetracycline 1 µg/mL, Erythromycin 2 µg/mL, Tylosin 2 µg/mL, Bacitracin 4 µg/mL and Neomycin sulfate 32 µg/mL); the treatment group C was provided tap water supplemented with 0.1 g GAE/L of BPE; and treatment group D was provided tap water supplemented with 0.1 g GAE/L of BPE and the treatment concentration was increased to 1 g

GAE/L during the last 72 h before euthanasia. The chicks were reared for 6 weeks and individual weights was recorded weekly. The data were analyzed with the Statistical Analysis System software (SAS, Institute Inc., Cary, NC, USA) using mixed effect Analysis of Variance (ANOVA) and Tukey's modification for multiple mean comparisons.

Sample collection and processing. After six weeks, all of the birds were euthanized with cervical dislocation followed by decapitation. Blood samples were collected in VACUETTE® Heparin Tubes (Greiner Bio-One, NC) and were analyzed with a ProCyte Dx® Hematology Analyzer (IDEXX, ME) according to the manufacturer's instructions. Chicken organs, e.g., spleen, liver, heart, and pancreas, were collected and weighed immediately. Ceca lengths were measured and contents from both ceca were thoroughly mixed followed by storage at -80˚C until DNA extraction for metagenomic analysis. Contents from 5 ceca from each group to a total of 20 ceca were randomly selected for metagenomic analysis. DNA extraction was carried out with QIAamp Fast DNA Stool Mini Kit (QIAGEN, CA) according to the manufacturer's instructions. Nextera DNA libraries were made for each of the 20 samples separately using Nextera DNA Library Preparation Kit and Nextera Index Kit (Illumina, San Diego, CA) followed by pooling into equimolar concentrations according to the manufacturer's instructions. Paired-end sequencing (2 X 151 bp) was conducted on an Illumina NextSeq 500 sequencing platform with a NextSeq 500/550 v2 High Output flow cell.

Analysis of metagenomics datasets. Data were demultiplexed using the BCL2FastQ program and PhiX reads were removed using DeconSeq (Schmieder and Edwards, 2011). Reads were further cleaned using Trimmomatic V 0.36 (leading 20, trailing 20, sliding 4:20, min len 36) (Bolger et al., 2014). Only paired data were further analyzed. After cleaning and curating the data the total reads in each sample ranged from 4.3×107 to 8.7×107 reads. Taxonomic labels were assigned to reads with taxonomic sequence classifier, Kraken, using Kraken-translate and --mpa format which reported levels of the taxonomy with standard rank assignments (Wood and Salzberg, 2014). The Kraken database was prepared using NCBI taxonomic information as well as the complete genomes in RefSeq for the bacterial, archaeal, and viral domains (https://www.ncbi.nlm.nih.gov/refseq/). Output files from Kraken were formatted with custom scripts to generate files containing taxonomic information and abundances at tab delimited csv

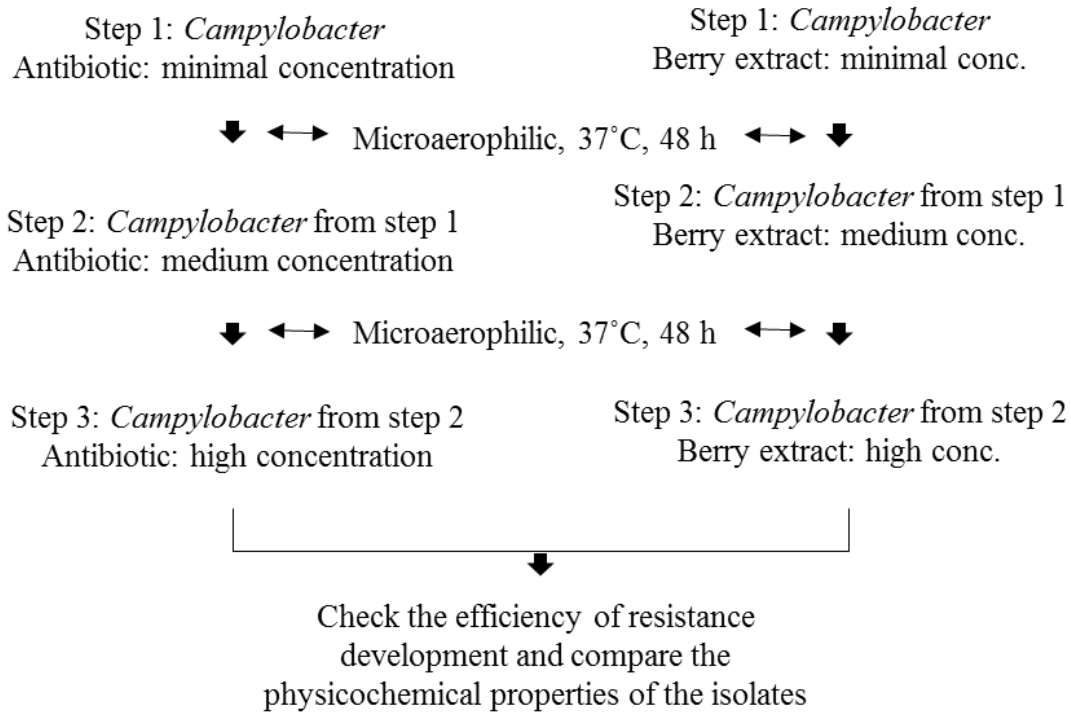
format which were ultimately fed into MEGAN (version 5.11.3, Huson et al., 2007) to generate matrices for sample comparisons. We determined relative abundances of microbial taxa in a cecum sample by dividing the number of reads of a specific taxon by the total number of reads in that sample. Initially the Bray-Curtis distance matrix and the Firmicutes/Bacteroidetes (F/B) ratio were determined for each sample in each treatment group (groups A, B, C, and D). Samples from a specific group that clustered together, contained cecal microbial profiles closest to the average of their respective groups, and possessed an F/B value ranging from 0.10 to 10.0 were considered to be the core microbiome representing that group and were used for further analysis (and we were left with a total of 12 samples, 3 from each group) (Mancabelli et al., 2016). Relative abundances from MEGAN-generated abundance matrices were used for statistical analysis, calculation of beta-diversity, and alpha-diversity with genus richness, Shannon, and Fisher's indexes. Rarefaction curves with genus richness, Shannon, and Fisher's indexes were calculated using package vegan in R (version 3.3.1).

For functional analysis, paired sequences were analyzed with metAMOS pipeline (Treangen et al., 2013). Output files from functional annotation section in metAMOS, which consisted UniProt ID of predicted proteins, were formatted with a custom scripts to generate assigned UniProt ID and abundances at tab delimited format. Retrieved UniProt entries were converted to the corresponding KEGG Orthology (KO) entries using Retrieve/ID mapping at http://www.uniprot.org/uploadlists/. A comparison matrix was prepared with Microsoft Query in Excel (version 2013) and fed to GraphPad Prism software (version 7) for statistical analysis. Interrogation of sequence reads for identity to known antibiotic resistance genes (ARGs) was performed using DIAMOND (Buchfink et al., 2015) (sequence identity > 90%, matched amino acid sequence length > 25, e-value < 10-5) and the database CARD (McArthur et al., 2013), which encompasses amino acid sequences of ARGs.

Artificial development of resistance in C. jejuni against BPE. In this experiment, artificial development of resistance against BPE were monitored. *Campylobacter jejuni* RM1221 (ATCC BAA-1062TM) were used in the current study. The bacterium were grown in Blood agar (Himedia, India) with 5% defibrinated sheep blood (Ward's Science) at 37˚C under microaerophilic (10% CO2, 5% O2, and 85% N2) condition. Bolton broth with 10% defibrinated blood to a final volume of 990 µL were prepared with varying concentrations of BPE or antibiotics. 10 µL bacterial

suspensions containing approximately 5×10^5 cfu/mL were added to each of the well of 24-wells plates. Plates were incubated for 48 hours at 37˚C under microaerophilic condition. Antibiotics commonly used for poultry growth promotion and therapeutics were used.

Figure: Artificial development of antibiotic resistance in the presence of antibiotic or bioactive berry extracts



Role of BPE on the expression of stress response genes of *C. jejuni*.

A. RNA extraction and cDNA synthesis. The extraction of RNA were carried out according to the protocol of ZR Bacterial RNA MiniPrep kit (Zymo Research Corp., Irvine, CA). RNA quantification were carried out using a NanoDrop spectrophotometer (Thermo Scientific Inc., West Palm Beach, FL). The cDNA synthesis were performed according to the protocol of qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). The eluted RNA (1 µg) were mixed with 4 µL of 5X qScript cDNA SuperMix containing optimized concentration of MgCl2, dNTPs, RNase inhibitor protein, qScript reverse transcriptase, random primers, oligo(dT) primer, and stabilizers and then incubated at 25˚C for 5 min, 42˚C for 30 min, and 85˚C for 5 min.

B. Quantitative RT-PCR assay. The mixture containing 10 µL of PerfeCTa SYBR Green FastMix, 2 µL of each primer (100 nM), 2 µL of cDNA (10 ng), and 4 µL of RNase-free water were amplified using an Eco RT PCR system (Illumine, San Diego, CA), which were denatured at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, 55°C for 15 sec, and 72°C for 10 sec. The custom-synthesized oligonucleotides (Erofins MWG Operon; Huntsville, AL) were used as primers to target conserved regions of *C. jejuni* are listed in Table 5.1. The relative expression levels of genes were calculated by the comparative method (Livak, and Schmittgen, 2001). The CT values of target genes in treated *C. jejuni* cells were compared to the CT values obtained from the control. The housekeeping gene, 16S ribosomal RNA, were used as the reference gene for normalization of target gene expression.

Results

Effect on chicken growth performance. Effect of supplemented diet on chicken growth promotion was measured at their 6 weeks of age (Fig. 5.1). Significant growth promotion (9.5% increase in mean body weight) was observed in chickens from group B who were provided with AGP as water supplement compared to the group A that were provided with only tap water (P = 0.001). Chickens from group C (provided with 0.1 g GAE/L of BPE) gained higher mean body weight by approximately 6% compared to group A (P = 0.029). No significant difference in weight gain was observed between group B and C (P = 0.242). Chickens from group D, where BPE concentration was increased to 1.0 g GAE/L during last 72 h before euthanasia, showed a mean body weight gain by more than 1% compared to group A (P = 0.488). However, when chickens were provided with 1.0 g AGE/L BPE for consecutive 6 weeks, mean body weight was decreased by 4% compared to group A (data not shown) which suggested a concentration dependent mechanism of BPE on chicken growth performance. Organ weight to whole body weight ratio remained unaltered for spleen (P = 0.844), liver (P = 0.548), heart (P=0.560), and pancreas (0.599) among chickens from all 4 groups (Table 5.2). However, a concentration dependent increase in mean cecum length (by more than 12%) in chickens was observed when provided with BPE (P = 0.037).

Hematologic analysis of five randomly selected chickens from each group showed no significant difference in tested parameters at alpha value of 0.05 (Table 5.3). Interesting to notice, mean RBC number of 2.8 million/μL in groups B and C compared to 2.5 million/μL in group A (P = 0.07). Similarly, 8.8 and 9.1 g/dL hemoglobin in groups B and C, respectively, compared to 8.2 g/dL in group A (P = 0.07). Any behavioral alteration or differences in eating habits were not noticed among chickens from any group.

Differential composition of gut microbiota. In order to assess the composition of gut microbiota in the 4 treatment groups, we analyzed cecal samples from 12 chickens (3 chickens from each group) with next-generation shotgun sequencing. The total number of reads in the cleaned and curated datasets from each sample ranged from 4.3×10^7 to 8.7×10^7.

Evaluation of rarefaction curves based on microbial genus richness on sample datasets indicated that all 12 curves tend to form a plateau which bear evidence of adequate sequence coverage for the vast majority of biodiversity contained within the samples (Fig. 5.2). Rarefaction

curves did not reveal a noticeable difference among genus composition in the cecal contents from 12 chickens. Shannon and Fisher's diversity indexes calculated at the highest rarefaction depth covered in all the samples indicated no significant difference in diversity among the 4 groups at genus level. A Neighbor-Joining tree generated using the Bray-Curtis distance matrix indicated that there were similar microbial communities in groups B and C, whereas group D mainly clustered with group A, though statistical significance was not detected among these relationships (Fig. 5.2). One-way ANOVA on relative abundances of microbial domains revealed that group B possessed significantly higher number of DNA viruses compared to the other groups (P = 0.031) whereas no significant difference in the number of bacteria was observed among all the groups (Fig. 5.2). A numeric reduction in the mean relative abundance of bacteria was noticed in group B compared to the other groups (P = 0.661). Unpaired t-test analysis on relative abundance of archaea demonstrated significantly higher numbers in groups B and C compared to group A (P = 0.049 and 0.031). Influence of archaeal communities in poultry gut is still an understudied area. Conversion of organic compounds to simple volatile fatty acids by fermentative bacteria leads to H2 accumulation in the gut that inhibits the growth in animals. Methanogenic archaea in GI tract consume H_2 and help to lower H_2 partial pressure in gut (Saengkerdsub and Ricke, 2013).

Assigned taxonomic profiles at the bacterial phylum level for pooled cecal samples revealed that Bacteroidetes was the dominant phylum (56.8%) in group A followed by Firmicutes (15%) and Proteobacteria (5%) (Fig. 3A). Relative abundances of bacterial phyla in individual samples are presented in supplementary file 3. In group A, relative abundances of Bacteroidetes, Firmicutes, and Proteobacteria ranged between 44.6 to 64.3%, 10.3 to 21.5%, and 5.0 to 9.1%, respectively (Supplementary file 3). In groups B and C, bacterial phyla distribution was dominated by Firmicutes (29.45 and 36.46%) followed by Bacteroidetes (23.62 and 31.11%) and Proteobacteria (21.29 and 7.55%). Differences in the community composition were observed among the different treatment groups. A higher relative abundance of Bacteroidetes in group A (P = 0.023) was detected, whereas a higher abundance of Firmicutes in groups B and C was observed compared to the other groups (P = 0.043). Group B possessed highest relative abundance of Proteobacteria, which was statistically significant at the alpha = 0.10 level. This finding was supported by Looft et al. (2012) who also reported that non-therapeutic concentration of chlortetracycline, sulfamethazine, and penicillin administered to piglets increased the prevalence of Proteobacteria. Important to note that Proteobacteria include a wide variety of pathogens, such

as Escherichia, Campylobacter, Salmonella, Helicobacter, Pseudomonas, and many other notable pathogenic genera.

Significant variation in the mean Firmicutes to Bacteroidetes (F/B) ratio (0.32, 1.32, 1.09, and 0.46 in groups A, B, C, and D, respectively) was noticed (P = 0.013). Pearson correlation coefficient indicated a moderately positive correlation between F/B ratio to chicken body weight (R2 = 0.483) (Fig. 5.3). This finding is supported by previous studies that showed increased F/B ratios were associated with AGP supplementation in feed and growth promotion in broilers (Singh et al., 2013; Mancabelli et al., 2016). In previous in vitro studies, we observed growth stimulation in Firmicutes, specifically probiotic Lactobacillus strains in the presence of berry extracts in broth, or chicken fecal medium that presented selective bias towards probiotic population when co-cultured with pathogens (Yang et al., 2014; Salaheen et al., 2015). These findings indicate increased Firmicutes level in chicken ceca might be one of the many factors responsible for growth promotion in chickens with BPE supplementation.

At the genus level a total of 670, 678, 691, and 683 bacterial taxa were identified in groups A, B, C, and D, respectively. As the reads were mapped to the lowest common ancestor (LCA) in this study, the identified number of genera in broiler cecum may not be comprehensive. Mancabelli et al., (2016) identified 252 taxa at genus level in broilers with 16s rRNA profiling. Sequencing methods, source of samples, and diet regimens may be responsible for variability in the number of bacterial genera in broilers. Significant differences at alpha value of 0.10 in the relative abundances of Bacteroides, Lactobacillus, Enterococcus, Escherichia, and Eubacterium genera were identified among treatment groups where relative abundance of Bacteroides was the highest in group A; Enterococcus and Escherichia in group B; and Lactobacillus and Eubacterium in group C (Fig. 5.3). Oakley and Kogut (2016) also reported significantly higher abundance of Bacteroides in ceca of broilers grown without AGP. Whereas, Viveros et al., (2011) reported higher abundance of Lactobacillus in broilers provided with plant extracts. Observed differences in the composition of bacterial genera may influence the dynamics of feed to energy conversion in chickens from different treatment groups.

There were no significant differences in the relative abundances of DNA viruses in the samples. However, based on presence or absence of a virus in a sample, bacteriophages e.g., Mu-like viruses, N15-like viruses, Phikz-like viruses, T7-like viruses, and Inoviruses were more prevalent in group B (Fig. 5.3). In addition, numerical (but not statistically significant) increase in

the mean abundances of Lamda-like viruses, Mardiviruses, and T4-like viruses was noticed in group B. Metagenomic studies in swine and mice model reported phage induction in gut due to oral supplementation with antibiotics (Allen et al., 2011; Modi et al., 2013). A collateral consequence of phage induction is gene transfer that promote both pathogen evolution and transfer of antibiotic resistance genes (Allen and Stanton, 2014).

Functional classification of chicken cecal microbiome. Assessment of the functional classification of open reading frames based on the KEGG Orthology (KO) database obtained from metagenomic datasets revealed significant variation in functional orthologs among the groups (Fig. 5.4). Analysis was directed towards enzymes that involved in carbohydrate metabolism and energy production and further sorted to enzymes showing variation among different datasets. Significant variations were observed in the relative abundance of several enzymes. Phosphoenolpyruvate carboxykinase, formate dehydrogenase major subunit, and 2-oxoglutarate dehydrogenase E1 component (involved in carbon metabolism, glyoxylate and dicarboxylate metabolism, methane metabolism, tryptophan metabolism, and lysine degradation) were more abundant in group C than in the other groups ($P < 0.10$). Whereas, L-xylulokinase, α-glucosidase, and tartronate-semialdehyde synthase (involved in pentose and glucuronate interconversions, ascorbate, aldarate, starch, sucrose, glyoxylate, and dicarboxylate metabolism) were highly abundant in group B ($P < 0.10$). Chicken cecal microbiome from group D possessed higher relative abundance of F-type H+-transporting ATPase subunit beta, and glycerol kinase ($P < 0.10$). These observations indicate metabolic diversions in chickens that were provided with AGPs or BPE, though both of these treatments resulted in higher body weight in chickens. These differences can be explained by the variability in the microbial communities, especially, Bacteroidetes and Firmicutes, that harbor important enzymes (e.g., pectinase, and cellulase) to hydrolyze cell wall components from plant based diets (Thomas et al., 2011).

Prediction of the cecal resistome profile. The core The core resistome of the microbial consortia residing in chicken cecal contents collected from various groups were generated by screening for known bacterial antibiotic resistance genes (ARGs) followed by sorting the genes based on their relative abundances (at-least one read per 10 million sequences) in the cecal samples. In silico analysis of the resistome profiles demonstrated a higher relative abundance of ARGs in group B

compared to group A (P = 0.067) (Fig. 5.5). Core resistomes of groups A, B, C, and D consisted 69, 103, 88, and 69 ARGs (from 3286, 5870, 3685, and 3081 reads per 10 million sequences), respectively. Both transferable ARGs and efflux pump mediatory ARGs were present in these core resistomes. Core resistomes of the treatment groups were generated this way: 112, 79, and 103 ARGs in group A; 129, 124, and 121 ARGs in group B; 108, 102, and 115 ARGs in group C; and finally 94, 111, and 80 ARGs in group D. Based on gene functions, the identified ARGs in individual samples were further assigned to 14 groups: resistance to aminocoumarin, aminoglycosides, beta-lactam, sulfonamides, bacitracin, chloramphenicol, fluoroquinolone (transferable element based), glycopeptide antibiotics, trimethoprim, macrolide-lincosamide-streptogramin B, polymyxin, streptothricin, tetracyclines, and efflux pump conferring resistance. Chloramphenicol acetyltransferase (cat) and CcrA beta-lactamase genes were found in all the samples but due to assigned abundance threshold, i.e., at-least one read per 10 million sequences, these genes were not listed in core resistome of group B. Higher relative abundance of beta-lactamases were previously reported in broilers grown with AGPs but absence of beta-lactam antibiotics in the AGP-mixture that was used in this study may be responsible for lower abundance of beta-lactamases (Laube et al., 2013; Mancabelli et al., 2016). Two out of 3 samples from group B contained trimethoprim resistance gene, dfrA1, hence missed from the group B resistome but no trimethoprim resistance gene was identified in other groups. Sulfonamide resistant dihydropteroate synthase genes (sul1 and sul2) were only observed in all the samples from group B but not in other groups. sul1 and sul2 are commonly associated with mobile genetic elements and predominance of resistant enteric bacteria associated with these genes has been reported in broilers grown with AGPs (Diarra et al., 2007; Simmons et al., 2016). In this study, percentages of ARGs associated with efflux pump mediated antibiotic resistance in core resistomes of groups A, B, C, and D were 39.1, 49.5, 46.6, and 39.1, respectively.

A total of 52 ARGs from 10 out of 14 ARG groups showed coexistence in all of the core resistomes. Relative abundances of aminoglycoside, bacitracin, fluoroquinolone (transferable elements), polymyxin, streptothricin, tetracycline and efflux pump conferring antibiotic resistance genes were higher in group B compared to the other groups (P < 0.05) (Fig. 5.5). Relative abundances of macrolide-lincosamaide-streptogramin resistance genes were also significantly higher in group B at alpha value of 0.10. No significant variation in the relative abundances of aminocoumarin and glycopeptide antibiotic resistance genes were observed. In this study,

incorporation of bacitracin (glycopeptide), erythromycin (macrolide), neomycin sulfate (aminoglycoside), oxytetracycline (tetracycline), and tylosin (macrolide), supplementation in chickens from group B is associated with higher relative abundances of ARGs in the cecal resistome. These findings are supported by previous studies that reported the association between the use of antibiotics in feed with development of ARGs in complex ecosystems (Andersson and Hughes, 2014; Levy 2014; Roca et al., 2015).

Based on the best-hit results from the DIAMOND analysis using the CARD database, the majority of identified ARGs were highly similar to those previously identified in Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Fig. 5.5). Among the ARGs similar to those identified in Actinobacteria, 42 to 54% were more specifically similar to those previously identified in the Bifidobacteriales order. Significantly lower ARGs associated with Bacteroidetes were identified in group B (0.14%) compared to groups A, C, and D (19.03, 11.01, and 15.64%, respectively) which can be explained by reduced relative abundance of Bacteroidetes in cecal microbiome of group B. Mancabelli et al., (2016) also observed high abundance of Bacteroidetes-associated ARGs in cecal resistomes of chickens grown without antibiotic supplementation. Alternatively, higher relative abundances of ARGs associated with Proteobacteria were observed in group B (41.93%) compared to the other groups. Higher relative abundances of Proteobacteria in chichen cecal microbiomes from group B were likely responsible for higher Proteobacteria-associated ARGs (Danzeisen et al., 2011).

Can C. jejuni become resistant towards BPE? In this study, with consecutive increase of antibiotic Kanamycin concentration in the growth medium, we developed *C. jejuni* strain possessing MIC value of > 100 µg/mL towards kanamycin. Consecutive increase of BPE concentration in the growth medium resulted in increased MIC to 0.9 mg GAE/mL from pre-documented MIC value of 0.3 mg GAE/mL. However, subculturing the BPE resistant *C. jejuni* in BPE-free medium resulted in revival of the original MIC of 0.3 mg GAE/mL.

Expression of strss response genes in C. jejuni with qRT-PCR showed significant downregulation of peroxide-sensing regulator (perB), Flavodoxin I (fldA), Superoxide dismutase (sodB), Alkyl hydroperoxide reductase (ahpC), Nonheme iron-containing ferritin (cft), Ferredoxin (fdxA), Ferric uptake regulator (fur), Bacterioferritin comigratory protein homolog (BCP), Rod shape-determining protein (mreB), membrane fusion protein (cmeA), inner membrane transporter

(cmeC), outer membrane lipoprotein (porA), ATP-dependent Clp protease ATP-binding subunit (clpA).

Discussion

Modulation of gut microbiota with antibiotic growth promoters (AGP) for enhanced performance in poultry can be justified until acquisition of antibiotic resistance in zoonotic bacterial pathogens through inter-bacterial transfer of antibiotic resistance genes (ARGs) in a complex microbial community is considered. In this study, we adopted mass-spec, phylogenetic, and metagenomice approaches to evaluate bioactive phenolic extracts (BPE) from blueberry (*Vaccinium corymbosum*) and blackberry (*Rubus fruticosus*) pomaces as alternative performance enhancer in poultry. We raised a total of 300 Cobb-500 broiler chicks receiving water W/ or W/O AGP (tylosin, neomycin sulfate, bacitracin, erythromycin, and oxytetracycline), or BPE supplementation for six weeks and discovered more than 6% increase in mean chicken weight gain with BPE supplementation compared to 9.5% with AGP. BPE caused an AGP-like pattern in bacterial community with comparative increase of Firmicutes and a concomitant reduction of Bacteroidetes in chicken ceca. This finding is supported by previous literature that showed increased F/B ratio in correlation with AGP supplementation in feed and growth promotion in broilers (Singh et al., 2013; Mancabelli et al., 2016). In previous in vitro studies, we observed growth stimulation in Firmicutes, specifically probiotic *Lactobacillus* strains in the presence of berry extracts in broth, or chicken fecal medium that presented selective bias towards probiotic population when co-cultured with pathogens (Yang et al., 2014; Salaheen et al., 2015). These findings indicate increased Firmicutes level in chicken ceca might be one of the many factors behind growth promotion in chickens with BPE supplementation.

AGP supplementation clearly caused phage induction and a richer resistome profile in cecal microbiome compared to BPE or regular tap water. Incorporation of bacitracin, erythromycin, neomycin sulfate, oxytetracycline, and tylosin, as water supplement in chickens from this group can be considered responsible for the phenomenon. Metagenomic studies in swine and mice model reported phage induction in gut due to oral supplementation with antibiotics (Allen et al., 2011; Modi et al., 2013). A collateral consequence of phage induction is gene transfer that

promote both pathogen evolution and transfer of antibiotic resistance genes (Allen and Stanton, 2014). Both AGP and BPE supplementation resulted in elevated relative abundance of archaea. Methanogenic archaea in GI tract consume H2 and help to lower H2 partial pressure in gut (Saengkerdsub and Ricke, 2013).

Conclusions

1. BPE supplementation in water increased the mean body weight of chickens by 6%.
2. BPE caused an AGP-like pattern in bacterial community with comparative increase of Firmicutes and a concomitant reduction of Bacteroidetes in chicken ceca.
3. AGP resulted higher prevalence of bacteriophages in the cecal microbiome compared to BPE.
4. BPE and AGP resulted in cecal microbiome with over-represented functional orthologs involved in carbon fixation in photosynthetic bacteria, carbon and amino acid metabolism, starch and cellulose metabolism, oxidative phosphorylation, and lipid metabolism.
5. Analysis of resistome profile of cecal microbiomes revealed higher predicted ARGs in chickens provided with AGP compared to BPE.
6. Sequential increase of BPE in growth medium doubled the MIC of BPE to *C. jejuni* but the strain became susceptible to BPE just after one subculte in BPE-free medium.
7. BPE significantly downregulated the *C. jejuni* genes involed in stress response.

List of Figures and Tables

Table 5.1. List of primers for stress response gene expression of *C. jejuni*

Function/protein	Gene	Primer	Sequences (5'-3')	References
Peroxide-sensing regulator	*perR*	Forward	TTCAATCTCTTTAGCGACGG	Xie et al., 2011
		Reverse	CACATTTGGCGCAAACAACA	
Flavodoxin I	*fldA*	Forward	TCCACCAAGACTAAGCCCTG	Xie et al., 2011
		Reverse	CAGAAGGAGCGGCTAATACAA	
Iron-binding protein	*dps*	Forward	CCACTAATGTTAATATGCGTTCC	Xie et al., 2011
		Reverse	TGTGCTTGATAATCTTGCGACAA	
Superoxide dismutase (Fe)	*sodB*	Forward	TGGCGGTTCATGTCAAAGTA	Xie et al., 2011
		Reverse	ACCAAAACCATCCTGAACCA	
Alkyl hydroperoxide reductase	*ahpC*	Forward	AGTTGCCCTTCGTGGTTCGT	Xie et al., 2011
		Reverse	ATCGCCCTTATTCCATCCTG	
Catalase	*katA*	Forward	ACCGTTCATGCTAAGGGAAG	Xie et al., 2011
		Reverse	CCTACCAAGTCCCAGTTTCC	
Nonheme iron-containing ferritin	*cft*	Forward	TTCTTCTTCGTGTTGTTCGC	Xie et al., 2011
		Reverse	GCTGGAGCCTTCTTGTTTGC	
Ferredoxin	*fdxA*	Forward	CCCCACTTCTCATATCAGCG	Xie et al., 2011
		Reverse	ATGCGTTGAATGCGTAGGAC	
Rubrerythrin	*rbr*	Forward	TGCAGCAGTTACTAGGTTTT	Xie et al., 2011
		Reverse	AGACATTTTAGAGAAGCGGC	
Ferric uptake regulator	*fur*	Forward	CCATTTCTTTTGGTTCAGCAG	Xie et al., 2011
		Reverse	TGCAATCAAGGCTTGCTGTC	
Carbon storage regulator	*csrA*	Forward	TCAAAGTCGTTCAAACAGGG	Xie et al., 2011
		Reverse	TCATTCTGAACAACAGAATGC	
Probable thiol peroxidase	*tpx*	Forward	GCCAGTTACAATGGTGCTGA	Xie et al., 2011
		Reverse	TTTGCCACAAAATCACTTGC	
Cochaperonin	*groES*	Forward	AAACAACAGCCTCAGGCATAA	Xie et al., 2011
		Reverse	TTCTGTTCCACCGTATTTAGCA	
Chaperonin	*groEL*	Forward	GCAGGCGATGGAACAACTAC	Xie et al., 2011
		Reverse	TCCATACCGCGTTTTACCTC	
Chaperone	*dnaK*	Forward	CGGTATGCCACAAATCGAAG	Xie et al., 2011
		Reverse	GCTAAGTCCGCTTGAACCTG	
Cochaperone	*dnaJ*	Forward	TTTAAAAGGCGGTGGATTTG	Xie et al., 2011
		Reverse	TTTTCTACGACGCGATGATG	
Bacterioferritin comigratory protein homolog	*BCP*	Forward	ACCCCAGGTTGTACTACAGAAG	Xie et al., 2011
		Reverse	AGCAATCTTACCTGTTTCATCG	
RelA/Spot family protein	*spoT*	Forward	GCCCCAATAGCCCATAGAC	Xie et al., 2011
		Reverse	ACCCCAAGCAAATCAAGAAC	
Rod shape-determining protein	*mreB*	Forward	GAGCCTTCTGTTGTGGCAGTT	Xie et al., 2011
		Reverse	AGCGGATCATTTTTTCAGTCAT	
RND efflux system; membrane fusion protein	*cmeA*	Forward	TATTACGCCGCTAACTTGAG	Xie et al., 2011
		Reverse	CAGCAAAGAAGAAGCACCAA	
RND efflux system; inner membrane transporter	*cmeB*	Forward	TAATCCAGGTATGGGAGGTA	Xie et al., 2011
		Reverse	GGAAAGATAGAAATGTAAGCG	

RND efflux system; outer membrane lipoprotein	*cmeC*	Forward	GGACGTTGAAGCAAGATGGT	Xie et al., 2011
		Reverse	AGTTGGCGCTGTAGGTGAAT	
Major outer membrane protein	*porA*	Forward	TTGATAGCGAACTTGATGAT	Xie et al., 2011
		Reverse	ATACGAAGTCAGCACCAACG	
Inner membrane protein	*yagU*	Forward	CTATTTCCATACCCCACAGC	Xie et al., 2011
		Reverse	CCTTTAATTGCAGAAGTTCC	
ATP-dependent Clp protease ATP-binding subunit	*clpA*	Forward	GTAGGAGCTGGAAGCACAGG	Xie et al., 2011
		Reverse	ACGGCGACTTAGGGGTTTAT	

Table 5.2. Relative organ to body weights and cecum length of broilers at day 42.

Organs	A	B	C	D	p-value
Spleen[1]	0.13_a	0.12_a	0.12_a	0.13_a	0.844
Liver[1]	2.18_a	2.22_a	2.07_a	2.08_a	0.548
Heart[1]	0.53_a	0.53_a	0.50_a	0.53_a	0.56
Pancrease[1]	0.86_a	0.79_a	0.79_a	0.79_a	0.599
Cecum[2]	17.24_a	18.64_a	19.15_b	19.43_b	0.037

[1]Ratio: Organ weight to total body weight during euthanasia;

[2]Length (cm)

Means with different letters (a-d) are significantly different ($p < 0.05$)

Table 5.3. Effect of BPE compared to AGPs on blood parameters and indices of broilers at day 42.

Blood parameters	A	B	C	D	p-value
RBC ($\times 10^6$/µL)	2.5±0.2[1]	2.8±0.2	2.8±0.1	2.7±0.1	0.07
WBC ($\times 10^3$/ µL)	551.5±212.6	644.2±159.2	565.7±100.3	774.6±87.3	0.11
Neutrophil ($\times 10^3$/ µL)	532.4±206.9	617.8±150.5	544.7±93.7	744.4±71.3	0.11
Lymphocyte ($\times 10^3$/ µL)	13.5±2.6	17.5±6.7	15.4±6.1	17.8±5.4	0.58
Monocyte ($\times 10^3$/ µL)	0.4±0.4	0.5±0.3	0.7±0.4	0.8±1.5	0.85
Eosinophil ($\times 10^3$/ µL)	2.5±3.2	5.8±5.8	3.7±5.3	8.6±13.5	0.64
Basophil ($\times 10^3$/ µL)	2.7±1.7	2.7±1.5	1.3±0.5	3.0±1.0	0.16
Hemoglobin (g/100mL)	8.2±0.5	8.8±0.6	9.1±0.4	8.8±0.3	0.07
Hematocrit (%)	29.4±2.1	30.8±1.7	31.4±1.5	30.7±0.9	0.26

[1]Values indicate Mean ± Standard deviation;

Fig. 5.1. Effect of berry pomace extracts compared to AGPs on the performance of broilers at day 42. Groups were assigned in the following manner; broilers from group A (negative control): only tap water, group B (positive control): tap water with AGP, group C: tap water with 0.1 g GAE/L of BPE, and group D: tap water with 0.1 g GAE/L of BPE for 39 days and 1.0 g GAE/L of BPE for last 3 days before euthanasia. * indicates significant variation compared to the negative control, group A ($p < 0.05$).

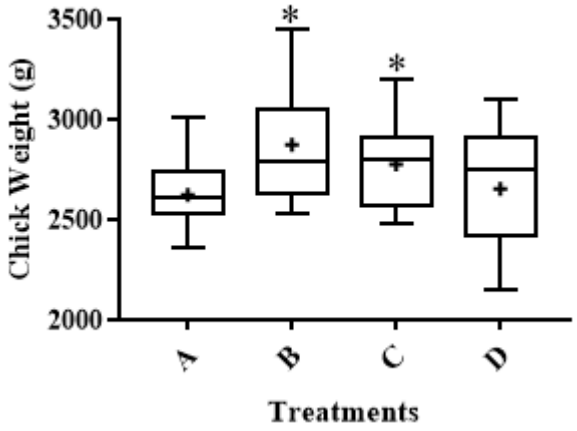
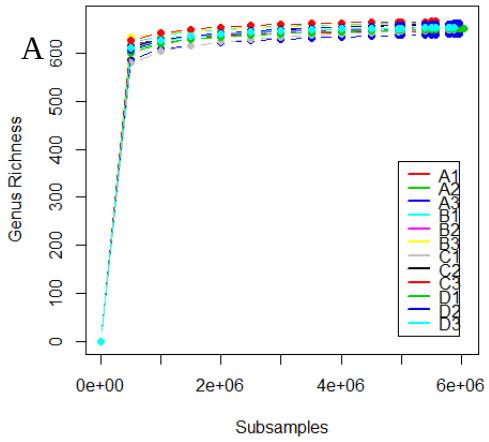
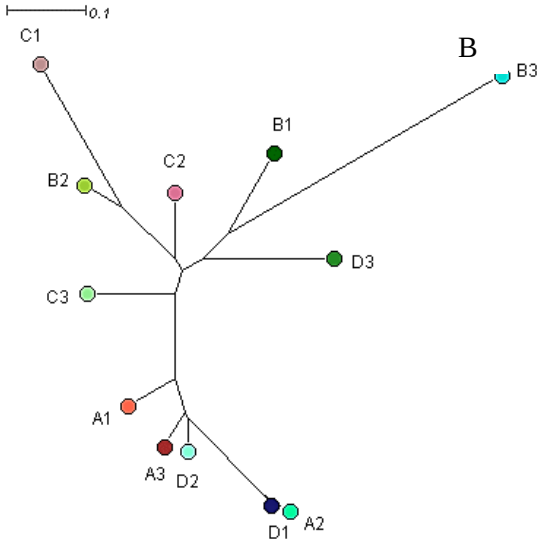


Fig. 5.2. Assessment of alpha-diversity in samples from the control and treatment groups. Panel A displays the rarefaction curve indicating genus richness at increasing sequencing depth of sample from groups A, B, C, and D. Panel B exhibits Neighbor-Joining tree based on Bray-Curtis distance matrix encompassing 12 datasets from all the groups. Panel C shows relative abundances of various taxa at super kingdom level in various groups. * indicates significant difference in a group compared to negative control at P value < 0.05.





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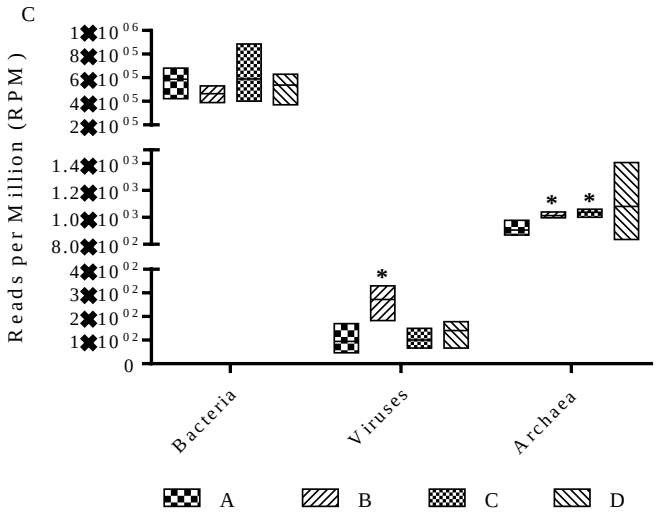
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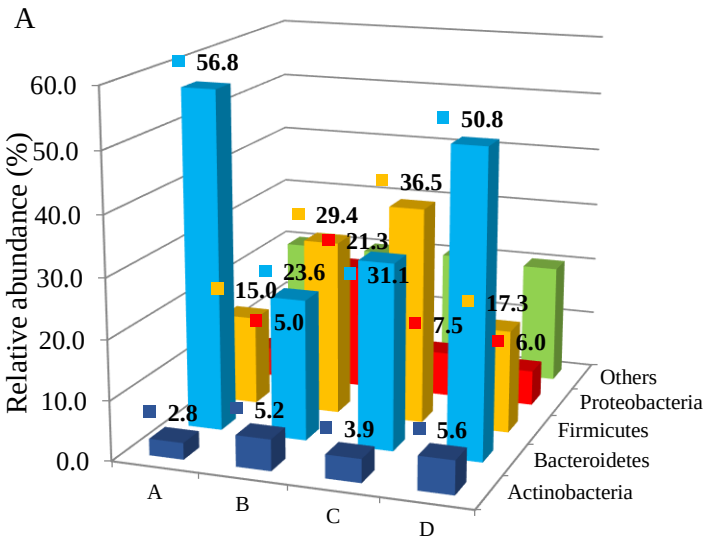
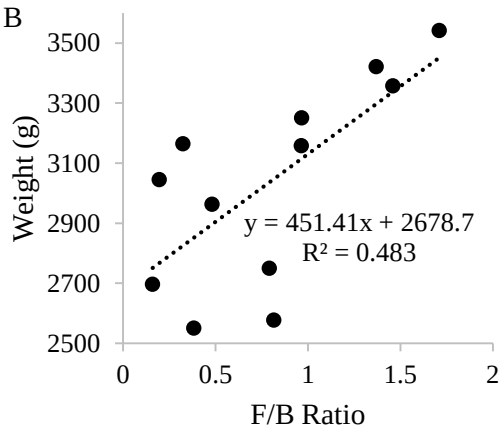
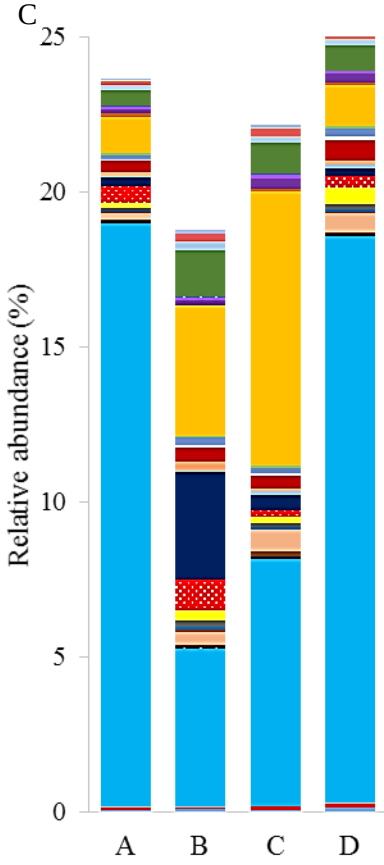
1.0✖10 03
8.0✖10 02
4✖10 02
3✖10 02
2✖10 02
1✖10 02
0
*
*
*
Bacteria
Viruses
Archaea
A
B
C
D

Fig. 5.3. Differential composition of chicken ceca microbiota. Panel A depicts bacterial distribution at phylum level in 3D plot with pooled datasets. Panel B demonstrates a scatter plot of Firmicutes to Bacteoirdetes (F/B) ratio to broiler weight at 42 days of age. Panel C shows bacterial distribution at genus level in all the broiler groups. Finally, panel D displays the variation in the presence or absence of DNA viruses at genus level in samples from groups A, B, C, and D.





C



Taxonomy	A	B	C	D
Adlercreutzia	0.05	0.12	0.08	0.14
Alistipes	0.10	0.03	0.10	0.13
Bacillus	0.03	0.05	0.02	0.04
Bacteroides	18.83	5.09	7.95	18.26
Bifidobacterium	0.05	0.05	0.09	0.09
Campylobacter	0.07	0.05	0.16	0.05
Clostridium	0.17	0.42	0.69	0.62
Corynebaterium	0.04	0.08	0.03	0.04
Desulfotomaculum	0.05	0.09	0.08	0.07
Desulfovibrio	0.07	0.19	0.10	0.16
Eggerthella	0.20	0.36	0.19	0.54
Enterococcus	0.52	0.99	0.24	0.37
Escherichia	0.27	3.43	0.48	0.23
Ethanoligenes	0.08	0.07	0.10	0.15
Eubacterium	0.12	0.28	0.12	0.10
Faecalitalae	0.37	0.43	0.40	0.69
Geobacter	0.05	0.11	0.07	0.09
Gordonibacter	0.13	0.26	0.18	0.24
Lachnoclostridium	0.06	0.03	0.05	0.08
Lactobacillus	1.15	4.19	8.86	1.32
Odoribacter	0.15	0.02	0.08	0.11
Oscillibacter	0.12	0.17	0.33	0.26
Peptoclostridium	0.06	0.07	0.11	0.09
Pseudomonas	0.04	0.04	0.03	0.03
Roseburia	0.52	1.49	1.02	0.82
Ruminococcus	0.14	0.29	0.14	0.18
Salmonella	0.03	0.01	0.03	0.01
Streptococcus	0.11	0.23	0.26	0.28
Streptomyces	0.04	0.03	0.06	0.06
Treponema	0.05	0.10	0.08	0.06

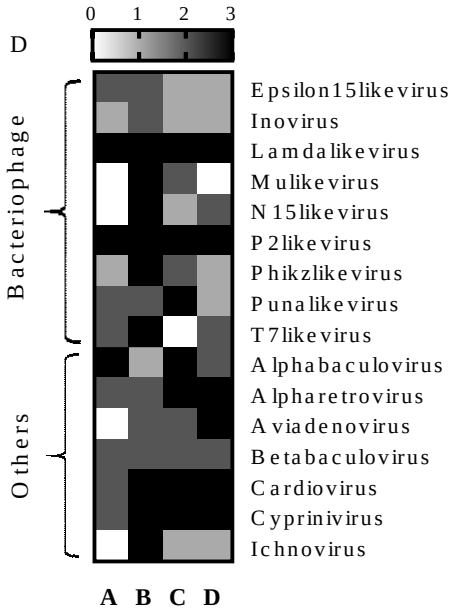


Fig. 5.4. Functional classification of chicken cecal microbiomes from groups A, B, C, and D. Relative abundances were calculated based on the numbers for every 100 million sequences. * and ** indicates significant difference in relative abundance of a KO functional orthog at alpha value of 0.10 and 0.05, respectively.

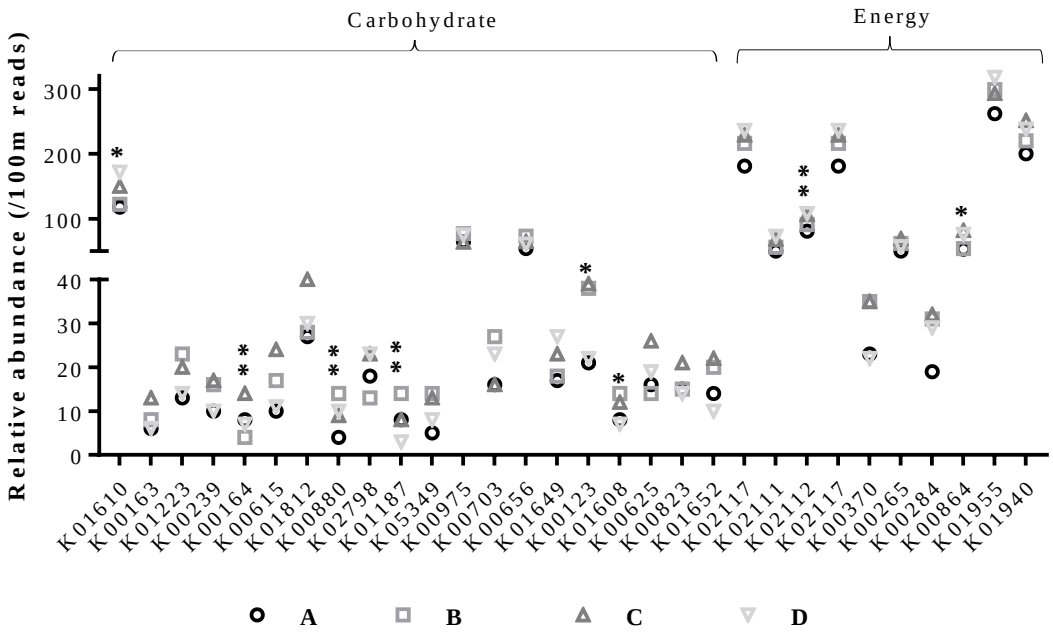
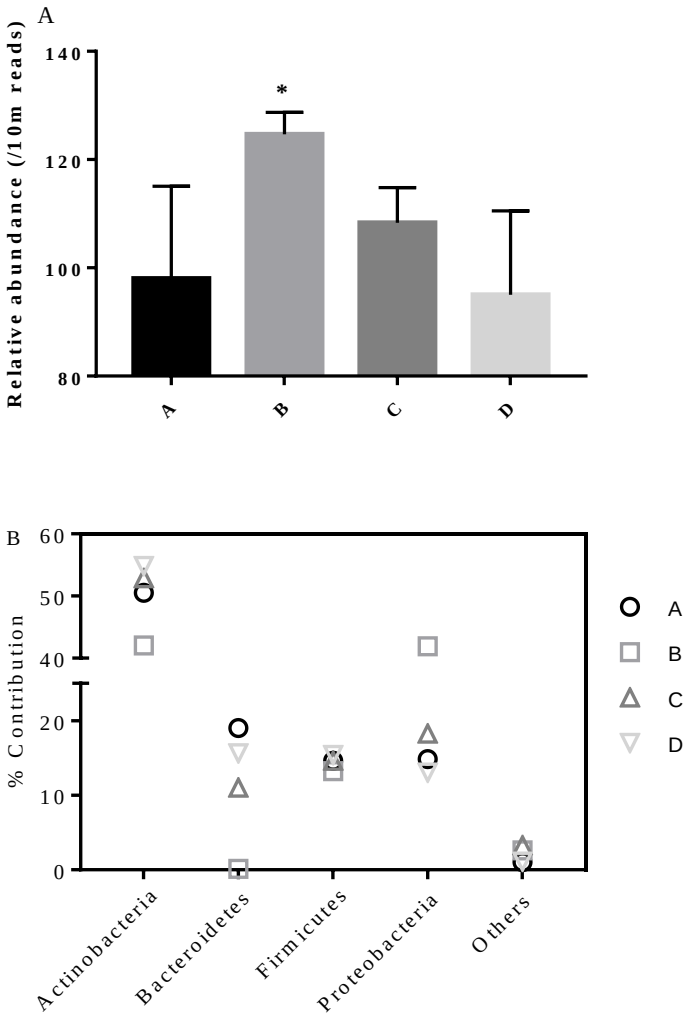


Fig. 5.5. Evaluation of resistome profiles in chicken gut microbiome. Panel A shows relative abundance (reads per 10 million sequences) of total Antibiotic Resistance Genes (ARGs) observed in core resistomes of broiler ceca from groups A, B, C, and D. Panel B depicts percent contribution of microbial taxa on relative abundances of ARGs in the core resistomes. Panel C exhibits a heatmap encompassing relative abundances of shared ARGs among the treatment groups in logarithmic scale.



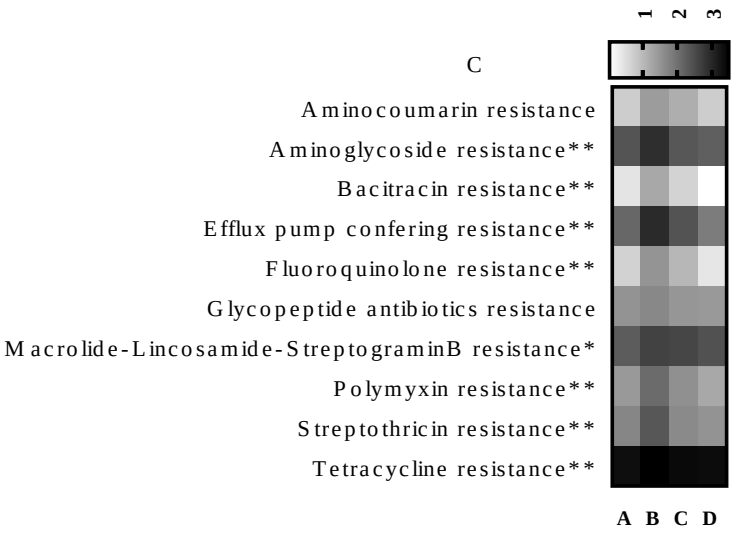
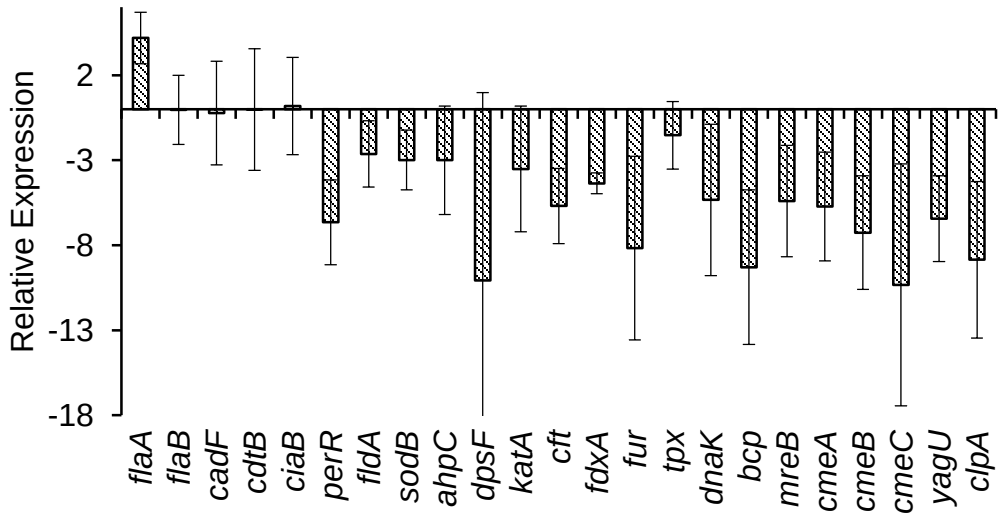

1
2
3
C
Aminocoumarin resistance
Aminoglycoside resistance**
Bacitracin resistance**
Efflux pump confering resistance**
Fluoroquinolone resistance**
Glycopeptide antibiotics resistance
Macrolide-Lincosamide-StreptograminB resistance*
Polymyxin resistance**
Streptothricin resistance**
Tetracycline resistance**
A B C D

Fig. 5.6. Expression of stress response genes in *C. jejuni* in the presence of BPE.



Overall conclusions

1. Berry pomaces are rich sources of bioactive phenolics. HPLC-MS analysis indicated that both blueberry and blackaberry pomace extracts contained thousands of compounds.
2. BPE inhibited *C. jejuni* growth *in vitro*, altered its pathogenicity, and physicochemical properties, as well as, its interactions with cultured host cells *ex-vivo*.
3. BPE inhibited the growth of commercially important poultry pathogens, e.g., *P. multocida*, *S.* Gallinarum, and *S.* Pullorum *in vitro*. BPE supported probiotic *L. plantarum* to competitively inhibit the growth of pathogenic *S.* Gallinarum, and *S.* Pullorum in a co-culture model.
4. 1.0 g GAE/L of BPE as water supplement reduced natural colonization of *Campylobacter* in broiler ceca by 5 logs at 6 weeks of age in floor pens, whereas 1.0 g GAE/L of BPE as water supplement for 72 h before euthanasia resulted approximately 2 logs reduced natural colonization of *Campylobacter* in chicken ceca at 6 weeks of age in floor pens.
5. 1.0 g GAE/L of BPE reduced marked *C. jejuni* colonization by 1 log upto 3 weeks of age while grown in isolated cageing system.
6. BPE supplementation in water increased the mean body weight of chickens by 6% compared to 9.5% with AGP supplementation.
7. BPE caused an AGP-like pattern in bacterial community with comparative increase of Firmicutes and a concomitant reduction of Bacteroidetes in chicken ceca. However, AGP was associated with increased relative abundance of Proteobacteria.
8. AGP resulted higher prevalence of bacteriophages in the cecal microbiome compared to BPE.
9. BPE and AGP resulted in cecal microbiome with over-represented functional orthologs involved in carbohydrate and energy metabolism.
10. Analysis of resistome profile of cecal microbiomes revealed higher predicted ARGs in chickens provided with AGP compared to BPE.
11. Sequential increase of BPE in growth medium doubled the MIC of BPE to *C. jejuni* but the strain became susceptible to BPE just after one subculte in BPE-free medium.
12. BPE significantly downregulated the *C. jejuni* genes involed in stress response.

Future directions

1. Supplementation of metagenomic data with metabolomic and metatranscriptiomic datasets.
2. Large scale growth promotion study at farm level.
3. Extensive cost-benefit analysis for practical applicability of BPE in farm animal production.

Glossary

Probiotics	Microorganism that are introduced into the body for beneficial purposes.
Metagenome	The total extractable DNA from an environment. The chicken cecal metagenome is the collection of the DNA of the chicken and the cecal microbiota. Metagenome is often referred to as microbiome.
Metagenomics	The study of the metagenome or microbiome. Metagenomics can be targeted study with 16S ribosomal RNA or shotgun sequencing based untargeted study.
Microbiota	The collective microbial (bacteria, virus, archaea) community that inhabit a specific environment, e.g., chicken cecal microbiota or human skin microbiota.
Microbiome	The collective microbial genomic contents. It also provides information on the the total genetic capacity of the microbial community.
Resistome	The collection of antibiotic resistance genes in a microbiome.
Zoonoses	Diseases transmissible from animal to humans.

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