INVESTIGATING THE MODE OF ACTION OF *BACILLUS SUBTILIS* C-3102 PROBIOTIC

IN BROILER CHICKENS

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NOTE TO READERS

This thesis is organized and formatted by following the University of Saskatchewan College of Graduate and Postdoctoral Studies guidelines for a manuscript-style thesis. Therefore, there is some repetition in content among chapters. Chapter 1 is a general introduction to motivation for this work and Chapter 2 provides a review of the literature supporting the presentation of the hypothesis and specific objectives. Chapter 3 to 5 will be submitted for publication in peer-reviewed scientific journals in the future and Chapter 6 contains a general discussion and overall conclusion. Tomohiro Hamaoka conceived the original idea, co-designed all experiments with Dr. A. Van Kessel, conducted all animal experiments with assistance of Mr. Jason Marshall, conducted all laboratory tissue analyses (with exception of mRNAseq), and completed statistical analysis of data. RNAseq sequence data was analyzed by Ms. Zoe Gillespie with support of Dr. C. Eskew. Mr. Hamaoka and Dr. Van Kessel interpreted the results and Mr. Hamaoka prepared all first drafts. References cited in each chapter were combined and listed in the List of References section at Chapter 7 of the thesis. Information on germ-free rearing systems utilized and further developed in the research chapters is provided in the Appendix section at the end of this thesis.

ABSTRACT

Bacillus probiotics have been utilized to improve body weight gain or feed conversion ratio in poultry production since 1970s, however, the mechanisms of action of *Bacillus* probiotics are not well delineated. Two major pathways were hypothesized; a direct pathway mediated by probiotic organism and an indirect pathway mediated via modified host intestinal microbiota. Two new gnotobiotic experimental systems were developed to differentiate these pathways. Firstly, the direct hypothesis was accessed by combination of *in ovo* inoculation and sterilized individual HEPA-filtered containers. *Bacillus subtilis* C-3102 spores (BS) were delivered to amniotic fluid at E17 by *in ovo* inoculation. Vegetative growth and mono-association by BS was observed at hatch. Multiple direct effects were observed including modified body and organ weight as well inflammation, barrier function, and nutrient digestion gene expression. The response to mono-association with other bacteria, including *Enterococcus faecalis* (ENT03), *Lactobacillus salivarius* (LCT01), *Escherichia coli* (ECL01 and ECL02), *Bacteroides fragilis* (BCT06), and *Clostridium butyricum* (CLS01), representing taxonomic families dominant in chicken were also investigated. Live but not heat-killed *E.coli* inoculation were lethal before E20. Other strains demonstrated variation in colonization density, and intestinal gene expression of inflammation and barrier function. Mixture of 5 bacterial species, Simplified Microbiota (SM) inoculant, were administered to 1-day-old germ-free birds in HEPA-filtered isolators. In 2 of 4 isolators, irradiated feed was supplemented with BS. At 14 day of age, culture of intestinal contents demonstrated colonization with all SM species without contamination. Vegetative BS was observed in BS supplemented SM birds associated with reduced abundance of ECL01 and ENT01. BS reduced relative organ weight, increased digestion and nutrient transport gene expression. Observations suggest probiotic actions of *B. subtilis* in broilers result from both direct and indirect mechanisms. Direct mechanisms increasing digestive and absorptive capacity are supported by observations *in ovo* and in SM model. Colonization patterns observed in SM model suggest competitive reduction of *E.faecalis* and *E.coli*, may be indirect mechanisms. The *in ovo* and SM models developed here to simplify the intestinal microbial environment were demonstrated to aid in the differentiation of direct and indirect mechanisms of action of gut modifiers such as probiotic bacteria.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AAFCO	American Association of Feed Control Officials
ANOVA	Analysis of variance
APN	Aminopeptidase N
BCT06	*Bacteroides fragilis* BCT06
BS	*Bacillus subtilis* C-3102
BW	Body weight
CDN1	Claudin 1
CDN4	Claudin 4
CDN5	Claudin 5
CFIA	Canadian Food Inspection Agency
CFU	Colony forming unit
cGH	Chicken growth hormone
CLS01	*Clostridium butyricum* CLS01
DFM	Direct-fed microorganisms
DNA	Deoxyribonucleic acid
ECL01	*Escherichia coli* ECL01
ECL02	*Escherichia coli* ECL02
EFSA	European Food Safety Authority
ENT03	*Enterococcus faecalis* ENT03
EU	European Union
FAO	Food and Agriculture Organizations
FCR	Feed conversion ratio
FDA	US Food and Drug Administration
GF	Germ-free
GI	Gastrointestinal
HEPA	High efficiency particulate air
IGF-I	Insulin like growth factor 1
IL-6	Interleukin 6
IL-8	Interleukin 8
LCT01	*Lactobacillus salivarius* LCT01
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
MUC2	Mucin 2
NGS	Next generation sequencing
PAFF	Plants, Animals, Food and Feed
PCNA	Proliferating cell nuclear antigen
PepT-1	Peptide transporter 1
PCR	Polymerase chain reaction
qPCR	Quantitative real-time PCR

RNA	Ribonucleic acid
S.E.	Standard error
SGLT-1	Sodium-glucose transport protein 1
SIF	Single Ingredients Feeds
SM	Simplified microbiota
TLR	Toll-like receptors
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
V:C	Villus height to crypt depth ratio
VDD	Veterinary Drugs Directorate
WHO	World Health Organization

1   GENERAL INTRODUCTION

The study of microorganisms known as microbiology, began in 1674 when Antonie van Leeuwenhoek observed bacteria in samples taken from the human mouth (Dock, 1928). Since their discovery, microbiology has revealed genetically diverse kingdoms with huge ecological roles in diverse environments from soil to deep oceans, to the digestive tract of animals affecting everything from soil fertility to climate change to animal health. Despite over 300 years of development of ever improving technologies to investigate microbial communities, knowledge of their composition and function remains limited and the whole truth of their biology and function is still beyond our reach.

Despite our lack of complete knowledge of microbial ecophysiology, man has successfully harnessed the "power" of microbial activity to enhance society. Numerous examples include early applications in the making of beer, wine and milk products, to the more recent harnessing of microbial biosynthetic capacity in making industrial and biomedical products with the application of genetic engineering. The first scientific report about the "power of microbial activity" in promoting health was made by a Russian scientist, Ilya Illych Mechnikov in 1907. He took his interest in the longevity of Cossacks in Bulgaria and suggested that their long life resulted from their high consumption of lactic acid bacteria fermented products (Metchnikoff, 1907). Since making this association, the relationship between human health and lactic acid bacteria has been extensively studied and exploited. One of the most extensively-investigated lactic acid strains might be *Lactobacillus casei* Shirota (Morotomi, 1996). *Lactobacillus casei* Shirota was isolated from human intestine in 1930 and has been marketed as a functional lactic acid bacteria (Yakult®, Yakult Honsha Co., Ltd., Tokyo Japan) since 1935 (Shirota et al., 1966). Not only in human life but also in animal production, bacterial strains have been utilized based upon traditional experience, anecdotal observation and scientific study in parts of Europe and the United States as well as in Japan for at least the most recent 40 years (Kozasa, 1989). These bacteria-based products have been identified using the term "Probiotic".

The term "Probiotic" is a composite word made from "Pro" meaning "for" in Latin and "Biotic" meaning "Life" in Greek. The word was first used by Lilly and Stillwell for describing substances secreted by one organism which stimulates the growth of another (Lilly and Stillwell, 1965). The probiotic preparations for human use are primarily based on lactic acid bacteria,

including members of the *Lactobacillus*, *Streptococcus* and *Bifidobacterium* genera (Fuller, 1991). In contrast, *Bacillus* spp. have often been selected and marketed as probiotic species for animal production due to their ability to form spores permitting high stability and observations of health and growth promoting effects (Cutting, 2011).

The *Bacillus* species that have been most extensively utilized as probiotic bacterial products include *B. subtilis*, *B. cereus*, *B. coagulans* and *B. licheniformis* (Cutting, 2011). The growth promoting effect of these *Bacillus* species started to be recognized in the early 1970s and several early products were launched in Japan in this decade (Kozasa, 1989; Y, 1979). Scientific reports about the effect of *Bacillus* probiotics on broiler productivity started to appear in the peer-reviewed literature in the 1980s (Nguyen et al., 1988; Sullivan et al., 1986). In addition to their growth promoting effect, *Bacillus* probiotics were observed to affect the composition of the intestinal microbiota (Ozawa et al., 1981) suggesting a potential mode of action. The relationship between the intestinal microbial community structure and broiler health and performance became an active area of research during 1990s because of growing concern regarding the development of antibiotic resistant bacteria linked to overuse of antibiotics in livestock production. Usage of antibiotics for livestock production continues to be a public concern and the application of probiotics is expected to serve as one alternative tool to limit pathogen colonization and promote bird health and performance.

Through microbial profiling of excreta and intestinal contents, oral supplementation of *B. subtilis* has been shown to exhibit an inhibitory effect on zoonotic bacterial pathogens colonizing the chicken gut (La Ragione et al., 2001; La Ragione and Woodward, 2003; Maruta et al., 1996a; Maruta et al., 1996b). The pre-harvest reduction of zoonotic pathogens in the intestinal tract of food animals, especially those important in foodborne illness such as *Salmonella* species, by *Bacillus* probiotics has also been observed in other food animal species such as piglet and turkey (Scharek-Tedin et al., 2013; Wolfenden et al., 2011). Nowadays, *Bacillus* probiotics are commonly marketed as effective in control of pathogenic bacteria such as *Salmonella* as a major component of their role as an alternative to antibiotics.

Bacillus probiotics have been reported as efficacious in promoting bird health and performance in several scientific reports (Cavazzoni et al., 1998; Cutting, 2011; Fuller, 1989; Hong et al., 2005; Kozasa, 1989). On the other hands, no clear significant benefit of *Bacillus* probiotic supplementation was also reported (Jenny et al., 1991; Samanya and Yamauchi, 2002). Because

of these variable results, much of the poultry industry remains skeptical about the efficacy of probiotics for growth promotion and pathogen control. Unfortunately, this problem remains an open question for scientists and manufacturers due to lack of knowledge about mechanism of action of *Bacillus* probiotics in both pathogen control and/or growth performance. In other words, while a number of empirical studies support the efficacy of probiotics for pathogen control and growth promotion, the lack of mechanistic knowledge leaves open the interpretation that studies demonstrating benefit can be attributed to normal biological variation.

We hypothesize that variation in the efficacy of probiotics is caused by differences of conventional background microbiota of test animals between each trial. In many culture-based studies, the chicken intestinal microbiota has been reported and published since the 1960s and combined show both an incomplete and highly variable representation of the taxonomic composition (Rehman et al., 2007). With the latest molecular biological techniques, recent studies have improved knowledge of the inventory of micro-organisms represented in the chicken intestine; however, large animal to animal variation in microbiota composition is still recorded even under carefully controlled trial conditions (Stanley et al., 2013b). Based on this knowledge, we also hypothesize the mechanism of action of *Bacillus* probiotics could be broadly separated into direct action on host physiology or indirect action via shifting the intestinal microbial composition. These mechanisms are not mutually exclusive and could be either additive or synergistic. Furthermore, the high bird-to-bird variation observed in the composition of the intestinal microbiota suggests that variability in efficacy of probiotic supplementation could be related to variation in the "starting" microbial composition in each setting.

To improve the opportunity to establish the mechanisms of action of probiotic *Bacillus* spp., a simplification and control of bacterial communities colonizing the gastrointestinal tract would be advantageous. Therefore, we have developed gnotobiotic models in the chicken based on either *in ovo* inoculation or HEPA-filtered isolator rearing. We anticipate these models will minimize variation observed in conventional birds and permit investigation and separation of the direct and indirect mechanisms of action of *B. subtilis*.

2   LITERATURE REVIEW

2.1   *Bacillus* probiotics

2.1.1   Characterization and commercial application of *Bacillus* spp.

B. subtilis is defined as the type species for the genus *Bacillus* according to the Bergey's Manual of Determinative Bacteriology Ninth Edition (Bergey and Holt, 1994). *B. subtilis* is a Gram positive bacterium with rod-shaped straight cells measuring 0.5-2.5 x 1.2-10μm, and growing in aerobic or facultatively anaerobic conditions (Holt et al., 1994). *Bacillus* spp. are well known to form spores which ensure long-term residence of the bacteria in inhospitable environments, such as conditions of high temperature, limited moisture, high pressure and chemical toxins (Nicholson et al., 2000). *Bacillus* spp. are primarily considered as a soil organism; however, sporulation ability allows members of this genus to spread into diverse environments and they are thus found in almost every terrestrial environment such as in decaying organic matter, on plant surfaces and the gut of insects, birds and mammals (Nicholson, 2002).

A couple of *Bacillus* spp., such as *B. cereus,* are well known as having the potential to cause foodborne illness (Van Doren et al., 2013), while others, such as *B. pumilus* and *B. subtilis* have been implicated as causative agents in foodborne disease but without clear consensus on their role in the illness (Logan, 2012). Generally, *Bacillus* spp. are recognized as non-harmful safe bacteria such that several species have been utilized historically as inoculants to make fermented foods (Chen et al., 2012). As a result, members of the *Bacillus* genus are often very familiar to people in lay environments, particularly those who live in eastern Asian countries. Natto is a traditional Japanese soybean food product which is fermented with *B. subtilis* var. natto. Interestingly, a health benefit of natto for humans was already recognized in the17[th] century in Japan (全国納豆協同組合連合会, 2004). The first scientific studies about the antagonistic function of *B. subtilis* var. *natto* against disease (dysentery) were reported in Bulletin of the Naval Medical Association at 1936 (Arima, 1936; Kozasa, 1989). More recently Natto was shown to have probiotic properties, such as enhancing body weight gain, suppressing number of *Candida albicans* and promoting growth of *Lactobacillu*s (Hong et al., 2005). *Bacillus* fermented products are also very popular in other Eastern Asian countries, where often unique products are associated

with specific regions such as Tempeh in Indonesia, Tuong in Vietnam, Doenjang in Korea and Chinese cheese in China and Taiwan (Chen et al., 2012).

A wide diversity of industrially important strains of *Bacillus* spp. are used for a variety of purposes, such as feed additives, insecticides, soil improvement agents and industrial enzymes (Schallmey et al., 2004; Su et al., 2020). *Bacillus thurigiensis* was originally recognized as a pathogen of the silkworm. Several strains of the species were genetically modified to enhance their toxic activity against target insect pests and utilized as insecticides since 1960s (Lucena et al., 2014). *Bacillus thurigiensis* is one of the most commercially successful entomopathogenic bacteria and is used in the biological control of insects in agronomical and industrial areas (de la Fuente-Salcido et al., 2013). Producing commercially valuable enzymes was also recognized as a major property of *B. subtilis* at the beginning of the twentieth century (Olivier, 1946). Amylase is one of the most popular industrial enzymes which is currently produced using *Bacillus* and large volumes are sold to the starch industry for production of syrups and ethanol, and to detergent manufacturers to enhance stain removal ability (Cherry and Fidantsef, 2003). *Bacillus* species are also widely used for production of other industrially important enzymes, such as proteases, lipases and phytases (Hasan et al., 2006). More relevant to the focus of this review, a number of *Bacillus* strains have been utilized as probiotic feed additives in animal production (Table 1.1) and recognized for their beneficial characteristics on poultry health and performance (Ramlucken et al., 2020). *Bacillus* spp. continue to serve many important functions in our modern life.

2.1.2   Definition of probiotics

The term probiotic was coined to contrast with the term antibiotic and was first used by Lilly and Stillwell describing substances secreted by one organism which stimulate the growth of another (Lilly and Stillwell, 1965). After the first appearance, the term has been used in several different ways and the definition has been modified many times.

In animal production, Parker started to use this word to describe growth promoting animal feed supplements and defined the term as "organisms and substances which contribute to intestinal microbial balance" (Parker, 1974). Fuller considered this wording imprecise and unsatisfactory as antibiotic compounds would be included (Fuller, 1991). So, he revised the definition as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal

microbial balance" (Fuller, 1989). Since 1989, many scientists have continued to redefine the term so that there are currently several definitions commonly utilized without significant consensus. According to several reviews that have examined the probiotic definition, it seems like one concept that has consensus among scientists is that probiotics consist of a live bacterium (Schrezenmeir and de Vrese, 2001).

An issue resulting in considerable debate regarding Fuller's definition has been the ability to define an improved "intestinal microbial balance". Nevertheless, this definition was groundbreaking because it focused on the interaction between intestinal microbiota as a mechanism of action of probiotics as well as emphasizing the importance of live cells as an essential component.

In 2002, a joint working group of the Food and Agriculture Organizations (FAO) of the United Nations and World Health Organization (WHO) published draft guidelines for the evaluation of probiotics in food. They offered adoption of the definition of probiotics as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO., 2012). Note that viability was still an essential requirement in this definition.

After 2010, several scientists and manufactures started to bring conflicting views on the necessity of the viable status of probiotic products. Lahtinen (2012) raised the question on viable status arguing several potential mechanisms of probiotic action that are not directly dependent on cell viability. However, products which consist of dead microorganisms are not recognized as probiotic at the time this thesis was written. The term 'inactivated probiotics' has been adopted by academics and the industrial communities to describe products based on killed microorganisms. Where such products show efficacy there is considerable potential advantage with respect to ease of product handling, processing and storage. Recently, this concept is slowly being accepted, and this change might affect the definition of the probiotics in the future.

Table 1.1. Example of *Bacillus* probiotic products marketed for poultry production.

Strain	Manufacturer	Product name	Reference
Bacillus subtilis DSM17299	CHR HANSEN	GALLIPRO®	https://www.chr-hansen.com/ Knarreborg et al. (2008), Knap et al. (2010), Knap et al. (2011)
Bacillus subtilis C-3102	Asahi Biocycle Co., Ltd.	CALSPORIN®	https://www.asahibiocycle.com/ Maruta et al. (1991), Maruta et al. (1996a), Fritts et al. (2000), EFSA (2006b), Jeong and Kim (2014) Liu et al. (2019), Rahimi et al. (2019),
Bacillus subtilis spp. (3 strains)	Novus International, Inc.	SORULIN®	https://www.novusint.com/
Bacillus cereus ver toyoi	RUBINUM	Toyocerin®	https://www.rubinum.es/
Bacillus subtilis DB9011	Idemitsu Kosan Co., Ltd.	Molucca	https://www.idemitsu.com/
Bacillus subtilis PB6	Kemin Industries Inc.	CLOSTAT®	https://www.kemin.com/ EFSA (2009), Jayaraman et al. (2013)
Bacillus subtilis BN	Meguro Institute Co., Ltd.	Growgen®	http://www.meguro-kenkyujo.co.jp/ Horie et al. (2018)
Bacillus subtilis DSM5750 *Bacillus licheniformis* DSM5749	CHR HANSEN	BioPlus2B®	https://www.chr-hansen.com/ EFSA (2016b)
Bacillus spp. (5 strains)	DuPont	Enviva® Provalen Plus	https://www.dupontnutritionandbiosciences.com/
Bacillus spp.	DSM	Blancius™	https://www.dsm.com/
Bacillus licheniformis DSM 28710	HuvePharma	B-Act®	https://www.huvepharma.com/ EFSA (2016a)

2.1.3 History of *Bacillus* probiotics in animal production

In multiple broiler markets around the world, *Bacillus* spp. are registered as a feed additive and utilized for broiler production (Silley, 2006). However, until recently *Bacillus* probiotics have not received extensive scientific study. For example, the first review about probiotics in poultry nutrition was written in 1985 (Jernigan et al., 1985). In this review, a total of 11 studies examining the effect of probiotics on broiler production were summarized, none of which examined the performance response to *Bacillus* probiotics. After the founding concept of probiotics by Metchnikoff (Metchnikoff, 1907), lactic acid bacteria and yeast have been the main species examined for probiotic properties in the English literature. This may reflect the broader use of these bacteria in Western cultures compared with *Bacillus* spp. which were commonly used in Asian cultures. One of the earliest reports examining the growth promoting effects of *Bacillus* probiotics was reported in 1986 (Sullivan et al., 1986) followed by a further report by Nguyen et al. (1988). As a consequence, a review article examining probiotics published in 1990 was the first to mention three *Bacillus* species (*B. cereus* toyoi, *B. subtilis* and *B. licheniformis*) as possible probiotic strains (Vanbelle et al., 1990).

In a review article about the effect of probiotics in poultry published in 1997, the author referenced reports in which no performance benefit was observed and described the effect of probiotics on poultry using the statement: "The lack of consistency in the results has caused many people to be skeptical about the positive effects of probiotics in chicken." (Jin et al., 1997). In contrast, more recent review articles summarizing avian probiotics referenced only positive reports on probiotic efficacy in poultry and did not identify lack of consistency as a concern (Khan and Naz, 2013; Smith, 2014). This change may signify a change in the scientific community regarding the potential of probiotic applications. Indeed, a literature search employing the key word "probiotic" returns over 4,000 original research articles in 2020 alone.

Probiotic industry participants indicate that the marketing and use of *Bacillus* spp. probiotics began at least by 1980 in Japan. An industry magazine article published in Japan in 1978 (Kimura, 1978) reported significant market penetration of *Bacillus* spp. probiotics in that country in the 1970s. In a peer-reviewed journal article published in Japanese, Kozasa (1989) cited several scientific publications examining the efficacy of *Bacillus* spp. probiotics in poultry dating back to the 1970s.

2.1.4 Regulations for *Bacillus* probiotics

It is often mis-stated that probiotic products are unregulated, and that quality and efficacy of the product is not tested scientifically and legally. In fact, probiotic products for animal production must be registered or approved to be on the market in most countries. Current regulations in major marketing areas are described briefly as follows.

In the European Union (EU), the Standing Committee on Plants, Animals, Food and Feed (PAFF Committee) plays a key role in animal health and welfare and is the regulatory authority responsible for feed additives (European Union, 2020). A European Food Safety Authority (EFSA) panel has authority to evaluate the probiotics and all probiotic products used in animal nutrition in the EU must be registered as microbial feed additives. The manufacturers must demonstrate data which include safety, efficacy and stability of their products (strains) by appropriate trials, and then all data should be documented and submitted to the EFSA scientific community according to regulation No. 1831/2003 of the European parliament and of the council of 22 September 2003 on additives for use in animal nutrition. Probiotics are categorized as zootechnical additives and a total of five strains of *Bacillus* are on the EU approval list as gut flora stabilizers which is one of functional groups in the category (European Union, 2014). In EU regulation, zootechnical additives are defined as "any additive used to affect favorably the performance of animals in good health or used to affect favorably the environment" and a functional subgroup termed "gut flora stabilizers" is described as "micro-organisms or other chemically defined substances, which, when fed to animals, have a positive effect on the gut flora" (European Union, 2003).

In the United States (US), the US Food and Drug Administration (FDA) is the primary federal agency responsible for regulation of animal feed. Under Memorandum of Understanding, American Association of Feed Control Officials (AAFCO) provides "model laws" and regulations that nearly all states have adopted as the basis for their feed-control program (U.S. Food & Drug Administration, 2019). Manufacturers are required to use the term of direct-fed microorganisms (DFM) instead of probiotics. A definition of DFMs can be found in section 36.14 Direct-Fed Microorganisms in the yearly Official Publication by AAFCO and appropriate bacterial species for use in animal feeds are listed. The scientific name of the bacteria in the DFM should be listed on the product label without their specific strain name unlike other countries. In addition, reference to the efficacy of DFMs (probiotics) for sales purpose in the US is strictly prohibited. Strain approval for sale is based on safety rather than efficacy. As of 2020, a total of six *Bacillus* species

are listed as reviewed species in section 36.14 Direct-Fed Microorganisms including *B. amyloliquefaciens, B. lentis, B. licheniformis, B. pumilus, B. coagulans* and *B. subtilis* (Association of American Feed Control Officials, 2020).

In Japan, probiotics for animal feed can be sold in two different formats, as probiotics (feed additive) and as a Mixed Feed. Ministry of Agriculture, Forestry and Fisheries is the administrator of all feed ingredient and feed additives for livestock. Selling the probiotic product as a feed additive is the first option for probiotic manufacturers because of several limitations on the sale of Mixed Feed. To be approved as a feed additive, scientific data about safeness of the strain and providing at least three trial results which shows effectiveness of the product on the target animal species are required. Specifications and safeness of the product strains must be shown through the data from both *in vivo* and *in vitro* studies. Efficacy of the product on body weight gain and feed conversion ratio must be shown in multiple field trials. Generally, these application processes are time and labor consuming and the process usually takes at least a few years until it is finished. In return for these cumbersome procedures, two claims are allowed for the product in the Japanese market, improving body weight gain and feed conversion ratio.

As noted above, probiotic products can as be sold as Mixed Feed in Japan. The registration process of Mixed Feed is simpler and easier compared with the process for registration of a feed additive, however there are two demerits to consider on this option. No efficacy claim is available for Mixed Feed and the product should be mixed into feed on site. This means products in Mixed Feed category are not allowed to be added to a premix of feed at the feed mill, thus only feed additives can be mixed into premix of animal feed at feed in Japan. As of 2014, a total of seven strains of *Bacillus* have been registered as a feed additive in Japan, *B. coagulans*, 3 strains of *B. subtilis*, 2 strains of *B. cereus* and *B. badius*.

In Canada, the Canadian Food Inspection Agency (CFIA) is the regulatory authority for feed ingredients. Single Ingredient Feeds (SIF) is defined as "any substance or mixture of substances that is assessed or evaluated as being acceptable for use in feeds and that is described in an item of Schedule IV or V" and listed ingredients in Part I have been evaluated for both safety and efficacy (Canada, 2020). In 2017, a new category was added in the ingredient list as "Gut Modifier". Feed claims, such as improves feed intake, improves feed efficiency, and improves daily weight gain, will be available for Gut Modifiers under the Feed Act and Regulations in Canada, presumably based on submission of supporting scientific data. The Veterinary Drugs

Directorate (VDD) and CFIA continue to work together to explore solutions and to formalize the registration process for the Gut Modifier (Illing and Price, 2017).

2.2 Efficacy of *Bacillus* probiotics in poultry production

2.2.1 Efficacy on body weight gain and feed conversion ratio of poultry

Improving body weight gain and feed conversion ratio (FCR) is a major claim and goal of *Bacillus* probiotics in broiler production. An examination of the influence of a dried *B. subtilis* culture on performance of broilers fed different protein levels (Sullivan et al., 1986), and of the effect of two commercial *Bacillus* probiotic products on broiler performance (Nguyen et al., 1988), were the earliest reports found about the growth promoting effect of probiotics. Jiraphocakul et al. (1990) fed 0.10% to 0.025% of dried *B. subtilis* culture to turkeys and reported significant improvement on body weight gain ($p<0.01$) and FCR ($P<0.05$) at 12 weeks of age. In these early studies, strain information and number of viable bacteria in the supplement were not considered as important parameters. Another early study shows 10 or 20 g/kg of *B. subtilis* culture supplementation improved feed efficiency, reduced nitrogen utilization and the ratio of abdominal fat or liver to body weight in female broiler chickens (Santoso et al., 1995). Cavazzoni et al. (1998), reported that their newly isolated *B. coagulans* improved efficacy in growth and feed conversion ratio of broiler chicken at day 49 comparable to that of the virginiamycin treatment group.

Again, a serious defect of these early studies was lack of information about the identity of the strain and optimal concentration of supplemented bacterium. This was a common fault of all early probiotic studies, not only for *Bacillus* probiotic studies. The importance of information on strain identity and dosage started to be recognized around the middle of the 1990s. Recent results indicated that effective concentration of *Bacillus* probiotics in feed is at least more than $1x10^5$ CFU per gram of feed. *B. subtilis* C-3102 ($3x10^5$ CFU/g of feed) significantly increased body weight gain to 42-days of age and improved FCR ($p<0.05$) from the 21- to 42-day period (Fritts et al., 2000). Improving growth performance of broilers by supplementation of *B. subtilis* C-3102 in feed continuously was reported by several investigators (Aliakbarpour et al., 2012; Gracia et al., 2008; Jeong and Kim, 2014). *B. subtilis* DSM17299 was supplemented into broiler feed with $8.5x10^5$ CFU/g and chick performance was significantly enhanced ($p<0.05$) at day 35 (Knarreborg et al., 2008). A probiotic product which contains three *Bacillus* strains showed significant improvement

on feed efficiency of 21-d-old broilers (Waititu et al., 2014). *Bacillus coagulans* NJ0516 significantly improved FCR when the final concentration of the probiotics were $2x10^6$ CFU/g of feed or $4x10^6$ CFU/g of feed (Wang and Gu, 2010). Molnar et al. (2011) conducted dose response trial of *B. subtilis* DSM17299 by using several dosages between $7.27x10^8$ CFU/g to $7.27x10^{11}$ CFU/g. All broilers fed *B. subtilis* supplemented diet had significantly higher body weights than the control group from day 7 to the end of growing period. In this case, no differences in body weight gain between supplementation levels of *B. subtilis* DSM17299 were reported in this trial.

In addition to results from publications in scientific journals, many data were also taken in the approval process in the EU. For example, *B. subtilis* C-3102 was registered as a zootechnical feed additive (functional group: gut flora stabilizer) in the EU in 2006. For the approval, efficacy of the product was demonstrated in member countries of the EU and in 2004 results were reported to the European Commission. A total of four trials were made with broilers in this case and there was a statistically significant benefit in final weight, daily weight gain and/or feed efficiency in three of the four trials (EFSA, 2006b). Other registered strains also provided data supporting a growth promoting effect and all data has been published in the EFSA Journal. *B. subtilis* PB6 improved final body weight and FCR in three studies (EFSA, 2009). Results from seven trials were provided by *B. subtilis* DSM17299 manufacturers and four showed significant weight gain improvement in treated group and improvement of FCR was observed in two trials (EFSA, 2006a). Based on these results from scientific research at academic and industry, effect of *Bacillus* probiotics on body weight gain and FCR can be claimed in several national markets in Europe or in Japan. These and other published studies indirectly support marketing in other countries such as the US, where performance claims are not permitted.

2.2.2 Effect of *Bacillus* probiotics on gut microbiota of broiler

In addition to growth promoting effects, the *Bacillus* probiotic has been observed to affect intestinal microbiota. Ozawa et al. (1981) demonstrated that administration of *B. subtilis* strain BN shifted the intestinal microbiota of weaning piglets. This ability to "shift" microbial populations by *Bacillus* supplementation has become one of the main topics of *Bacillus* probiotic studies since late 1990s. Maruta et al. (1996a) observed a large change in the intestinal microbial population of broiler chickens following *B. subtilis* C-3102 supplementation. After two weeks continuous

feeding of $3x10^5$ CFU/g of *B. subtilis* C-3102, a significant increase in the number of lactobacilli and a decrease in the number of *Clostridium* spp. and *Salmonella* spp. was reported in the broiler gut. Similarly, a significantly larger population of *Lactobacillus* was found in the small intestine of chickens fed with feed incorporated with 0.1% of *B. subtilis* CIP5832 culture product (Jin et al., 1996b). Increasing the relative proportion of lactobacilli in the poultry intestine is considered one of the major effects of *Bacillus* probiotics supported by a number of studies using several different strains of *Bacillus*. For example, freeze dried *B. subtilis* KD1 increased lactobacilli concentrations in the rectum of the 21 d-old broilers and 42 d-old broilers (Wu et al., 2011). Further, increased *Lactobacillus* following *B. subtilis* probiotic supplementation in the feed has continued to be reported in several publications as listed in Table 1.2.

Reduction of the number of coliforms and *Campylobacter* spp. on the broiler carcasses was also observed when $3x10^5$ CFU/g of *B. subtilis* C-3102 was supplemented in the feed during the entire production period (Fritts et al., 2000). The addition of *B. subtilis* KD1 significantly reduced the *E.coli* concentrations in broilers (Wu et al., 2011). *B. subtilis* C-3102 decreased number of intestinal pathogens of chicken in the field, such as Enterobacteriaceae, *Clostridium perfringens*, *Salmonella* and *Campylobacter* (Maruta et al., 1996a). Reduction of *Clostridium* by *B. subtilis* PB6 was confirmed under *Clostridium perfringens* challenge conditions (Jayaraman et al., 2013). At day 35, broilers fed diets supplemented with *B. subtilis* LS1-2 showed a significant decrease in caecal *Clostridium* and coliform counts (Sen et al., 2012a).

Effect of *Bacillus* probiotics on intestinal microbiota might have wide diversity and more strains and species could be affected by *Bacillus* supplementation. Modulation of ileal microbial communities by *B. subtilis* DSM17299 was observed in broilers and complex and diverse bacterial composition in the ileum was seen in the majority of chickens fed with the test strain (Knarreborg et al., 2008). *B. subtilis* C-3102 modulates microbiota composition dramatically in the *in vitro* gastrointestinal model and at least 58 strains of 400 investigated bacterial groups and species were influenced (Hatanaka et al., 2012).

Since Metchnikoff hypothesized an important role of lactobacilli on human lifespan, lactobacilli have been generally recognized as a beneficial bacterium for human and animal health. In several articles, a lactobacilli dominant microbiota is often described as an indicator of good health status and a microbial "balance" skewed toward lactobacilli is generally considered as beneficial for animals and humans. Although *Lactobacillus* spp. have been recognized by the

scientific community as lacking pathogenic species and proinflammatory characteristics (Willing and Van Kessel, 2009), work clearly establishing a performance or health benefit associated with increased colonization remains limited. There is the possibility that the changes we could see in these reference works might be just a part of the phenomenon and *Bacillus* might have more impact on the chicken microbiota.

Table 1.2. Shift of microbiota in the gut of broiler observed with *Bacillus* probiotic supplementation in scientific publications.

Reference	Test Animal	Age	Region	Probiotic strain	Increase	Decrease
Jin et al. (1996b)	Broiler	21	Intestine	*B. subtilis*	*Lactobacilli*	
		28	Intestine	*B. subtilis*	*Lactobacilli*	
Maruta et al. (1996a)	Broiler	14	Feces	*B. subtilis* C-3102	*Lactobacillus*	
		49	Feces	*B. subtilis* C-3102		*C. perfringens* *Salmonella*
Wu et al. (2011)	Broiler	21	Rectum	*B. subtilis* KD1	*Lactobacillus*	*Escherichia coli*
	Broiler	42	Rectum	*B. subtilis* KD1	*Lactobacillus*	*Escherichia coli*
Jeong and Kim (2014)	Broiler	35	Cecum	*B. subtilis* C-3102	*Lactobacillus*	*Escherichia coli* *Salmonella*
			Excreta	*B. subtilis* C-3102	*Lactobacillus*	*Escherichia coli* *Salmonella* *C. perfringens*
Sen et al. (2012b)	Broiler	35	Cecum	*B. subtilis* LS1-2		*Clostridium spp.* *Coliforms*
Ahmed et al. (2014)	Broiler	35	Cecum	*Bacillus amyloliquefaciens*		*Escherichia coli*
Lei et al. (2015)	Broiler	21	Excreta	*Bacillus amyloliquefaciens*	*Lactobacillus*	*Escherichia coli*
	Broiler	42	Excreta	*Bacillus amyloliquefaciens*	*Lactobacillus*	*Escherichia coli*
Hossain et al. (2015)	Broiler	35	Ileum	*B. subtilis* DSM17299[*1]	*Lactobacillus*	*Escherichia coli*
			Cecum	*B. subtilis* DSM17299[*1]	*Lactobacillus* *Bifidobacteria*	*Escherichia coli* *C. perfringens*
Park et al. (2017)	Broiler	42	Small intestine	*B. subtilis* C14, RX17[*2]	*Lactobacillus*	*Salmonella*

*1 Tested as combination product of *Bacillus subtilis* DSM 17299, *Clostridium butyricum* and *Lactobacillus acidophilus*.

*2 Tested under *S. gallinarum* ATCC9184 challenge condition.

2.2.3 Effect of *Bacillus* probiotics on *Salmonella* infection

2.2.3.1 *Relationship between Salmonellosis and poultry production*

Controlling the number of zoonotic pathogens in animal production is an important issue for society, given the significant incidence of human food borne illness. *Shigella*, *Listeria*, *Escherichia coli* O157:H7, *Salmonella* and *Campylobacter* are all well-known agents of food-borne illness. Among these bacterial pathogens, the risks of *Salmonella* contamination can be one of the most serious infections for consumers and have devastating effects for the industry. In the early 20th century, *Salmonella* groups of organisms were already recognized as predominant in food poisoning outbreaks (Savage, 1929). Over 90 years has already passed, and *Salmonella* continues to be a major foodborne illness for human society. Results from surveillance programs by Centers for Disease Control and Prevention in United States indicated that *Salmonella* is estimated to cause over one million illnesses in the United States, including 19,336 hospitalizations and 378 deaths (Batz et al., 2012). It is estimated that *Salmonella* spp. caused 11% of foodborne illness and 28% of foodborne illness-related deaths (Scallan et al., 2011). In fact, a total of 6,647 outbreaks of foodborne disease were recorded in United States during 1998 to 2002 and *Salmonella* serotype Enteritidis accounted for the largest number of outbreaks and outbreak-related cases (Lynch et al., 2006). In England, over 740,000 laboratory reports of *Salmonella enterica* infection were received and almost 43% were for *S. enterica* ser. Enteritidis during 1945 to 2011 (Lane et al., 2014). *Salmonella* has been estimated to cause 627,200 cases of infection and cost 846.2 million CAD per year in Canada (Todd, 1989). Salmonellosis has become a worldwide public health hazard.

Unfortunately, it is well known that poultry products, specifically meat and eggs, have been the most common source linked to *Salmonella* infection. Surveys in the US indicated that poultry was known as a vehicle of *Salmonella* in 20.9% of food borne illness caused by *Salmonella* from 1999 to 2008 (Batz et al., 2012). A total of 37,557 *Salmonella* Enteritidis infections were reported by the National Veterinary Services Laboratories in US from 1968 to 2011 and chicken was the source of the infection in 15,526 clinical cases (Centers for Disease Control and Prevention, 2013a). In addition, direct contact with a farm animal, a chicken, is also recognized as a transmission route for Salmonellosis (Cummings et al., 2012). Since the 1990s, a total of 45

Salmonella outbreaks have been linked to live poultry and which causes 1563 illnesses, 221 hospitalizations and five deaths (Centers for Disease Control and Prevention, 2013b). According to Humphrey (2006), for the last 20 years, there has been a pandemic of *Salmonella* Enteritidis infection in almost all parts of the world. Control of food-borne *Salmonella* infection is one of the major public-health goals and an important task in poultry production.

To develop effective strategies for reducing the risk of *Salmonella* from poultry products, several feed additives have been investigated such as organic acids, probiotics, prebiotics and certain specific carbohydrates, egg proteins, essential oils and bacteriophages (Berge and Wierup, 2012). *Bacillus* probiotics is also considered a candidate for *Salmonella* infection measures and it has gained much attention as alternative to antibiotics (Knap et al., 2011; Lee et al., 2011; Tellez et al., 2012).

2.2.3.2 Efficacy of Bacillus probiotic on Salmonella infection of poultry

Efficacy of *Bacillus* probiotics on *Salmonella* infection of broiler chicken has been studied since early 1990s and reduction of number or detection ratio of *Salmonella* in the chicken gut by administration of *Bacillus* probiotics has been reported by several scientists.

Maruta et al. (1996a) reported that seven weeks of continuous feeding of 3×10^5CFU *B. subtilis* C-3102 per gram of feed significantly decreased detection ratio of *Salmonella* from excreta of broiler chickens in a field trial. Detection ratio of *Salmonella* was 20/20 in control group and 10/20 in *Bacillus* supplemented group. Also, the number of *Salmonella* decreased from 4.07 log CFU/g in control group to 3.31 log CFU/g in treatment group. The same strain of *Bacillus* has been tested at the University of Arkansas where researchers examined carcass microbiological status of broiler chickens at the end of the trial. All 94 pre-chilled carcasses of birds fed control diet were positive for *Salmonella*, while 41 of 96 carcasses of birds fed *B. subtilis* C-3102 were positive at this trial (Fritts et al., 2000). In another Leghorn chicken trial, only 38% of birds from the *Bacillus cereus* toyoi supplemented group were *Salmonella* positive, whereas 63% of birds were still *Salmonella*-positive in the untreated control group at three week after inoculation (Vila et al., 2009). Knap et al. (2011) investigated fecal shedding of *Salmonella* at their broiler trial and tested birds fed with *B. subtilis* DSM17299 showed only 58% *Salmonella*-positive birds compare with control birds, which had 100% presence of *Salmonella*. *B. subtilis* B2A supplementation was

significantly associated with reduced intestinal *Salmonella* burden in day 28 old broilers (Park and Kim, 2014). Menconi et al. (2013) isolated seven strains of *Bacillus* from soil and poultry sources and indicate seven days continuous feeding of one isolate or a combination of two isolates significantly reduced the number of *Salmonella* in the crop and in the ceca of broiler chicken. In some cases a single oral inoculum of $1x10^9$ spores of *B. subtilis* was sufficient to suppress colonization and persistence of *Salmonella* Enteritidis in 20-day-old specific pathogen free chickens (La Ragione and Woodward, 2003).

2.3 Mechanisms of actions of *Bacillus* probiotics

Exact mechanisms of action of *Bacillus* probiotics, for example, how does *Bacillus* probiotic reduce the number of *Salmonella* in the chicken gut, is still not fully explained just like mechanisms for other effects of the probiotics. This is a common challenge for all probiotics beyond *Bacillus* probiotics. Currently, no probiotic effect is perfectly understood or controlled. According to the variability in results from many reported studies, the mechanism might not be only one and there may be several complex pathways. Also, the effect might be the result of an accumulation of small changes from several different mechanisms (Dumonceaux et al., 2006). To approach this expected complex system, we need to classify the phenomenon from *Bacillus* probiotic trial very carefully. We hypothesized that at least four categories of mechanisms of actions might be available, two of direct responses and two of indirect responses. In this section, the hypothesis will be explained by using the *Salmonella* reduction as example for the action of the *Bacillus* probiotics.

2.3.1 Direct response

2.3.1.1 Direct effect of Bacillus probiotics on the number of Salmonella in the gut

The biological properties of *B. subtilis* against other bacteria has been well known since 1940s (Olivier, 1946). The potential of *B. subtilis* as an antibiotic substance producer has been recognized for over 50 years and it was reported that the bacteria is able to produce more than two dozen antibiotics with an amazing variety of structures (Stein, 2005). Consequently, it is well known that several *Bacillus* species show antagonistic activity against other bacterial species in

co-culture. It is reported that 15 strains in 117 *Bacillus* isolates were found to have the ability to inhibit the growth of *Salmonella* Enteritidis DMST 15676 and *Salmonella* Typhimurium TISTR 292 (Thirabunyanon and Thongwittaya, 2012). Another article reported that all of seven *Bacillus* strains isolated from the environment showed antagonistic activities and three of them showed strong anti-*Salmonella* activity against 21 different *Salmonella* sero-types (Moore et al., 2013). According to results from an intestinal epithelial cell culture model using Caco-2 cells, *B. subtilis* culture medium itself significantly inhibited *Salmonella* Enteritidis invasion of epithelial cells in the model (Thirabunyanon and Thongwittaya, 2012). Live *B. subtilis* were removed from the cell culture media before *Salmonella* was added suggesting that *B. subtilis* secreted antimicrobial compounds into the media. However, the exact substance responsible for the antagonistic activity has not been identified. Indeed, each *Bacillus* strain appears to produce a unique cocktail of antibiotic substances (Katz and Demain, 1977). A few antibiotics have been found to be produced by a great variety of *B. subtilis* strains (e.g. subtilosin, surfactin, bacilysin), on the other hand, some antibiotics (e.g. lantibiotic sugtilin, ericin, mersacidin) are produced by specific strains of *Bacillus* (Stein, 2005). In particular, the antimicrobial activity of biosurfactants have received attention recently from several scientists due to their role as anti-adhesive agents to pathogens (Singh and Cameotra, 2004). Surfactin is one of those biosurfactants produced by some *B. subtilis* strains and known as strong antimicrobial substance against Gram negative bacteria (Hsieh et al., 2004). Results from the above-mentioned studies are often shown as collateral evidence to support existence of a direct effect of *Bacillus* probiotics against *Salmonella* in the gut. However, direct response theory does not have full consent from scientists despite a strong and simple logic. At least three unsolved questions might need to be solved before reaching consent on direct effect theory.

The first question is related to the differences in the number of active cells of *Bacillus* in the gut versus the plate medium. The spot test and paper disc diffusion method are popular methods to measure Minimum Inhibitory Concentration (MIC). These two methods employ high concentrations of vegetative *Bacillus* cells in close proximity to target bacteria (Moore et al., 2013). In contrast, *Bacillus* concentration in chicken feed is, at the most, between 10^5 CFU to 10^7 CFU of spores and recovery of vegetative *Bacillus* cells in intestinal contents is typically relatively low (2-5 log CFU/g). Therefore, *in vivo*, the ratio of *Bacillus* to target (pathogenic) bacterium is

considerably lower than during culture tests. Furthermore, these *in vitro* culture test rarely if ever are conducted in the context of a complex microbiota as observed in the gut environment.

A second question, as alluded to above, is the germination ratio of *Bacillus* in the gut. *Bacillus* probiotic products consist of a spore powder. The strains need to germinate to become metabolically active and produce antibiotics (Marahiel et al., 1993). Approximately 50% of *Bacillus subtilis* in a colony is still vegetative cells on plate medium after 24 h incubation (Gomez-Aguado et al., 2013). Based on this, it is considered that over 10^8 CFU of *Bacillus* strains are involved in the production of antimicrobial materials as vegetative cells during *in vitro* inhibition trials. Even if all *Bacillus* spores in the feed germinate in the gut, a supposition that is unlikely (Casula and Cutting, 2002; Hamaoka et al., 2010), total counts (vegetative plus spores) only reach around 10^5 CFU to 10^6 CFU per gram of contents. Combined, these findings regarding the relative abundance of vegetative cells in the gut environment suggest that a direct antimicrobial effect of *Bacillus* probiotics against *Salmonella* or other target bacteria is unlikely.

A third question which needs to be considered is a lack of dose response effect. The dose response relationship is not clearly confirmed in *Bacillus* probiotic trials. Park and Kim (2014) reported an effect of dietary *Bacillus subtilis* B2A on intestinal *Salmonella* populations in broiler chicks with different supplementation levels, such as 1.1 x 10^4, 1.1 x 10^5 and 1.1 x 10^6 CFU per gram in diet. In this trial, *B. subtilis* B2A significantly reduced number of *Salmonella* in small and large intestine both, however the response was not dose dependent. In another feeding trial, five different doses of *B. subtilis* DSM17299 between 1.56 x 10^5 CFU/g to 2.08 x 10^8 CFU/g were administered in feed. *Bacillus* supplementation was demonstrated to lower colonization by *E. coli*, but again the effect was not dose dependent (Molnar et al., 2011). If there is a direct pathway to suppress number of *Salmonella* or other pathogens, the effect of *Bacillus* probiotics could be expected to be dose dependent.

Direct antagonistic effect against *Salmonella* is a simple and strong theory and it could be part of the mechanisms of action of *Bacillus* probiotics. However, further information about the life cycle (germination and sporulation) of *Bacillus* in the gut will be needed to address the three points described above.

2.3.1.2 Direct effect of Bacillus probiotics on host physiology

The direct stimulation of the immune system by supplemented *Bacillus* probiotic is another possible hypothesis of the mechanisms of action. It is known that several immune mechanisms play a role in systemic clearance of *Salmonella* Enteritidis in chicken gut (Desmidt et al., 1998).

The innate immune system is one of the primary defense systems against bacterial infections. The Initial event at intestinal cells in *Salmonella*-infected chicken is detection of lipopolysaccharide (LPS) by toll-like receptors (TLR) (Barrow, 2007). LPS is known as a component of the outer membrane of *Salmonella* and other Gram-negative bacteria. TLR4 is one of the most extensively studied pattern recognition receptors and known as the LPS receptor (Albiger et al., 2007). Rodent studies have demonstrated that vegetative cells of *B. subtilis* can stimulate expression of the toll-like receptor genes for TLR2 and TLR4 (Huang et al., 2008). This pathway could amplify phagocytic function in the intestine and reduce number of *Salmonella* in the chicken gut. Lee et al. (2011) demonstrated that four *Bacillus* strains enhanced *Salmonella* phagocytic activity of macrophages on *Salmonella* Enteritidis in broilers. In this article, it was also reported that the effect was strain dependent and they could confirm the enhancement by four of the nine test *Bacillus* strains.

Other data also suggest stimulation of innate immune response by *Bacillus* probiotic strains. Effect of *B. subtilis* var. *natto* on T and B lymphocytes from the spleen of test chicken was reported in 1986 (Inooka et al., 1986). *B. subtilis* were able to stimulate proliferation of cells in gut-associated lymphoid system of mice (Huang et al., 2008). These data might suggest a higher immunological activity of the mucosa of *Bacillus* treated animals. In another chicken trial, response to Newcastle Disease vaccination was significantly higher in the *Bacillus* treatment group (Molnar et al., 2011). The significant increase of Newcastle disease antibody titer by a probiotic, which included *Bacillus cereus*, was also confirmed in chicken by a different research group (Li et al., 2009).

These data are still insufficient and very fragmentary; however, several studies suggest the existence of a direct effect of *Bacillus* probiotics on the host immune system in chicken and it might have roles on the clearance of *Salmonella* or other pathogens in the gut.

2.3.2 Indirect response

As described above, several studies have reported that *B. subtilis* can alter the intestinal microbial composition, such as increasing *Lactobacillus* and reducing several detrimental bacterial groups (Jeong and Kim, 2014; Jin et al., 1996b; Maruta et al., 1996a; Song et al., 2014). These changes in microbial composition could be an indirect mechanism of action for *Bacillus* probiotics. For example, the altered microbial composition could result in enhanced competitive exclusion, a widely accepted mechanism considered to protect chickens from *Salmonella* infection as originally presented in the early 1970s (Nurmi and Rantala, 1973). Alternatively, the *Bacillus*-altered microbial composition could mediate changes in host immune responses. This indirect pathway may be more consistent with the variable results reported on efficacy of *Bacillus* probiotics. These variable results suggest the existence of uncontrollable factors in the trial design, one of which could be the variable starting microbiome reported among individual birds and rearing environments (Stanley et al., 2013b).

2.3.2.1 *Indirect effects via modified host intestinal microbiota*

As described above, increasing the number of lactobacilli in the gut contents is often reported as one of the beneficial effects of *Bacillus* probiotics. The mechanism of the increase is still unclear; however, this shift of microbiota could be one of the pathways of *Bacillus* action in the gut.

Lactobacillus is a commonly detected genus in the gastrointestinal tract of chicken and known as a suppressor of other bacteria. Intestinal *Lactobacillus* genus have wide antagonism against several food poisoning bacterial species in the small intestine of chicken (Nakphaichit et al. (2011). An antagonistic effect of intestinal lactobacilli against *Salmonella* has also been well documented. Jin et al. (1996a) isolated twelve *Lactobacillus* strains from three-week-old broilers and indicated that all twelve *Lactobacillus* strains showed inhibition against five different serotypes of *Salmonella* isolated from chicken. In a study where one-day old broiler chicks were administered a *Lactobacillus* probiotic and subsequently challenged with *Salmonella* Enteritidis, administration of the *Lactobacillus* probiotic strains caused a reduction of *Salmonella* enumerated in ceca 24h after the challenge (Higgins et al., 2007; Higgins et al., 2010). Reduction of *Salmonella* in broiler chickens and turkeys by 11 different *Lactobacillus* strains was also reported (Menconi

et al., 2011). *L. reuteri* R-17485 and *L. johnsonii* R-17504 significantly decreased the colonization of *Salmonella* Enteritidis in ceca, liver and spleen of challenge chicks (Van Coillie et al., 2007). Therefore, a significant body of evidence would support that the effect of *Bacillus* probiotics on colonization of the chicken gut by *Salmonella* and other bacterial pathogens could be mediated indirectly by increasing colonization by members of the *Lactobacillus* genus.

Two mechanistic questions that could be posed here are how does *Bacillus* increase lactobacilli in the gut and how does the increased lactobacilli suppress the *Salmonella* in the gut? Several potential mechanisms might be available for both questions.

Hosoi et al. (1999) reported that *B. subtilis* var. *natto* has growth-promoting effect on lactobacilli when they are co-cultured aerobically *in vitro* (Hosoi et al., 2000). In this case, only intact *Bacillus* spores enhanced the growth of *Lactobacillus* and autoclaved spores did not (Hosoi et al., 1999). This data suggests the importance of germination to enhance *Lactobacillus* in the gut. Microbiology textbooks have described *B. subtilis* as a strict aerobe, but other studies have shown that *B. subtilis* can grow anaerobically by using nitrate or nitrite as a terminal electron acceptor (Nakano and Zuber, 1998). Even so, *B. subtilis* consume massive amounts of oxygen when they grow and all oxygen in liquid medium (Tripticase Soy broth) which includes saturated dissolved oxygen was consumed within 2.5 hours by only 3×10^5 CFU/ml of *B. subtilis* inoculation (Unpublished 2003 Hamaoka). Making anaerobic conditions by consuming oxygen in the environment could be one of the pathways to increase lactobacilli and other anaerobic bacterium.

How does *Lactobacillus* reduce the number of *Salmonella* in the chicken gut? A direct effect of *Lactobacillus* on *Salmonella* is confirmed by several *in vitro* studies (Fazeli et al., 2009; Jin et al., 1996a; Kezerwetter-Swida and Binek, 2005). It is reported that all eight *Lactobacillus* strains isolated from gastrointestinal tract of chicken have the ability to inhibit *Salmonella* spp. using the agar spot test (Hutari et al., 2011). In another study, 43 tested strains of *Lactobacillus*, including 24 strains isolated from excreta of chickens, showed inhibition against *Salmonella* Enteritidis and Typhimurium (Yamazaki et al., 2012). A total of 53 *Lactobacillus* strains were isolated from the cloaca and vagina of laying hens and evaluated by spot test for antagonistic function against 20 different *Salmonella enterica* strains. There was a difference in the strength of the inhibition; however, almost all lactobacilli strains showed inhibition against *Salmonella* strains in anaerobic condition (Van Coillie et al., 2007). These data suggest that inhibition against *Salmonella* is a common ability among lactobacilli although the strength is strain dependent.

Unlike *Bacillus* species, *Lactobacillus* is free from the argument on concentration and metabolism in the gut environment. According to results from 13 different microbial profiling studies, the concentration of lactobacilli in the ileum of chicken is around 8.0 log CFU/g (Rehman et al., 2007). Sometimes, over 9.0 log CFU/g of lactobacilli have been observed in field studies (Jin et al., 1996b; Maruta et al., 1996a).

The concept of competitive exclusion also includes competition for nutrients, adherence to mucosal binding sites, as well as generating an antimicrobial environment through acidification (organic acid synthesis) or producing antimicrobial substances. Lactobacilli produce organic acids in anaerobic condition, such as lactic acid and acetic acid. Production of lactic acid might relate to *Salmonella* inhibition. The organic acid could make an intestinal environment with low pH which might suppress growth of *Salmonella*. In fact, the amount of produced lactic acid and strength of inhibition corresponded quite well in *in vitro* tests (Van Coillie et al., 2007).

 Ability to adhere to the host mucus layer is also considered a possible pathway to reduce *Salmonella,* by blocking the adhering sites where *Salmonella* would attach. Adhesion to epithelial cells is important in predicting gastrointestinal colonization (Spivey et al., 2014) and a single strain of *Lactobacillus acidophilus* significantly reduced attachment of *Salmonella* to ileal epithelial cells of chicken (Jin et al., 1996c). Valeriano et al. (2014) reported *Lactobacillus* strain LM1 showed good adhesion and significant displacement of *Salmonella* on cell surface in *in vitro* trials.

Lactobacillus is known as a major dominant intestinal bacterium strain in the chicken gut (Wei et al., 2013) and again, increasing *Lactobacillus* by *Bacillus* probiotic supplementation is recognized as one of the effects of the probiotics. Involvement of lactobacilli in mechanisms of *Bacillus* probiotics against *Salmonella* infection could be a leading hypothesis.

2.3.2.2   *Indirect effect via modified host intestinal microbiota and host physiology*

This is a difficult hypothesis to approach because of the factorial relationship between the diverse bacterial species present in the gut and how it is affected by the addition of the *Bacillus* probiotic and subsequently which of the many pathways by which bacteria may affect the host mediating a change in host physiology or immunity (Willing and Van Kessel, 2009).

In the case where *Bacillus* increases abundance and/or metabolism of *Lactobacillus* spp., the relationship between lactobacilli and the host immune system is increasingly well studied.

According to review articles about the effect of lactobacilli on host immune system, lactobacilli possibly affects secretory-IgA levels, IgA-secreting cells, cytokine-producing cells, toll like receptor expression, villi structure, mucin secretion, antimicrobial peptide productions immunoglobulin titers, macrophage function and leucocyte counts (Ashraf and Shah, 2014; Cunningham-Rundles et al., 2011; Kemgang et al., 2014; Valeriano et al., 2014). Similar host immune system responses have been described for other potentially probiotic organisms (Hanchi et al., 2018; Maldonado Galdeano et al., 2019). Thus, it is plausible that the increase in *Lactobacillus* species previously reported following *Bacillus* spp. supplementation (Jeong and Kim, 2014; Jin et al., 1996b; Maruta et al., 1996a; Wu et al., 2011) could mediate changes in host response.

Of course, other species in the gut may also be altered by the supplementation of *Bacillus.* Any of these bacteria could mediate direct beneficial changes in host physiology (digestive function, immunity). One approach to investigating this route is the use of transcriptomic, proteomic and/or metabolomics analyses to assess changes in host response pathways, first in a conventional environment, and subsequently in simplified environments including potentially mono-associated environments. Pathways activated in a conventional environment with *Bacillus* supplementation could be examined for activation by other bacteria in a simplified environments to establish which bacteria species are capable of pathway activation. Again though, such an approach is not feasible to test all commensal bacterial species found in the poultry gut and potentially modulated by *Bacillus*.

2.4   Life cycle (germination and sporulation cycle) of *Bacillus* in the chicken gut

Investigation of the life cycle of *Bacillus* (germination-sporulation cycle) in chicken gut is a crucial factor to clarify mechanisms of action of *Bacillus* probiotics, because this information is basic to assessing the metabolic activity of *Bacillus* in chicken gut and thus the possible mechanisms of action. For example, it is well known that *Bacillus* spp. produce several antibacterial compounds (Olivier, 1946) such that colonies of cultured (vegetative) cells show a zone of inhibition of growth against several foodborne pathogens such as *Salmonella*, *E. coli* and *Clostridium* on agar media (Thirabunyanon and Thongwittaya, 2012). Without knowledge of the extent and localization of germination of *Bacillus* spores in the chicken gut when supplemented in

the diet, the relevance and extent of this direct anti-pathogen effect cannot be assessed. Indirect mechanisms of pathogen control may also be affected by the location and extent of spore germination. Hosoi et al. (1999) reported lactobacilli was increased by the supplementation of intact spores of *B. subtilis* var. *natto*, but that this effect was absent when the spores were autoclaved. Thus, germination might be key to altering the intestinal microbial composition and an indirect mechanism of action.

Several research data indicate that at least part of the *Bacillus* probiotic spore can germinate in the gastrointestinal tract. In mice, *B. subtilis* strains are able to carry out their entire life cycle in the gut (Tam et al., 2006) and a significant number of spores germinate in the jejunum and ileum and may colonize the small intestine briefly (Casula and Cutting, 2002). Both studies conclude that *Bacillus* has a potential to colonize the mouse gut.

In chicken, the number of shedding spores in the excreta decreased with time in case of 10^9 CFU spores of *B. subtilis* SC2362 administered to 1-day-old chick by a bolus oral gavage (Cartman et al., 2008). Shedding number of *Bacillus* spores in the gut was over 10^7 CFU/g 6 hour after the inoculation and the number decreased to around 10^3 CFU/g 168 hour later. In another study, the reduction was also confirmed when a single dose of 10^6 spores of *B. subtilis* PHL-NP22 was gavaged per chick on day of hatch (Latorre et al., 2014). Continuous feeding of 10^6 spores of *B. subtilis* PHL-NP22 to one-day old chicks was also tested and the number of recovered *B. subtilis* was consistently about 10^5 spores per gram of digesta (Latorre et al., 2014).

In Japan, the regulations requires clarification of the distribution and excretion of the feed additive candidate in the gut of target animals. As of 2014, a total of 6 *Bacillus* products were registered as a feed additive in Japan. This means investigation about the life cycle of six different *Bacillus* strains has already been reported to the Ministry of Agriculture, Forestry and Fisheries in Japan when the strain was approved. However, the data is not available to the public.

One of the strong limitations to approaching the life cycle of *Bacillus* spp. in chicken gut is the limitation on the analytical technology. Culture-based methods, based on the resistance of spores to heat inactivation, are available to observe the proportion of cells in spore or a vegetative state. The advantage the culture-based method is only viable target cells in the sample can be detected and the target cells, *Bacillus* strains in this case, can be kept for further analysis if it is necessary. On the other hand, identification of the target bacteria strain relies on morphology and some bacteria strain is still unculturable. Molecular approaches have been investigated to improve

upon challenges to the culture-based approach to differentiation of spore and vegetative cells. For example, Casula and Cutting (2002) developed a test based on RT-PCR by creating a genetically engineered chimeric gene which is strongly expressed only in vegetative cells. Quantitative RT-PCR detection of the expressed gene would serve as a proxy for vegetative cell number. Other scientists have tried to measure germination and sporulation in the mouse gut by quantitative expression analysis of natural germination and sporulation genes (Tam et al., 2006). Indeed, a semi-quantitative RT-PCR using this approach was developed to detect vegetative cells of *B. subtilis* SC2362. The problem with RT-PCR is PCR worked accurately for vegetative cells, but not for spores due to low efficacy on extracting DNA from spores. Furthermore, the absolute detection limit of the assay was found to be 3.33×10^5 cells per gram of tissue (Cartman et al., 2008).

A new reliable method to enumerate spores of *Bacillus* has not been developed yet and the culture-based method is still one of the most potent tools to count total number of *Bacillus* in the sample. Therefore, there is still no clear conclusion about the life-cycle of *Bacillus* probiotics strains in the chicken gut and further investigations are required to clearly identify the site of *Bacillus* germination and the management and nutritional practices which optimize germination. In addition to germination, it is starting to be recognized that sporulation is a major *Bacillus* activity in the gut as a form of survival and propagation in the gut environment (Serra et al., 2014). Information on the site of germination/sporulation and optimization conditions are important considerations in establishing the role of germination and the mechanisms of action that support the highest probiotic effect.

2.5 Characterization, function and control of chicken intestinal microbiota

2.5.1 Intestinal microbiota of chicken

The composition and diversity of intestinal microbiota in chicken gut has been investigated by culture-based methods for over 70 years. In the last 20 years, advanced molecular technologies started to be utilized for microbial community profiling and providing a more detailed picture of chicken gut microbiota for scientists.

Chicken embryos developing inside the eggs laid by healthy hens are microbiologically sterile (Furuse and Okumura, 1994). Colonization of bacterium starts right after hatching due to

exposure to the environment around neonatal chicks, then the intestinal microbiota increases in complexity over the first four to six weeks (Barnes, 1979). Basically, bacterial density in the chicken gut increases with age (Benno and Mitsuoka, 1986) and is ultimately dominantly comprised of Bacteroidaceae, Clostridiaceae, Lactobacillaceae, Enterococcaceae and Enterobacteriaceae families (Lu et al., 2003). It is currently well-known that diversity and composition of intestinal microbiota can be affected by feed, age, feed additives and other environmental factors (Guan et al., 2003; Stanley et al., 2013b). In the adult, anaerobes are dominant over aerobes throughout the gastrointestinal tract of chicken. Based on knowledge from several microbial profiling studies using a culture-based method, the number of intestinal bacteria is highest in ceca and lowest in the gizzard (Rehman et al., 2007). Concentration of microbiota in ceca is roughly estimated around 10^{11} bacteria per gram of digesta by direct microscope cell count (Barnes, 1979; Salanitro et al., 1974b). According to a summary of 18 studies about cecal microbiota in chicken, the total count of anaerobes by culture-based method was maximum 10.9 $\log^{10}$ CFU and minimum 9.5 $\log^{10}$ CFU per gram of intestinal content (Rehman et al., 2007). This result indicates that roughly a minimum of 10% to a maximum of 90% of the bacteria population in the chicken gut are culturable strains and the remaining bacteria are not to be detected by culture-based method.

There is consensus that the upper GI tract of chicken is dominated by lactobacilli. Lu et al. (2003) reported that 68% of sequences from the ileum were related with *Lactobacillus* and the remaining part of the composition was occupied by *Clostridiaceae*, *Streptcoccus* and *Enterococcus*. The highest diversity of *Lactobacillus* spp. is confirmed in the crop (Gong et al., 2007). Indeed, several different studies illustrated that lactobacilli were the main bacteria genes in the upper intestinal tract of chicken in the trials and abundance was a maximum of 99% in the jejunum (Amit-Romach et al., 2004; Lu et al., 2008; Rehman et al., 2007; Stanley et al., 2012).

In contrast, species in the *Clostridiaceae* family are the most abundant group in chicken cecum. The dominant species in ceca was Clostridia-related sequences and *Clostridium* occupied 40% of tested sequences (Gong et al., 2007). Lu et al. (2003) and Dumonceaux et al. (2006) also reported that bacteria in the Clostridiales order was the most abundant group detected in chicken ceca and occupied over 65% of the total number of sequences from ceca. Studies at an early era also indicated that *Clostridia* were constituted around 20% of cecal microbiota (Salanitro et al., 1974a; Salanitro et al., 1974b).

A total of 33,598 16S rRNA gene sequences of bacteria found in chicken gut were retrieved from available databases by Wei et al. (2013). Analysis of sequences showed a total of 915 operational taxonomic units were found in the chicken and 117 established bacterial genera were included with most genera belonging to the phyla *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* (Wei et al., 2013). It is also known that 4% to 25% of the strains in chicken gut are still recognized as unclassified (Gong et al., 2002; Wei et al., 2013). These data indicate the probable presence of unknown functional bacterium in the chicken gut.

In recent years, several scientific reports have indicated the existence of embryonic, pre-hatchling, microbiota in the chicken by next generation sequencing (NGS) method (Akinyemi et al., 2020; Lee et al., 2019). Authors suggest the chicken's gut microbiota may be seeded before hatching. On the other hand, making "germ-free" chickens as test animals is a well-established technique (Guitton et al., 2020) which argues against pre-hatch colonization. In addition, as described above, establishment of the complexity of the gut microbiota after the hatch is also a well-studied topic in the past even by next generation sequencing (Videnska et al., 2014). There was consensus on sterility of well-managed chicken embryos and the development of postnatal microbiota before. To fill the gap between new findings on embryonic microbiota and traditional understanding on sterility in the embryonic stage, quantitative information may be necessary for further discussion in addition to composition data shown by NGS. For example, Kizerwetter-Świda and Binek (2008) reported that number of Enterococcus found in 18 day old chicken embryo was between 10^2 to 10^4 cfu/g in yolk sac. Egg yolk is suitable medium for bacterial growth and Enterococci reach to above 10^7 CFU/g in the yolk within 24 hours after inoculation or contamination and could grow above 10^8 CFU/g 48 hours later (Imai, 1980). Septicemia is also known as a fatal factor for the avian embryos (Amer et al., 2017; Hansen et al., 2015b; Orajaka and Mohan, 1985). Therefore, further investigation may be required to understand whether the microbiota establishment in the embryonic stage is normal for chicken or not.

2.5.2 Function of intestinal microbiota of chicken

Recently, several scientists started to investigate the functional impact of changes in the composition of the intestinal microbiota. One idea to approach the functional impact of the microbiota is to transfer whole microbiota from a donor animal demonstrating a particular

phenotype, to a recipient germ-free animal to determine whether all or part of the phenotype of the donor is transferred to the recipient. For example, it was reported that intact uncultured microbiota taken from obese mice, when transferred to germ-free mice, conveyed significantly greater increases in body mass and adiposity of recipient mice compared with microbiota taken from lean individuals (Turnbaugh et al., 2006). Controlling body weight and composition is a major interest in human and animal health science and these studies have confirmed a relationship between microbiota composition or metabolism and adiposity (Muscogiuri et al., 2019). In poultry, studies have also identified links between the composition of the intestinal microbiome and growth performance. The differences in intestinal microbiota between chickens with high FCR and low FCR were analyzed by high-throughput sequencing methods and 24 unclassified bacterial species were found to be significantly differentially abundant between high and low performing birds (Stanley et al., 2012). The same research team compared high performance and low performance birds in the same flock and they found a bacterial genus, *Bacteroides* was associated with high energy efficiency birds, a phenotype consistent with members of this genus recognized as cellulose and starch degraders (Stanley et al., 2013a). The linkage between adiposity and ratio of two different bacterial phyla, the Bacteroidetes and Firmicutes, has also been recognized at human and animal trials. The relative proportion of Bacteroidetes was decreased in obese people compare with lean people (Ley et al., 2006). Association between the *Firmicutes* and *Bacteroidetes* ratio and weight gain was also reported in chicken and duck trials and higher *Firmicutes/Bacteroidetes* ratio was confirmed with significant body weight gain in the *Lactobacillus* spp. probiotic treatment group (Angelakis and Raoult, 2010).

Kogut (2019) indicated a possible link between microbial composition in the gut and metabolic or immune functions that could have wide-ranging implications for poultry health. Development of the avian immune system begins at the embryonic stage and continues until a few weeks of age post hatch (Panda et al., 2015). As reviewed by Kau et al. (2011), gnotobiotic studies have confirmed that the gut microbiota affects development of both immune defense and inflammatory responses. The presence of commensal bacteria promotes the maturation of the intestinal immune system in comparison to GF chickens and influences the immune cells present in the intestine (particularly T-cells), the expression of cytokines, the expression of toll-like receptor proteins, and activation and priming of the acquired immune system (Oakley et al., 2014; Kogut et al., 2019). Manipulation of commensal microorganisms in the gut may provide

new opportunities for enhancing immunity in the gut (Atarashi et al., 2011; Ivanov et al., 2009) and adding beneficial bacteria to the intestine by probiotics and prebiotics are considered as a methodology to manipulate the gut microbiome for the potential benefits on immune systems (Kogut, 2019).

2.5.3 Controlling the intestinal microbiota of chicken for research

The term gnotobiotic means a defined microbiota. Currently, the only approach to a completely defined microbiota in animals is to first generate a germ-free animal and subsequently, in a controlled environment, expose the animals to a defined bacteria or group of bacteria. The first efforts to make germ-free chicks were done at the end of 19 century and successes to maintain germ-free chicks for a short period was recorded in 1913 by Schottelius (Reyniers et al., 1950). After that, the gnotobiotic chicken has been utilized to investigate the effect or function of individual bacterial strains by comparison between germ-free chicken and mono-associated chickens. Germ-free birds were reported to have improved growth and feed efficiency compared to conventional birds when highly digestible diets are provided, a response attributed to lower maintenance requirements associated with lack of immune stimulation (Furuse and Okumura, 1994). In contrast to germ-free mice, the microbial synthesized vitamins are not utilized in chicken (Coates, 1973) and vitamin supplementation did not increase the growth rate of germ-free chicken (Reyniers et al., 1950).

Morishita et al. (1971) inoculated 11 different *Lactobacillus* strains to ex-germ-free birds and found that non-intestinal lactobacilli, such as strains isolated from Swiss-cheese starter or pickled cabbage, failed to be established in the gut of germ-free chicken. Watkins and Miller (1983a) reported an attachment of *Lactobacillus* strains on intestinal epithelia through physical contact through observation from *Lactobacillus acidophilus* mono-associated chicks using electron microscopy. Further, *Salmonella* challenge in the *Lactobacillus acidophilus* mono-associated chicken resulted in significantly less shedding of *Salmonella* in excreta compared with the germ-free birds (Watkins and Miller, 1983b). Inhibitory effects of *Lactobacillus acidophilus* against pathogenic *Escherichia coli* (Watkins et al., 1982) and *Salmonella* reduction in *Escherichia coli* mono-associated chicken were also confirmed by similar type of trials (Fukata et al., 1989;

Fukata et al., 1991a). Another study showed the mixture of *E. coli* and *Lactobacillus* sp. suppressed *Salmonella* colonization most effectively in gnotobiotic chickens (Baba et al., 1991).

The use of a germ-free environment allows the effects of bacterial strains to be observed in a mono-associated state. An advantage of the approach is that differences between germ-free animals and mono-associated animals is wholly ascribable to the inoculated strain. On the other hand, the disadvantage of the mono-associated condition is that colonization occurs without competition affecting colonization density (Morishita et al., 1981) and potentially activated metabolic pathways (Luczynski et al., 2016). Furthermore, differentiation of responses attributable to the inoculant strain from generic responses to any (most) bacteria is difficult. Faith et al. (2014) adopted an interesting approach to overcome these challenges. This group isolated 17 major abundant strains from obese human feces and tested the effect of 94 combinations with a subset size 7.6 ± 3 strains made by random selection. Measurements on the immune system, adiposity and several facets of metabolism were made in mice associated with each of the 94 combinations compared with germ-free mice. Computer simulation provided efficient trial combinations. The effect of test strains alone and combined were investigated and estimated from the results. For most parameters investigated, the response to a single strain was not unique and additional response were typically not observed following colonization of 3-5 strains. These results indicated that most test strains show a significant effect on host physiology in mono-associated condition and there is no guarantee that such effects will be observed when in combination with other bacteria.

Another approach to minimize variation in intestinal microbiota of test animals is to inoculate starter strains at the beginning of the trial to stabilize background microbiota in each trial. A primitive idea to achieve this concept might be done by inoculating gut contents to transfer "normal" intestinal microbiota from adult broiler to chick in early stage of their life (Nurmi and Rantala, 1973; Rantala and Nurmi, 1973). However, recreating the same composition of conventional microbiota in an open environment is a great challenge and still not achieved. For example, a mixture of 48 different bacterial strains from adult birds were given to one day old chicken (Impey et al., 1982). It was reported that adult-type microbiota was established by the inoculation, but the composition of cecal microbiota were different among three repeated trials. Yin et al. (2010) made three groups of inoculants with different bacterial composition and inoculated the mixtures by oral gavage within 4 h after hatch. The results indicated that the three

different inoculums could lead development of different bacterial communities in the gut and a different composition were still observable 15 days after the inoculation. However, reproducibility of this unique balance for each inoculant in subsequent studies was not reported. Finally, Stanley et al. (2013b) reported high variation of microbiota in the chicken among three similar trials under carefully controlled conditions. Recreating the same composition of conventional microbiota in open environment has yet to be achieved.

2.6 *In ovo* inoculation as test model

The technique of *in ovo* inoculation was first tested by Sharma and Burmester (1982) as an embryonic vaccination. In the current US broiler industry, *in ovo* injection is in use in more than 90% of hatcheries (Peebles, 2018) and widely accepted as a safe and effective vaccination method for Marek's disease (Sarma et al., 1995; Sharma and Witter, 1983), Infectious Bursal Disease (Newswire, 1997; Sharma, 1985), Newcastle disease (Sanling et al., 2020; Stone et al., 1997), and Avian influenza (Breedlove et al., 2011). The *in ovo* technique is used not only for vaccination purposes but also utilized for nutritional supplementation of minerals (Ahmadzadeh et al., 2019; Vaibhav Bhagwan et al., 2020; Yair et al., 2015), vitamins (Hayakawa et al., 2019; Zhu et al., 2019), amino acids (Kop-Bozbay and Ocak, 2019; Nazem et al., 2019), and carbon sources (Ghanaatparast-Rashti et al., 2018; Retes et al., 2018; Zhai et al., 2011).

Chicken embryos are also used as a laboratory animal model (Ruijtenbeek et al., 2002). For example, the chicken embryo was reported as fast and robust model to test the function of muscle fusion genes (Daniel et al., 2017). Also, the embryo has been shown to be a suitable bioassays for the impact assessment of endocrine disrupting chemicals on reproductive tissues (Jessl et al., 2018). The *in ovo* technique has been utilized as a method for the assessment of biological activity of oligosaccharides (Gulewicz, 2004; Villaluenga et al., 2004), assessment of the effect of hormones to control bird's aggressive behaviors (Ahmed and Essa, 2020) and toxicity of a total of 80 rubber chemicals (Hemminki, 1983).

The *in ovo* technology has also been used as a method to introduce probiotic bacteria prior to hatch. Three different purposes can be seen with probiotic *in ovo* inoculation, such as enhancing growth performance, manipulation of intestinal microbiota, and stimulation of the innate immune system. Safeness and benefit of *Lactobacillus. reuteri in ovo* inoculation was first reported in the

1990's (Edens et al., 1997). *In ovo* inoculation was investigated as a possible delivery method for commercial probiotics (de Oliveira et al., 2014; Huff et al., 2015) and positive effects of *in ovo* inoculation on growth performance were reported with *Lactobacillus acidophilus* (Triplett et al., 2018), *Enterococcus faecium* (Claudia et al., 2020; Skjøt-Rasmussen et al., 2019), *Lactobacillus animalis* (Beck et al., 2019), *Bifidobacterium longum* (Abdel-Moneim et al., 2020; Beck et al., 2019), *Bifidobacterium bifidum* (El-Moneim et al., 2020), *Bifidobacterium animalis* (Abdel-Moneim et al., 2020; Triplett et al., 2018) and a cocktail of probiotic strains (de Oliveira et al., 2014; Majidi-Mosleh et al., 2017b; Pender et al., 2017). Injection of *Bacillus* spp. probiotic into broiler embryos was also tested by Arreguin-Nava et al. (2019) and significant body weight gain at day 7 was confirmed in *Bacillus* treatment.

2.7   Conclusions

The gut microbiome plays an important role in host physiology including impacts on health, digestive function and energy metabolism. The oral administration of *Bacillus* probiotics has been shown to impact health and performance outcomes in the bird and may do so through manipulation of the gut-microbiome interface. The detailed mechanisms that contribute to health and performance benefits following *Bacillus* administration are not understood. Understanding the mechanisms involved are critical to refining the application of *Bacillus* probiotics to establish conditions under which benefit is expected and to inform development of improvements in this technology.

Two different mechanistic pathways could be hypothesized for effect of *Bacillus* probiotic in chicken production. *Bacillus* probiotics may show some efficacy mediated by direct effects on the host such as direct stimulation of immune system or digestive functions. On the other hand, the latest developments in molecular biological technology have revealed a complex intestinal microbiota in the chicken gut and probiotic studies show a shift of gut microbiota composition by *Bacillus* probiotics supplementation in the feed. This shifted microbiota may cause changes on host physiologies as an indirect effect of *Bacillus* probiotics. For these two different hypotheses, making gnotobiotic chicken could be a powerful approach to separate outcomes mediated directly by the probiotic compared with those mediated by indirectly by changes to the microbial composition. Firstly, the direct mechanisms could appear and be observed in comparison between

germ-free birds and *Bacillus* mono-associated birds. Secondly, the complexity, dynamics and variability of the intestinal microbiota provide a challenge to investigating indirect mechanisms of action of *Bacillus* probiotics. Therefore, approaches to simplifying the microbiota to assess the indirect mechanisms should be valuable.

2.8   Overall objectives and Hypothesis

Dietary supplementation of *B. subtilis* has been shown to improve growth performance and shift intestinal microbiota by lowering detrimental bacterial colonization in broiler chickens (Knap et al., 2011; Maruta et al., 1996a; Tellez et al., 2012). We hypothesize that the mechanisms of action of *B. subtilis* C-3102 include both direct and indirect components. Direct mechanisms could include direct inhibition of the detrimental bacterium or direct stimulation of host functions such as mucosal barrier, improvement in digestion or absorption resulting in better growth performance. Alternatively, indirect mechanisms could include *B. subtilis*-mediated shifts in gut commensal microbial populations such that members of the altered population either exclude detrimental bacteria or enhance the immune or digestive functions of the host, which could contribute to improved growth performance.

Knowledge regarding the mode of action of probiotics such as Bacillus probiotics is important given the variation in response reported in the literature and commercially. By understanding mode of action we can better predict when probiotic administration will be beneficial or make appropriate adjustments to the dosage, timing, nutritional balance of the host, and combination with other feed additives in order to support efficacy. In addition, mode of action studies may reveal new facts to select or develop the next generation of probiotics with new strains or new combinations of the bacterium. Therefore, we expect this series of research will be a step in the path to more efficacious probiotic products.

The overall objective of this research is to establish gnotobiotic models to control microbial colonization of the chicken gastrointestinal tract as an approach to determining the mechanism of action by which supplementation of B. subtilis C-3102 spores in feed of broiler chickens improves growth performance as an alternative to antibiotics. Specific objectives include:

1. To develop an *in ovo* bacterial inoculation model to investigate the effect of *B. subtilis* C-3102 on development of the gastrointestinal tract of the chicken.
2. To isolate representatives of major taxonomic groups in the chicken gastrointestinal tract and employ an *in ovo* bacterial inoculation model to compare effects of different bacteria on gastrointestinal development.

3. To establish a simplified microbiota model in chicken.

4. To characterize the effect of simplified microbiota on chicken physiology with and without *B. subtilis* C-3102 supplementation.

5. To characterize mechanisms by which *B. subtilis C-3102* affects the health and performance of broiler chickens when supplemented as a feed additive.

3   EFFECT OF *BACILLUS SUBTILIS* C-3102 ON INTESTINAL DEVELOPMENT IN THE CHICK EMBRYO

3.1   Abstract

To investigate the direct effect of a probiotic on intestinal development, healthy chicken embryos were mono-associated with *Bacillus subtilis* C-3102. The exterior of fertilized eggs was sanitized and incubated under standard conditions in a HEPA-filtered sanitized incubator. At day 17 of incubation (E17), the amniotic fluid was injected (100 µl) with sterile saline (Germ-free; n=20) or saline containing 6.7×10^6 spores of *B. subtilis* C-3102 (BS; n=20). The eggs were then placed into sterilized individual heated containers with HEPA-filtered humidified air supply until study completion. Chicks were killed either immediately following hatch (n=10) or exactly 24 hours after hatch. Body weight, organ size, ileal gene expression, *Bacillus* spores and *Bacillus* vegetative cells in intestinal contents were measured and analyzed by two-way ANOVA using BS and Age as main effects. Culture confirmed germination and replication of BS in chicken intestinal tract without evidence of microbial contamination. BS increased ($P < 0.05$) incubation time to hatch, yolk sac weight and relative liver weight, but decreased ($P < 0.05$) yolk sac free body weight at hatch and 24 hours after hatch. Ileal expression of interleukin *(IL)-6*, toll-like receptor (*TLR) 2* , *TLR 4*, peptide transporter-1 (*PepT1*), aminopeptidase N (*APN*), insulin-like growth factor-1 (*IGF-1*), growth hormone (*GH*) and mucin-2 (*MUC2*) were increased ($P < 0.05$) with age, whereas mono-association with BS upregulated ($P < 0.01$) expression of *TLR* 2 and *4*, tended ($P < 0.10$) to upregulate *PepT1*, *APN*, *IGF-1* and *GH*, and downregulated ($P < 0.01$) Claudin 4. Age and BS interacted ($P < 0.05$) such that *IL-8* transcript abundance was higher in BS birds only at hatch and proliferating cell nuclear antigen (*PCNA*) was lower in BS birds only at 24 hours. An interaction for *MUC2* indicated that BS initially increased expression at hatch and decreased expression at 24 hours. Pathway analysis of RNA sequencing (RNAseq) data confirmed enrichment *Bacillus* enrichment of Chemokine pathways at both hatch and 24 h of age, and enrichment of digestion and absorption pathways at 24 h post hatch. The mechanisms of action of *Bacillus* probiotics in the gut may include metabolic activities associated with bacterial germination and vegetative metabolism. Although there was evidence of advanced maturation of digestive function, *in ovo B.*

subtilis C-3102 modified body and organ weight as well as intestinal gene expression primarily associated with unfavorable effects on embryonic development.

3.2   Introduction

Bacillus subtilis has been recognized as a growth promoter for broiler production at least since 1970 (Kozasa, 1989; Mitsuoka, 1991) and strains of *Bacillus* spp. are regulated and registered as probiotics for animal production in the EU (EFSA, 2006b; Silley, 2006), the USA (Pendleton, 2015) and Japan (Kojima, 1995). Despite an over 40-year history of use, the mechanisms that underlie performance benefits observed with *Bacillus* probiotics are not fully elucidated.

Multiple mechanisms have been proposed including secretion of antimicrobial substances (Katz and Demain, 1977; Stein, 2005), modulation of protective immunity (Desmidt et al., 1998; Huang et al., 2008; Lee et al., 2011), reduction of detrimental bacteria (Fritts et al., 2000; La Ragione and Woodward, 2003; Maruta et al., 1996a; Vila et al., 2009) and increased intestinal *Lactobacillus* populations (Hosoi et al., 1999; Jeong and Kim, 2014; Maruta et al., 1996a; Wu et al., 2011). These potential mechanisms can be classified into two categories from an intestinal microbiological point of view, including a direct effect of *Bacillus* probiotics on host physiology and an indirect effect mediated via a shift intestinal microbial composition. However, it is impossible to separately observe these direct and indirect effects in a conventional environment with a diverse gut microbiota.

Gnotobiotic poultry models have been reported as early as 1950 (Reyniers et al., 1950) and have been used previously to elucidate probiotic mechanisms (Fukata et al., 1991b; Morishita et al., 1971; Watkins and Miller, 1983a). While gnotobiotic models are powerful tools, they are very difficult to establish and commonly experience contamination problems (Cheled-Shoval et al., 2014; Phillips et al., 1962). We therefore elected to take advantage of the germ-free state of the developing embryonic digestive tract (Furuse and Okumura, 1994) and of previously established methods for *in ovo* delivery of vaccines (Sarma et al., 1995), antibiotics (Bailey and Line, 2001), minerals (Ahmadzadeh et al., 2019; Vaibhav Bhagwan et al., 2020; Yair et al., 2015), nutrients (Dong et al., 2013; Kadam et al., 2013; Retes et al., 2018; Saeed et al., 2019), and selected bacteria (Arreguin-Nava et al., 2019; de Oliveira et al., 2014; Majidi-Mosleh et al., 2017b; Pender et al.,

2017; Triplett et al., 2018). The *in ovo* inoculation model offers a relatively simple model to assess the direct effect of selected bacteria (alone or in combination) on chick gastrointestinal development. However, extrapolation to post-hatch conditions will be disadvantaged by the incomplete development pre-hatch (Geyra et al., 2001) .

The objectives of this experiment were to further develop the *in ovo* inoculation model to permit isolation and hatching of individual eggs under gnotobiotic conditions and to extend the analysis of a gnotobiotic environment on gut development to the 24-hour post hatch period. This model was employed to investigate the direct effects of *in ovo* inoculation with *B. subtilis* C-3102 (BS) at day 17 of incubation (E17) on the development of chick embryos at the time of hatch and 24 h after hatch. We hypothesized that BS inoculation would hasten gut development resulting in a more robust chick at hatch.

3.3   Material and Methods

3.3.1   Animal Care

All animal experiments were conducted with the approval of the University of Saskatchewan Animal Research Ethics Board (Protocol # 20150017) according to the guidelines of the Canadian Council on Animal Care.

3.3.2   Animals, Experimental Design and Sample Collection

Fertilized eggs (White Leghorn, ISA Bovans White x Lohmann LSL-Lite) were obtained from the University of Saskatchewan Poultry Research and Teaching Unit (Saskatoon, SK). Fertilized eggs (n=132; 55 g to 65 g), free of cracks and other defects, were collected on the day of lay and stored at 13 °C overnight. The selected eggs were soaked in 0.5% of sodium hypochlorite at 30 °C for 12 minutes and then transferred to a HEPA-filtered sterilized incubator at 37.8 °C. At the end of day 17 (E17), eggs containing undeveloped chicken embryos and with weight loss greater than the mean weight loss for all incubated eggs plus one standard deviation were discarded. Of the remaining eggs, 40 were randomly selected and assigned to four treatment

groups balanced for weight at the beginning of the incubation (E0) and subject to further decontamination by soaking 0.5% sodium hypochlorite for three seconds at 30 °C. Subsequently, a small hole was made in the shell at the top of each air cell using a Dremel® rotary tool (Racine, WI, U.S.) with a diamond tip. Using a pipette, sterile saline (100 µl) was injected into amniotic fluid of eggs in two germ-free (GF) treatment groups and saline containing 6.7 x 10^6 CFU/100 µl (6.8 log CFU) of *B. subtilis* C-3102 was injected into 2 *B. subtilis* (BS) treatment groups. The hole in the shell was sealed using a food industry grade 100% silicone sealant (Kitchen grade 100% silicone, DAP®, Canada) and eggs were placed in a sterile, HEPA-filtered hatcher (Robbins®, Robbins Incubator Co., California, USA) for 10 min until the silicone solidified. The eggs were then placed into sterile individual sterile hatching jars and sealed. The temperature and humidity in the individual hatching jars were maintained at 38 °C and around 50% relative humidity by circulation of humidified air through a 0.45 µm filter (Nalgene Syringe Filter 0.45µm SFCA, Thermo Scientific) using an air pump (Whisper® Aquarium Air Pump, Tetra, Blacksburg, VA, U.S.A.). Time of hatch was recorded for all birds. One GF and one BS treatment group (n=10 / group) were killed immediately following hatch and chicks in remaining two treatment groups (n=10 / group) were killed 24 hours after the time of hatch.

Following euthanasia, body weight, yolk sac weight and weight of the gizzard, bursa of Fabricius and liver were recorded. Length of the small intestine including the duodenum (duodenal loop), jejunum (proximal end of duodenal loop to Meckel's diverticulum), and ileum (Meckel's diverticulum to ileal-cecal junction) were also measured and recorded. Cecum contents and gizzard contents were collected to confirm microbial status. For samples collected 24 hours after hatch, microbial status of the gizzard was determined using a cotton swab as liquid contents were insufficient to permit direct collection. Segments from the middle location of the small intestinal region and the cecum were placed in 10% formalin to permit histological analysis (1 cm) or snap frozen in liquid nitrogen to permit analysis of gene expression.

3.3.3 Culture of Intestinal Contents and Spore Enumeration

To confirm germ-free or *Bacillus* mono-associated status in intestine of newly hatched chicks, fresh gizzard or cecal content (50 µL) was diluted (1:10 w/v) in 0.1% peptone water and

spread on BBL^{TM} Trypticase^{TM} soy broth with 2% agar (TS agar; Becton, Dickinson and Co. Sparks, MD, USA) and incubated at 37 ˚C for 24 hours aerobically, or spread on BL agar (Eiken Chemical Co., Ltd., Japan) and incubated at 37 ˚C for 48 hours anaerobically (Anaeropack System, Mitsubishi Gas Chemical Company, INC., Tokyo, Japan). Remaining contents for gizzard and cecum were heat treated at 65 ˚C for 35 min to kill vegetative cells prior to plating to enumerate spores.

3.3.4 Preparation of *Bacillus subtilis* C-3102 inoculant.

Bacillus subtilis C-3102 (DSM 15544) was cultured on TS agar at 37 ˚C for 24 hours and kept at 5 ˚C for 24 hours to ensure spore formation. Colonies were collected aseptically by sterilized platinum loop, placed into plastic tubes containing ice cold sterile saline and homogenized using POLYTRON® Model K Homogenizer (Kinematica, Luzern, Switzerland) at full speed for 30 sec. The homogenized solution was centrifuged at 10,000 g for 20 min, the supernatant discarded and the spore-containing pellet re-dissolved into fresh ice cold sterilized saline. This washing process was repeated until the supernatant became clear. Pellets were transferred to filter paper and dried overnight at 60 °C to kill vegetative cells. Dried remaining spores were finely ground using a sterilized mortar and pestle and number of *B. subtilis* C-3102 spores per gram dry weight enumerated by aerobic culture on TS agar at 37 °C for 24 h. Dried spores were separated into aliquots (1.0 g) and heated to 60 °C for 2 hours to ensure destruction of any contaminating vegetative bacteria. The spore powder was kept at room temperature until the trials and number of viable *B. subtilis* C-3102 per g of powder was re-confirmed by culture-based enumeration before use. For *in ovo* inoculation, the spore powder was dissolved into sterile saline and adjusted to 6.7 x 10^7 CFU per ml (6.7 x 10^6 CFU per bird) 10 min prior to the injection and kept at room temperature.

3.3.5 Histochemistry

After formalin-fixation in 10% neutral buffered formalin at least for 24 hour, tissue samples were submitted to the Prairie Diagnostic Services Laboratory (Saskatoon, SK, Canada) for paraffin embedding, sectioning (5 μm thickness) and staining with hematoxylin and eosin (H&E). A total of at least four cross sections were prepared from two different regions of each section for each

bird. Using an observer blinded to treatment, at least 10 well oriented villi and 10 crypts were measured per bird under Axiostar plus light microscope (Carl Zeiss Canada Ltd., Toronto, ON) and AxioVision 4.1 measurement software (Carl Zeiss Canada Ltd.). Mean values were recorded for each sample.

3.3.6 RNA extraction from ileum tissue

Frozen ileal tissues were finely ground and mixed using a mortar and pestle under liquid nitrogen. Total RNA was extracted from 30 mg of ground tissue using the RNeasy Mini Kit (Qiagen, Mississauga, ON) incorporating RNase-Free DNase (Qiagen, Mississauga, ON) to remove genomic DNA from the sample. Total RNA concentration was quantified by optical density at 260 nm using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington DE). Only samples with a 260/280 ratio between 1.80 and 2.00 were retained for gene expression analysis. The total RNA (5 µg) was reverse transcribed with random hexamer primers using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA, USA) and the cDNA was stored at -80 °C until gene expression analysis was performed by quantitative real-time PCR (qPCR).

3.3.7 Quantitative real-time PCR gene expression analysis

Specific transcript abundance was measured using quantitative real-time PCR (qPCR) carried out using CFX96 real-time PCR detection system on a C1000 thermal cycler (Bio-Rad Laboratories, Inc., California, U.S.A.). As a template for each reaction, 2.0 µL of a 1/100 dilution of cDNA was utilized and mixed with 1.0 µL of 10 µM forward and reverse primer (Table 3.1), 10.0 µL of SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc., California, USA) and 6.0 µL of nuclease free water. The qPCR reaction conditions were 95 °C for 2 min followed by 40 cycles at 95 °C for 5 seconds and annealing at 53-61 °C for 5 seconds (see Table 3.1). A melting curve analysis was conducted at the completion of amplification cycles by increasing temperature from 65 °C to 95 °C in 0.5 °C increments for 5 seconds each. Standard curves were prepared using 1.0 µL of a 5-fold dilution series of pooled cDNA in triplicate assigning an arbitrary value to the

highest concentration standard. All standard curves demonstrated a PCR efficiency between 98.8% and 117%. All samples were analyzed in duplicate and duplicates with greater than 10% coefficient of variation were repeated.

A total of eight different candidate genes were tested as housekeeping genes for this study (Table 3.2). Mean threshold value for each sample was not different ($P >0.05$) among treatment groups for *GAPDH, SDHA* and *TFRC*, such that for each sample, the mean of the arbitrary value for each of these housekeeping genes, interpolated from the standard curve, was divided by the arbitrary value of the gene of interest to normalize expression values (Livak and Schmittgen, 2001). Fold change was calculated relative to the mean normalized arbitrary value for the Germ-free treatment at hatch and 24 hours of age.

Table 3.1 Primers used for qPCR quantification of genes of interest

Symbol	Gene name	Accession no.	Ori	Primer sequences (5'-3')	Temp[3]
IL-6	Interleukin-6	JN639849	F[1]	GAAATCCCTCCTCGCCAATCTGA	55
			R[2]	TGAAACGGAACAACACTGCCATCT	
IL-8	Interleukin-8	AJ009800	F	ATGAACGGCAAGCTTGGAGCT	61
			R	TCACAGTGGTGCATCAGAATTGA	
TLR2	Toll-like receptor 2	NM_204278.1	F	GGCTGTGAACCTGAGAACC	55
			R	CTGATGACTGCTGAGAATACG	
TLR4	Toll-like receptor 4	NM_001030693	F	ATCACTTCTGTCTGTCTCC	53
			R	CTGTTGCCACTCCTTATCTTG	
APN	Aminopeptidase-N	NM_204861.1	F	GTCCAACAGAGCCACTTCC	54
			R	CGTCCACCAGCCAATACC	
SGLT-1	Sodium glucose co-transporter 1	AJ236903.1	F	GTCTACCTGTCAATCCTTTCAC	52
			R	GGCATCATACCCTCCAACC	
PepT-1	Peptide Transporte-1	AY129615.1	F	ATGTTCCTTGCTGGTCTGG	52
			R	TGCGTATTGCTGCTTATTGAG	
cGH	Chicken Growth hormone gene	HE608816	F	CACCACAGCTAGAGACCCACATC	58
			R	CCCACCGGCTCAAACTGC	
IGF-I	Insulin-like growth factor-I	JN942578	F	GGTGTCGAGCTGGTTGATGC	58
			R	CGTACAGAGCGTGCAGATTTAGGT	
CDN1	Claudin-1		F	TGGAGGATGACCAGGTGAAGA	58
			R	CGAGCCACTCTGTTGCCATA	
CDN4	Claudin-4	GI: 363741048	F	CGGGATCCGATGGCCTCCATGGGGCT	58
			R	GTGGAATTCCTTACACGTAGTTGCTG	
CDN5	Claudin-5		F	CAGAAGCGGGAGATAGGGG	58
			R	TACTTGACGGGGAAGGAGGT	
PCNA	Proliferating Cell nuclear antigen	NM_204170	F	GGGTTCGGGCGGCATCAG	57
			R	TCTTCATTTCCAGCACACTTCAG	
MUC2	Mucin 2	XM_421035	F	CCTGTGCAGACCAAGCAGAAA	58
			R	CCTCTGTTTTTCAGCAAAGAACAC	

[1] F: Forward, [2] R: Reverse, [3] Annealing temperature

Table 3.2 Primers used for qPCR quantification of housekeeping genes.

Symbol	Gene name	Accession no.		Primer sequences (5'-3')	Temp[3]
GAPDH	Glyceraldehyde-3-phospate dehydrogenase	NM_204305	F[1]	GTGAAAGTCGGAGTCAACGGA	60
			R[2]	AAGGGATCATTGATGGCCAC	
RPL30	Ribosomal protein L30	NM_001007479	F	GAGTCACCTGGGTCAATAA	56
			R	CCAACAACTGTCCTGCTTT	
SDHA	Succinate dehydrogenase complex, subunit A	XM_419054	F	CAGGGATGTAGTGTCTCGT	58
			R	GGGAATAGGCTCCTTAGTG	
18S	18S ribosomal RNA	AF173612	F	CGAAAGCATTTGCCAAGAAT	58
			R	GGCATCGTTTATGGTCGG	
PGK1	Phosphoglycerate kinase 1	NM_204985	F	AAAGTTCAGGATAAGATCCAGCTG	58
			R	GCCATCAGGTCCTTGACAAT	
RPS7	40S ribosomal protein S7	XM_419936	F	TAGGTGGTGGCAGGAAAGC	58
			R	TTGGCTTGGGCAGAATCC	
TFRC	Transferrin receptor protein 1	NM_205256	F	GGAACTTGCCCGTGTGATC	58
			R	GTAGCACCCACAGCTCCGT	
YWHAZ	14-3-3 protein zeta/delta	NM_001031343	F	GTGGAGCAATCACAACAGGC	58
			R	GCGTGCGTCTTTGTATGACTC	

[1] F: Forward, [2] R: Reverse, [3] Annealing temperature

3.3.8 Whole transcriptome analysis using mRNAseq

Total RNA extracted from ileal tissue was adjusted to 100 ng RNA per µL by diluting in nucleic acid free water. Quality and integrity were assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) at National Research Council Canada (CNRC: Saskatoon, SK, Canada) prior to reverse transcription. Barcoded cDNA libraries were sequenced on a HiSeq® 2500 Sequencing System (Illumina, Inc., San Diego, CA, USA). Sequencing was pair-ended providing 125 base pairs in read length and samples (8 samples per treatment) were arranged to give an expected read depth of 10.9 M reads per sample. Quality of the raw sequence results were examined by FastQC (Babraham Bioinformatics) and adaptors/poor quality sequences were trimmed with Trim Galore! (Babraham Bioinformatics). The trimmed reads were aligned to the *Gallus gallus* reference genome GRCg6a_v95 using HISAT2 v. 2.2.0 (Kim et al., 2015). The resultant bam files were imported into SeqMonk v1.47.2 (conditions: duplicate reads not removed, minimum mapping quality 20, primary alignments only, RNA-seq data, paired end), probes generated (RNASeq Quantitation Pipeline: transcript features mRNA, library type: non-strand specific, libraries are paired end, merge transcript isoforms), manual quantitation correction 0.05, and reads normalized against control samples.

Pathway analysis of genes identified as changing ≥3 fold was conducted using PANTHER (www.pantherdb.org) Over Representation Test (Released 2020-07-28, Reactome version 65 Released 2020-11-17) with FISHER test. Gene Ontology (GO) terms were identified with PANTHER and GO Ontology database DOI: 10.5281/zenodo.4081749 (Released 2020-10-09). Dot plots were generated using ggplot2 in R.

3.3.9 Statistical analysis

All results were expressed as the mean ± standard error (S.E.). All data were analyzed with SAS for Windows version 9.4 (SAS Institute Inc., Cary, NC, USA) by using the Proc Mixed procedure with factorial arrangement using *Bacillus subtilis* C-3102 (BS), time of sampling (Age) and their interaction as sources of variation. The individual bird was the experimental unit.

Age was considered a random effect, and BS a fixed effect. In the case where a significant interaction was observed, multiple comparisons were made using Tukey HSD after the main effects were combined as a single variable. For all tests, a level of 0.05 was used to determine statistical differences and a level of $0.10 \geq P \geq 0.05$ was indicated as a trend.

3.4 Results

3.4.1 Bacterial colonization

No growth was detected for aerobic or anaerobic culture of cecal contents harvested from GF eggs confirming germ-free status at both collection times. Uniform colonies characteristic of *Bacillus subtilis* C-3102 were observed for all cecal samples collected from chicks arising from eggs in the BS group and cultured aerobically, whether collected at hatch or at 24 hours of age. No growth was observed on anaerobic culture of cecal samples from chicks in the BS group indicating no contamination with anaerobic bacteria.

3.4.2 Enumeration of *Bacillus subtilis* C-3102

Bacillus subtilis C-3102 was enumerated in cecal and gizzard contents of the BS treatment group (Table 3.3). *Bacillus subtilis* counts were 6.74 log CFU/g in the gizzard at hatch, however, by 24 hours after hatch no contents could be recovered from the gizzard. Swabs taken from the gizzard mucosa confirmed presence of *Bacillus* spp. At both hatch and 24 hours after hatch, the majority of cultured colonies were heat resistant (77-88%) consist with inoculation of spores. The number of *B. subtilis* C-3102 in the cecum was 7.11 log CFU/g contents at hatch and increased to

7.54 log CFU/g by 24 hours after hatch. Germination of *B. subtilis* C-3102 was confirmed in the cecum by the low abundance of heat resistant colonies indicative of spores (33-36% spores).

3.4.3   Incubation time and percent hatchability.

All experimental eggs resulted in viable chicks at hatch such that hatchability was 100%. The effect of treatment on incubation time to hatch is shown in Figure 3.1. Mean incubation time to hatch was not different for birds euthanized at hatch and at 24 hours post hatch, however, *in ovo* inoculation of *B. subtilis* significantly increased ($P < 0.05$) incubation time to hatch. For GF groups euthanized at hatch and 24 hours post hatch, incubation time was 496.6 ± 1.7 h and 499.5 ± 1.4 h respectively. Mean incubation time for all GF birds was 498.0 ± 1.1 h. For BS groups euthanized at hatch and 24 h post hatch, incubation time was 505.4 ±3.0 h and 503.4 ± 3.4 h, respectively. Mean incubation time for all BS treated birds was 504.5 ± 2.2 h.

3.4.4   Body weight, organ mass and length

Body weight significantly decreased ($P < 0.001$) between hatch and 24 hours of age in both GF and *Bacillus* mono-inoculated birds (Table 3.4). A decrease in yolk sac weight was the primary contributor to the age-dependent reduction in body mass, however, the yolk sac-free body mass was also reduced in the 24 h period. *B. subtilis* inoculation tended to reduce ($P < 0.10$) body weight at both ages. Interestingly, this was associated with a significant ($P < 0.05$) increase of remaining yolk sac weight at both ages and a significant ($P < 0.01$) decrease in yolk sac-free body weight relative to the GF controls.

Table 3.3 Mean (±SE) number (log CFU/g) of *Bacillus subtilis* C-3102 in gizzard and cecum contents at hatch and 24 hours after hatch for birds in the BS treatment group.

	Gizzard			Cecum		
	Total Colonies (log CFU/g)	Heat Resistant Colonies (log CFU/g)	Percent spores (%)	Total Colonies (log CFU/g)	Heat Resistant Colonies (log CFU/g)	Percent spores (%)
At hatch	6.49 ± 0.10	6.34 ± 0.09	76.9	7.11 ± 0.19	5.96 ± 0.11	36.2
24h post hatch	Present[1]	Present[1]	87.8	7.59 ± 0.39	6.84 ± 0.49	33.3

[1] Gizzard were empty at 24 hours after the hatch and samples were collected by swab.
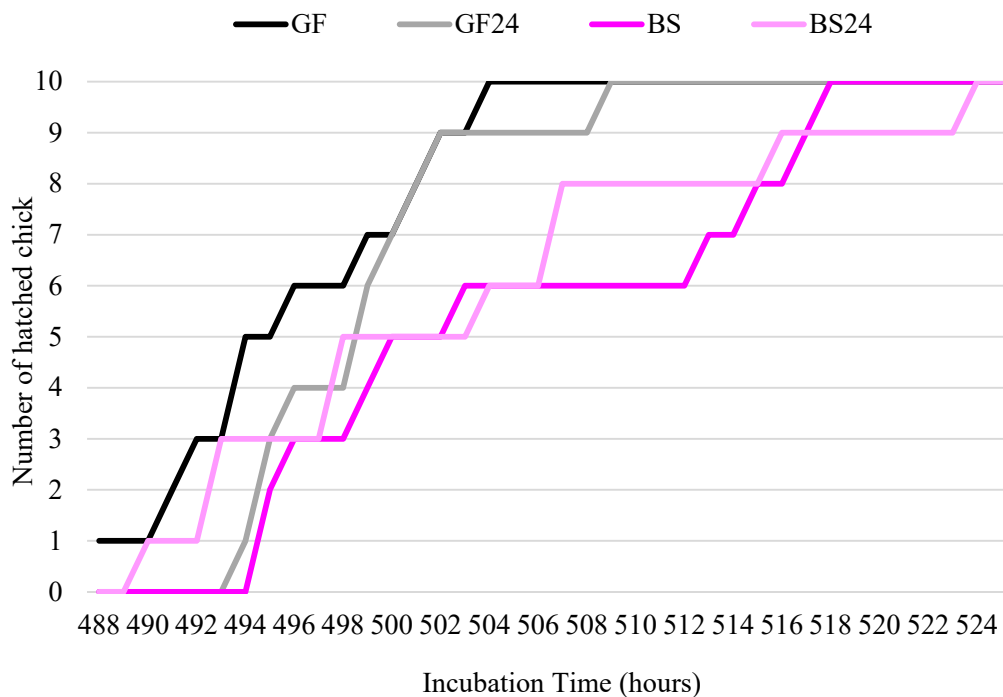
Figure 3.1 Incubation time until hatch for germ-free (GF) and *Bacillus*-inoculated (BS) birds harvested at hatch (GF and BS) or 24 hours after hatch (GF24, BS24).

Table 3.4 Mean (±SE) egg weight (EW) at the start of incubation (E0) and at day 17 of incubation (E17) and mean body weight (BW), yolk sac weight and yolk sac-free body weight for Germ-free (GF) and *Bacillus* inoculated (BS) birds at hatch and 24 hours after the hatch.

| | | | At sampling | | |
| | | | | Yolk sac weight | Yolk sac-free body weight |
Treatment	E0 EW (g)	E17 EW (g)	BW (g)	(g)	(g)
Germ-free	61.8 ± 0.60	54.8 ± 0.59	44.8 ± 0.56	6.13 ± 0.33	38.7 ± 0.37
Germ-free (24h)	61.8 ± 0.58	54.7 ± 0.47	41.3 ± 0.47	3.57 ± 0.34	37.7 ± 0.65
Bacillus	61.8 ± 0.59	54.4 ± 0.62	43.8 ± 0.55	6.38 ± 0.24	37.2 ± 0.48
Bacillus (24h)	61.7 ± 0.56	55.1 ± 0.53	40.8 ± 0.28	4.67 ± 0.39	36.2 ± 0.43
Age	*0.9538*	*0.5863*	*0.0001*	*0.0001*	*0.0578*
Bacillus	*0.9064*	*0.9021*	*0.0905*	*0.0488*	*0.0044*
Bacillus x Age	*0.9172*	*0.4874*	*0.4402*	*0.2085*	*0.9224*

Relative mass of the gizzard and bursa of Fabricius increased ($P < 0.05$) dramatically with age but were not affected by *Bacillus* inoculation (Table 3.5). Relative liver mass also increased dramatically with age. *In ovo* inoculation with *Bacillus* significantly increased ($P < 0.01$) relative liver weight at both hatch and 24 hours of age.

Table 3.5 Mean (±SE) relative weight (g/100g of BW) of gizzard, Bursa of Fabricius and liver in Germ-free (GF) and *Bacillus* inoculated (BS) birds at hatch and 24 hours after the hatch.

Treatment	Gizzard (g/100g BW)	Bursa (g/100g BW)	Liver (g/100g BW)
Germ-free	3.13 ± 0.12	0.14 ± 0.02	1.90 ± 0.04
Germ-free (24h)	4.78 ± 0.24	0.21 ± 0.02	2.57 ± 0.07
Bacillus	3.10 ± 0.11	0.16 ± 0.02	2.18 ± 0.06
Bacillus (24h)	4.45 ± 0.14	0.19 ± 0.03	2.69 ± 0.08
Age	*0.0001*	*0.0175*	*0.0001*
Bacillus	*0.2722*	*0.8964*	*0.0036*
Bacillus x Age	*0.3506*	*0.3133*	*0.2154*

Marked increases ($P < 0.0001$) were observed in the length of all small intestinal segments in the first 24 hours after the hatching (Table 3.6). Mean of relative length of total small intestine was 579 mm/100g BW at hatch in GF birds and elongated to 755 mm/100g BW at 24 hours of age

indicating an elongation rate of approx. 2.00 mm/100g BW of small intestinal length per hour. *Bacillus* inoculation significantly increased ($P < 0.05$) length of the duodenum, however no other segment length nor total small intestinal length were affected by *Bacillus* inoculation.

Table 3.6 Mean (±SE) relative intestinal segment length (mm / 100g BW) in Germ-free (GF) and *Bacillus* inoculated (BS) birds at hatch and 24 hours after the hatch.

Treatment	Duodenum	Jejunum	Ileum	Length of total small intestine	Cecum
			(mm / 100g of body weight)		
Germ-free	118 ± 4.2	231 ± 7.3	230 ± 8.4	579 ± 18.0	64 ± 1.2
Germ-free (24h)	149 ± 3.1	310 ± 8.0	296 ± 13.0	755 ± 21.6	79 ± 2.2
Bacillus	129 ± 5.4	246 ± 5.4	246 ± 5.5	620 ± 12.4	63 ± 1.3
Bacillus (24h)	168 ± 8.7	300 ± 9.4	294 ± 8.2	763 ± 18.3	79 ± 2.4
Age	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*
Bacillus	*0.0143*	*0.7365*	*0.4465*	*0.1797*	*0.9170*
Bacillus x Age	*0.4850*	*0.1210*	*0.3436*	*0.3496*	*0.7475*

3.4.5   Intestinal histology

Villus height and thickness of the *muscularis* mucosa in duodenum, jejunum and ileum is given in Table 3.7. As crypts were rudimentary at hatch (Geyra et al., 2001) and not morphologically discernable, only villus length and *muscularis* mucosa were recorded (Figure 3.2). Villus height increased dramatically in the first 24 hours in all intestinal locations. The thickness of the *muscularis* mucosa decreased ($P < 0.05$) in all small intestinal regions with age.

Bacillus inoculation decreased ($P < 0.05$) villus height in jejunum but increased ($P < 0.05$) villus height in the ileum. Regarding the thickness of the *muscularis* mucosa, *Bacillus* decreased ($P < 0.05$) thickness at hatch but not at 24 h of age in the duodenum. No effect of *Bacillus* on *muscularis* mucosa thickness was observed in jejunum, however, a significantly thinner ($P < 0.05$) *muscularis* mucosa was observed in the ileum of *Bacillus* treatment at both time points.

At hatching   *At 24 hours after hatching*
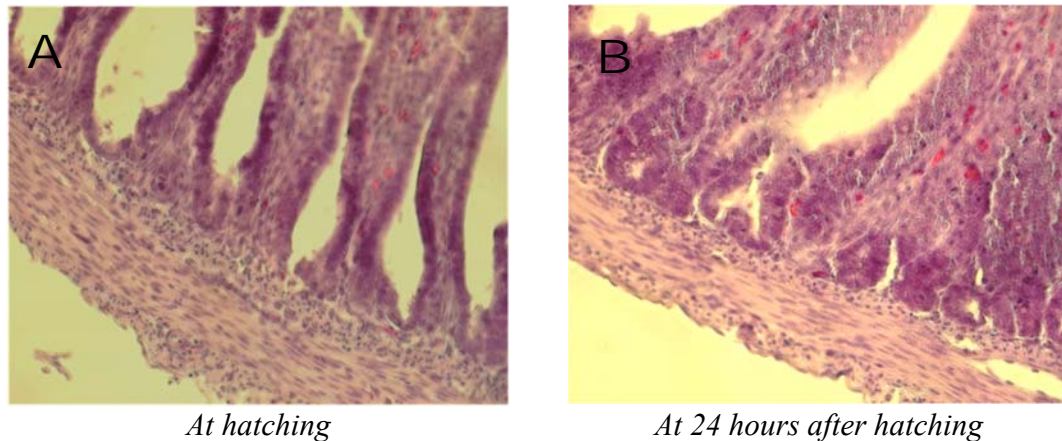
Figure 3.2 Sample picture of crypt of duodenum at hatching (Panel A) and at 24 hours after the hatching (Panel B).

3.4.6   Gene expression analysis using qPCR

Several genes of interest were differentially expressed in the ileum tissue between GF and BS birds at hatch and 24 hours after hatch (Table 3.8). The biggest fold change was observed in *IL-8* transcript abundance which was nearly 15-fold higher ($P < 0.05$) in BS birds at hatch. However, a significant *IL-8* response to BS was no longer present by 24 hours post hatch. Higher expression was also observed in BS birds for *TLR 2* ($P < 0.0001$) and *4* ($P < 0.01$) at both ages. A significant interaction for *MUC2* expression indicated *Bacillus* initially increased *MUC2* expression at hatch and deceased expression at 24 h post hatch. No altered response to *B. subtilis* C-3102 *in ovo* inoculation was shown for *CDN1* and *CDN5* at hatch and 24 hours after the hatch. *Bacillus subtilis in ovo* inoculation significantly decreased ($P < 0.001$) *CDN4* abundance at both hatch and 24 hours of age compared with germ-free birds. Significant upregulation ($P < 0.001$) by age can be seen in the expression of *IL-6, TLR-2, TLR-4, APN, SGLT-1, PepT-1,* and *cGH*. Interestingly, *PepT1* and *APN* gene expression tended ($P < 0.10$) to be higher with *Bacillus* inoculation. For *PCNA*, a significant interaction ($P < 0.01$) indicated *B. subtilis* C-3102 *in ovo* inoculation did not alter ileal expression at hatch but decreased expression 24 hours post hatch.

Table 3.7 Mean (±SE) villus height and thickness of *muscularis* mucosa in duodenum, jejunum, and ileum in Germ-free (GF) and *Bacillus*-inoculated (BS) birds at hatch and 24 hours after hatch.

Treatment	Duodenum		Jejunum		ileum	
	Villus height	*Muscularis Mucosa*	Villus height	*Muscularis Mucosa*	Villus height	*Muscularis Mucosa*
			(μm)			
Germ-free	381 ± 17.5	57 ± 1.6[a]	264 ± 9.9	47 ± 2.3	217 ± 7.7[c]	56 ± 2.7
Germ-free (24h)	598 ± 25.9	45 ± 1.8[b]	376 ± 19.5	40 ± 1.1	285 ± 11.3[b]	46 ± 3.4
Bacillus	359 ± 16.0	45 ± 2.5[b]	248 ± 11.8	44 ± 2.3	218 ± 11.6[c]	44 ± 2.3
Bacillus (24h)	631 ± 14.4	42 ± 1.8[b]	330 ± 9.4	40 ± 2.9	342 ± 18.1[a]	41 ± 3.3
Age	*0.0001*	*0.0009*	*0.0001*	*0.0346*	*0.0001*	*0.0348*
Bacillus	*0.7654*	*0.0006*	*0.0268*	*0.5741*	*0.0345*	*0.0130*
Bacillus x Age	*0.1574*	*0.0322*	*0.2896*	*0.6111*	*0.0449*	*0.2363*

[ab] Values in same column with different superscripts are significantly different ($P < 0.05$)

Table 3.8 Mean (±SE) fold change in expression of genes in the ileum for Germ-Free (GF) and *Bacillus*-inoculated (BS) birds at hatch and 24 hours after the hatch.

Gene	Hatch		24 h of age		*P value*		
	Germ-free	*Bacillus*	Germ-free	*Bacillus*	Age	*Bacillus*	*Age x Bacillus*
IL-6	1.0 ± 0.37	0.7 ± 0.31	2.8 ± 0.70	3.3 ± 1.10	*0.0038*	*0.8336*	*0.5645*
IL-8	1.0 ± 0.12[b]	14.7 ± 3.38[a]	0.8 ± 0.05[b]	4.4 ± 1.90[b]	*0.0117*	*0.0001*	*0.0155*
TLR2	1.0 ± 0.05	2.4 ± 0.20	2.4 ± 0.37	3.8 ± 0.44	*0.0001*	*0.0001*	*0.9431*
TLR4	1.0 ± 0.09	2.4 ± 0.22	4.1 ± 0.57	5.6 ± 0.63	*0.0001*	*0.0027*	*0.8192*
APN	1.0 ± 0.03	1.3 ± 0.08	2.6 ± 0.18	3.2 ± 0.41	*0.0001*	*0.0819*	*0.5120*
SGLT-1	1.0 ± 0.05	1.4 ± 0.13	2.4 ± 0.23	2.5 ± 0.21	*0.0001*	*0.4151*	*0.7205*
PepT-1	1.0 ± 0.07	1.7 ± 0.31	4.5 ± 0.61	5.2 ± 0.26	*0.0001*	*0.0698*	*0.9201*
cGH	1.0 ± 0.13	1.6 ± 0.30	2.6 ± 0.48	3.6 ± 0.70	*0.0004*	*0.0933*	*0.6736*
IGF-I	1.0 ± 0.09	0.9 ± 0.09	1.3 ± 0.08	1.1 ± 0.10	*0.0119*	*0.0565*	*0.3267*
CDN1	1.0 ± 0.08	0.9 ± 0.19	1.2 ± 0.20	0.9 ± 0.12	*0.4997*	*0.2113*	*0.5680*
CDN4	1.0 ± 0.07	0.8 ± 0.04	1.1 ± 0.11	0.7 ± 0.10	*0.7269*	*0.0009*	*0.1988*
CDN5	1.0 ± 0.08	1.2 ± 0.21	1.5 ± 0.21	1.0 ± 0.11	*0.3138*	*0.4123*	*0.0609*
PCNA	1.0 ± 0.06[b]	1.1 ± 0.07[ab]	1.3 ± 0.08[a]	1.0 ± 0.05[b]	*0.0920*	*0.2035*	*0.0078*
MUC2	1.0 ± 0.09	1.7 ± 0.29	3.0 ± 0.36	2.1 ± 0.26	*0.0001*	*0.6689*	*0.0059*

[ab]*Values within a row with different superscripts are different.*

3.4.7 Whole transcriptome analysis using mRNAseq

A total of 129 genes were upregulated ≥3-fold in the ileum when comparing GF to BS at hatch, whereas at 24 h of age, 110 genes were upregulated. Of these, 52 genes were upregulated with the BS treatment at both hatch and 24 h of age. A total of 54 genes were ≥3-fold downregulated in BS compared to GF at hatch, whereas 31 genes were downregulated in BS at 24 after hatch. Of these, 11 were downregulated at both hatch and 24 h after hatch (Figure 3.2). At hatch, pathway enrichment analysis indicated BS treatment enriched the chemokine pathway. The pathway is a subset of the other pathways identified in Figure 3.3 panel A, including according to decreasing level of specificity pathways identified as Peptide Ligand-Binding Receptors, Class A/1 (Rhodopsin-Like Receptors, and GPCR Ligand binding (Figure 3.3). At 24 h after hatch, the Chemokine Receptors Bind Chemokines pathway was also enriched in BS birds. Additionally, BS enriched the Digestion and Absorption pathway at 24 hours after hatch (Figure 3.4).
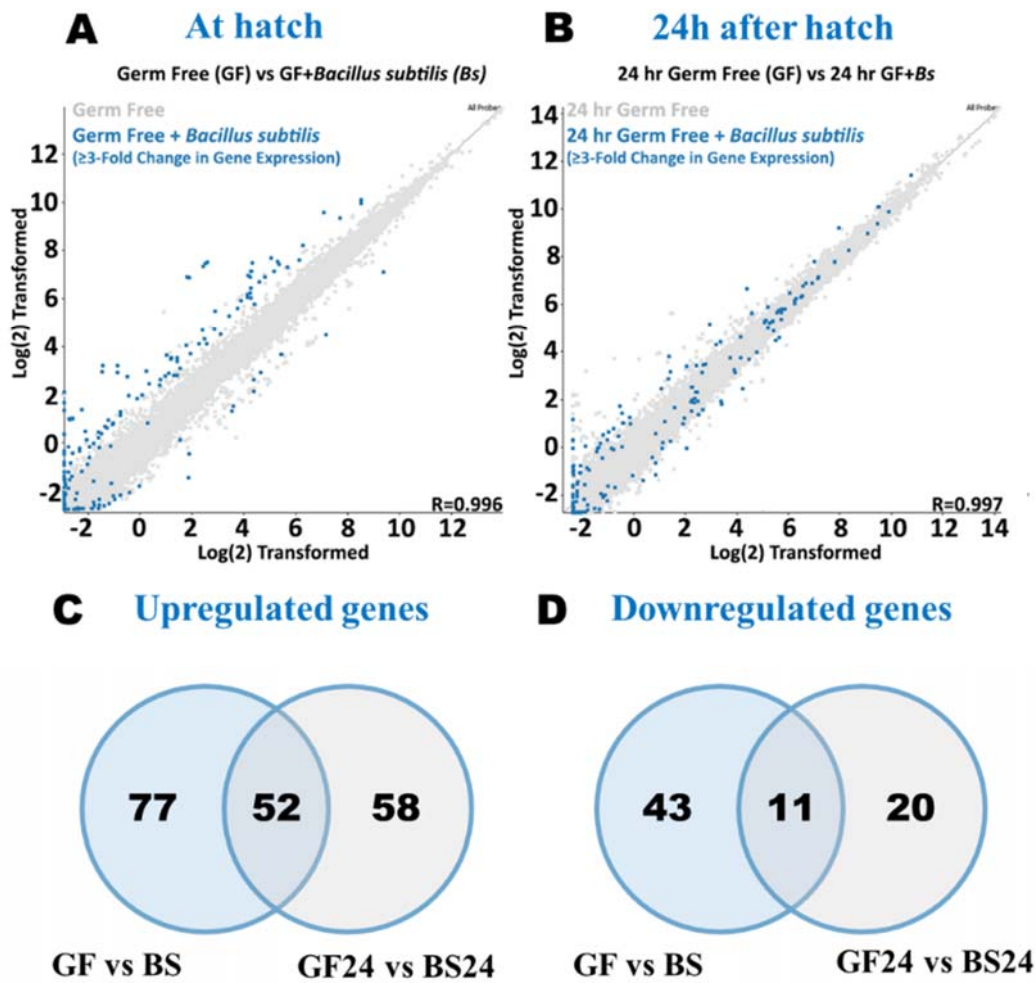
Figure 3.3 Scatterplots comparing the expression of individual genes in the ileum for Germ-Free (GF) and *Bacillus*-inoculated (BS) birds at hatch (A) and 24 hours after the hatch (B). Overlap in genes changing expression ≥3-fold between at hatch and 24 hours after hatch, upregulated (C) or downregulated (D).
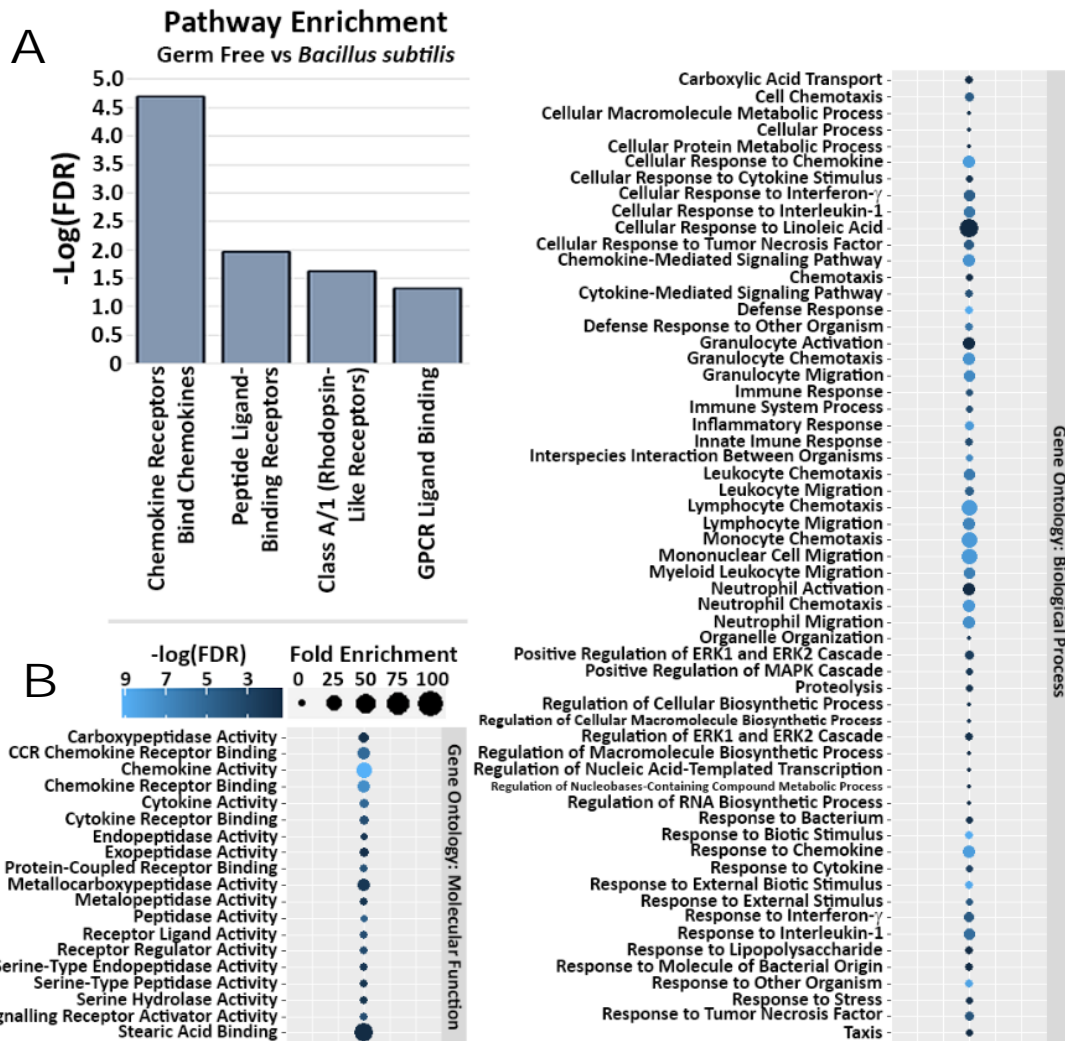
Figure 3.4 Identification of enriched pathways (A) and enriched gene ontology terms for biological processes (B) in the ileum of Germ-Free (GF) and *Bacillus*-inoculated (BS) birds at hatch.
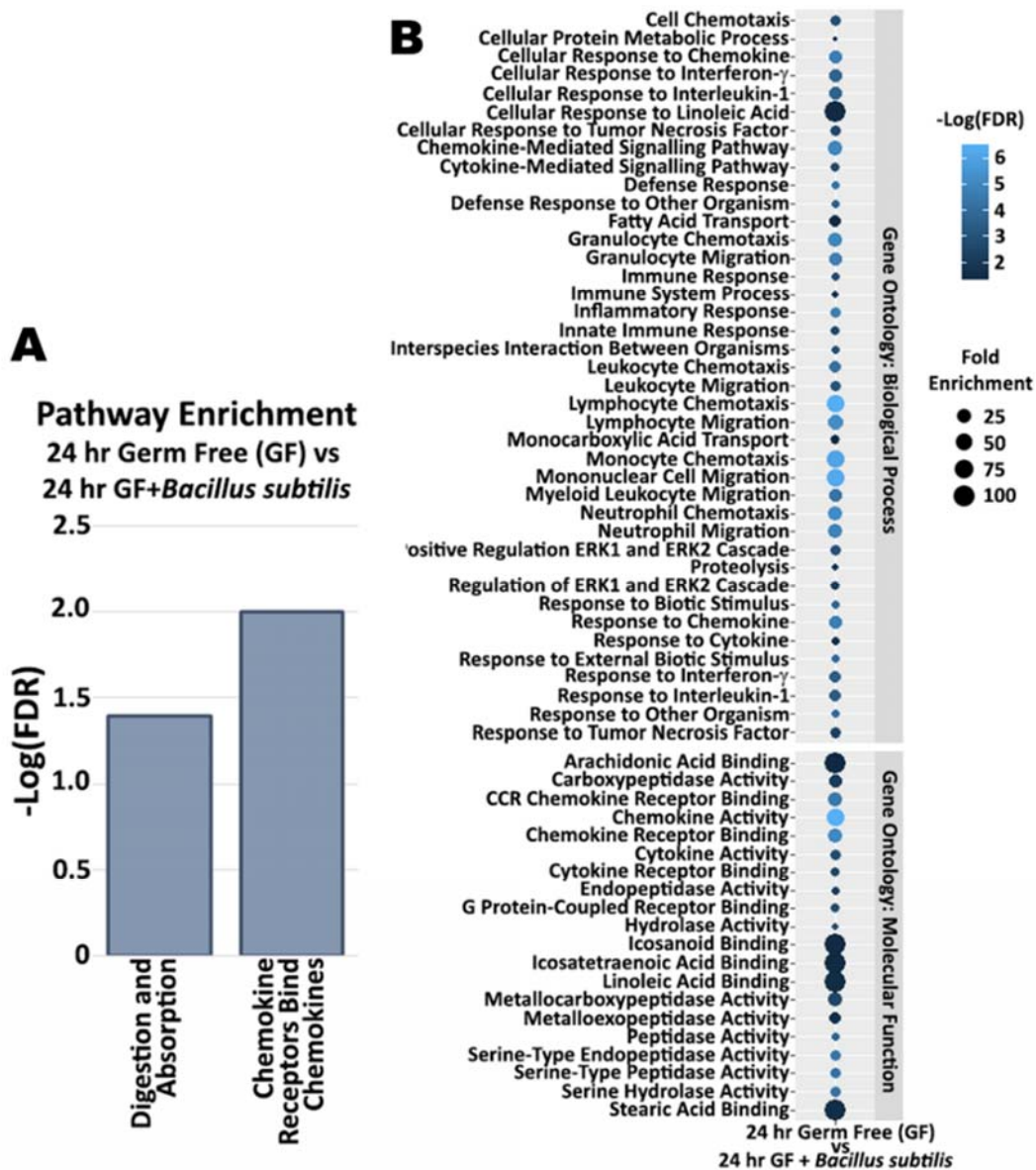
Figure 3.5 Identification of enriched pathways (A) and enriched gene ontology terms for biological processes (B) in the ileum of Germ-Free (GF) and *Bacillus*-inoculated (BS) birds at 24 hour after hatch

3.5 Discussion

A persistent question regarding the mode of action of *Bacillus* probiotics administered as spores, is whether germination of spores occurs during passage through the gastrointestinal tract (Cartman et al., 2008; Latorre et al., 2014). Although spores or microbial products of sporulation remaining in the spore preparation may influence gut microbial composition or host response, germination to vegetative cells is required for proliferation and *in situ* bioactivity. In the present study, germination of *Bacillus subtilis* C-3102 in the gut of the chicken embryo was evidenced by the low proportion of spores recovered at all locations, particularly in the cecum, at time of hatch and at 24 hours of age. Some germination of spores may have occurred in gizzard (Bernardeau et al., 2017) since greater than 10% of cells recovered from the gizzard were not heat resistant. Interestingly, *Bacillus* counts recovered in cecal contents increased by 0.6 log CFU/g as compared to the gizzard. Combined, these data suggest that at least a portion of administered spores, germinated and proliferated in the *in ovo* chicken gut.

Others have also reported evidence of germination of *Bacillus* spores in the post-hatch chicken gastrointestinal tract. Vegetative cells of the *Bacillus* strain were detected throughout the GI tract of White Leghorn chicks at 20 hours post oral gavage of *B. subtilis* SC2362 spores at 1 day of age. Indeed, 62% to 96% of the *Bacillus* detected in the ceca in that study were recorded as vegetative cells (Cartman et al., 2008). Similarly, Latorre et al. (2014) reported a 90% germination rate for *B. subtilis* PHL-NP122 when supplemented in feed of chicks from day of hatch based on comparison of spore numbers in crop and ileum contents.

Interestingly, in both studies the number of spores recovered from the intestine declined over time when spores were administered as a single gavage indicating that if vegetative cells replicate in the gastrointestinal tract, the rate of replication is insufficient to compensate for the loss of cells through peristalsis (Cartman et al., 2008; Leser et al., 2008). Peristaltic washout in the *in ovo* model would be far less of a factor accounting for the higher recovery of *Bacillus* cells here 4-5 days following *in ovo* delivery of a single dose. Furthermore, *Bacillus* cell counts numerically increased from the time of hatch until 24 hours after hatch in the present study suggesting a "washout" effect had not yet occurred within this timeframe and further supportive of *Bacillus* cell proliferation. Finally, the high proportion of vegetative cells found in the cecum in the mono-associated *in ovo* model reported here, could reflect a limited rate of re-sporulation given the abundance of substrates,

compared to conventional chickens, where re-sporulation is hypothesized to occur in ceca (Bernardeau et al., 2017).

The current work demonstrates that specific nutrients essential for germination, such as L-alanine, L-valine, and L-asparagine for *Bacillus* spores in nature (Setlow, 2014) are present to support *in ovo* germination. In agreement with others, our observations suggest metabolic activities associated with vegetative cells, likely contribute to host responses to spore-based *Bacillus* probiotics (Bernardeau et al., 2017; Cartman et al., 2008; Latorre et al., 2014)

The finding of vegetative cells here supports the potential of the *in ovo* model system for evaluation of the direct effect of *Bacillus* probiotics on intestinal development and function. Furthermore, modifications to the model to permit incubation and hatching of birds in individual HEPA-filtered and climate-controlled containers provided several major advantages. Firstly, the system minimized the impact of a potential microbial contamination event to an individual bird (although we had no occurrence of contamination in the present study). Secondly, the use of clear containers allowed the precise timing of hatch and permitted tissue collection precisely at hatch without contaminating the environment of remaining yet-to-hatch eggs. Finally, we were able to ensure that sample collection occurred at a precise time following hatch (24 hours) and we were able to maintain a sterile or mono-associated environment during that period. Future developments in the model will be the delivery of sterilized feed and water to evaluate development in a controlled environment over a longer post-hatch period.

In ovo inoculation with BS at E17 did not affect hatchability but resulted in negative effects on chick growth and development including increased incubation time to hatch, reduced yolk sac-free body weight at hatch and changes in intestinal morphology and gene expression associated with barrier function, growth and nutrient absorption. Pathway analysis applied to RNAseq data confirmed BS-activated chemokine and nutrient absorption pathways. Our findings of no effect on hatchability agrees with results from others investigating *in ovo Bacillus* probiotic inoculation (Arreguin-Nava et al., 2019; Majidi-Mosleh et al., 2017a; Majidi-Mosleh et al., 2017b). However, there have been reports of reduced hatchability following *in ovo Bacillus* inoculation (de Oliveira et al., 2014; Triplett et al., 2018). Further, our findings regarding detrimental effects on body and organ weight contrast an increase body weight at hatch following *in ovo* inoculation of *Bacillus* spp. reported by (Arreguin-Nava et al., 2019). Yolk sac weight was not recorded by others and

contrasting changes in yolk sac weight and yolk sac-free body weight observed with BS inoculation here and described below may explain some of this variation.

As expected, without access to water or feed, chicks lost body mass in the first 24 hours after hatch associated primarily with utilization (disappearance) of yolk sac mass (Incharoen et al., 2015; Jacobs et al., 2016) but also loss of yolk sac-free body mass. However, *Bacillus* inoculation appeared to decrease utilization of the yolk sac, both pre and post hatch, as indicated by an increased yolk sac mass and decreased yolk sac free body weight relative to the germ-free control. A decrease in yolk sac utilization at hatch is somewhat surprising given the prolonged incubation time associated with *in ovo Bacillus* inoculation which presumably would require additional nutrients. The reduction in yolk sac-free body weight with *Bacillus* inoculation could reflect chick increased metabolic activities such as those associated with barrier function and possibly a pro-inflammatory response (see below) in BS birds resulting in partitioning of nutrients away from muscle and skeletal growth. Nutrient repartitioning and inflammatory responses (Klasing, 2007) may also have reduced nutrient assimilation from the yolk increasing remaining mass.

An alternative explanation of the increased mass of remaining yolk sac at hatch is that vegetative *Bacillus* cell metabolism or *Bacillus*-induced metabolic activities in the embryo may have utilized growth and development-limiting essential nutrients present in the albumen or in the yolk and leading to extended incubation time. For example, the yolk is the major origin of essential minerals including Mn, P, Fe, Ca, Cu, and Zn (Yair et al., 2015; Yair and Uni, 2011), which may have been utilized by *Bacillus* cells. Further, changes in nutrient requirements associated with pro-inflammatory and barrier responses in birds are well recognized (Klasing, 2007).

A final explanation for increased yolk sac mass in BS-inoculated chicks could be fluid infiltration into the yolk sac associated with an inflammatory response. The yolk sac is heavily vascularized (Sheng, 2010) to support direct absorption of nutrients across the yolk sac epithelium (Bauer et al., 2013). The late stage embryo is capable of developing an inflammatory response (Schilling et al., 2018), and could lead to fluid accumulation in the yolk in response to microbial stimulation. Unfortunately, we did not examine the composition of the remaining yolk sac contents to differentiate these possibilities.

Although local inflammation in the yolk sac could not be confirmed, a significant increase in relative liver weight, reduced villus height in jejunum and upregulation of *IL-8, TLR2,* and *TLR4*

expression in the ileum was observed in BS birds. Chemokine pathway enrichment was confirmed at both hatch and 24 hours of age by RNAseq analysis consistent with qPCR gene expression and suggests that *Bacillus in ovo* inoculation induced an intestinal and potentially systemic (based on liver weight) inflammatory response. This contrasts our previous findings showing no increase in inflammatory gene expression (including *IL-8*) in 14-d-old birds mono-associated with *Bacillus subtilis* C-3102 from the day of hatch (Hamaoka et al., 2011). This contrast in results may reflect the limited development of intestinal barrier function in the chick embryo permitting easier bacterial translocation.

The well-established success of *in ovo* immunization against several viral diseases (Steel et al., 2008) confirms the ability to prime an adaptive immune response in the embryo. Also, others have recorded changes in lymphoid organ morphology at hatch following *in ovo* bacterial inoculation indicative of embryonic immune-responsiveness (Madej et al., 2015). On the other hand, a functionally mature adaptive immune system does not occur until around 1–2 weeks of age such that protection of the embryo and chick relies primarily on the innate immune system (Bavananthasivam et al., 2018; Schilling et al., 2018). Indeed, activation of innate immune responses in the embryo has been confirmed by a number *in ovo* injection experiments including glucose (Bhanja et al., 2015), selenium (Lee et al., 2014), Campylobacter antigens (Kobierecka et al., 2016), and *Salmonella* flagellin (Vaezirad et al., 2018). One hypothesis is that early immune stimulation by *in ovo* administration of bacterial antigens or live bacteria will advance development of innate protective systems providing increased protection following hatch. For example, upregulation of *IL-4, IL-6, IFN-β,* and *IL-18* was observed after *in ovo* inoculation with *Lactobacillus acidophilus* and *Streptococcus faecium* (Slawinska et al., 2014). On the other hand, *in ovo* inoculation of *L. salivarius* and a *Pediococcus* sp. as a mixed inoculant decreased inflammatory status at hatch compared with conventional chicks (Wilson et al., 2019b). Similarly, down regulation of *TLR2, TLR4, IL-4* and *IL13* were reported with *in ovo* inoculation of a combination probiotic product consisting of *L. acidophilus*, *L. casei*, *E. faecium*, and *Bifidobacterium bifidum* (Pender et al., 2017). Whether a downregulation or upregulation of inflammatory responses in the embryo results in post hatch protection against infection requires further study.

Interestingly, a downregulation of *CLDN4*, a key tight junction protein which acts to regulate epithelial permeability (Wang et al., 2020), was observed here which could indicate loss of barrier function following *Bacillus* inoculation. There is little information regarding the effect of probiotic bacteria on expression of tight junction proteins *in vivo*; however, in a recent *in vitro* study using Caco-2 cells, a strengthening of barrier function was reported suggested by increased transepithelial electrical resistance and upregulated *CLDN1* expression following *B. subtilis* Bs29784 (Rhayat et al., 2019). This upregulation of barrier function was observed in association with a limiting of inflammatory response to other stressors.

The secreted mucin *MUC2*, is also a marker of barrier function. We observed upregulation of *MUC2* expression in the first 24 h after hatch in agreement with Zhang et al. (2015). Interestingly, the increase in *MUC2* occurred here in GF birds that were not exposed to post-hatch bacterial colonization or feed-origin stimulants. The expression of *MUC2* at hatch was increased by *Bacillus* inoculation, but inoculation appeared to suppress the post-hatch increase in *MUC2* expression observed in GF birds. The pre-hatch increase in MUC2 is consistent with increased pre-hatch expression of other inflammatory mediators. It is not clear why *Bacillus* blunted the post-hatch increase in *MUC2* expression compared with germ-free birds.

Higher ileal *MUC2* gene expression by *in ovo* inoculation with *Bacillus* was observed by Majidi-Mosleh et al. (2017b) at E21 and, in contrast to this study, 3 days post-hatch. In the study by Majidi-Mosleh et al. (2017b), birds were provided feed in a conventional environment where *MUC2* upregulation after the hatch is affected by feeding (Proszkowiec-Weglarz et al., 2020). The presence or absence of other inflammatory stressors in the *in ovo* and post-hatch environment may therefore influence the immune and barrier response to *Bacillus* inoculation.

Upregulation of expression of SGLT-1 and PepT1 has been shown in the first 30 hours after hatching (Yalcin et al., 2016) consistent with observations here and consistent with rapid post hatch development of intestinal absorptive function (Jha et al., 2019). Interestingly, *in ovo Bacillus* inoculation tended to increase expression of *APN* and *PepTI,* both at hatch and 24 hours after hatch. In agreement with mRNA expression results from qPCR, enrichment of Digestion and Absorption pathway was also shown by RNAseq analysis at 24 h post hatch. These data indicate that *in ovo Bacillus* may have accelerated digestive and absorptive maturation. As such, post birth microbial colonization is associated with maturation of the intestinal epithelium in pigs (Willing and Van

Kessel, 2010) and poultry (Cheled-Shoval et al., 2014; Schokker et al., 2015). Whether the response observed here is specific to *B. subtilis* C-3102 or a general response to microbial colonization needs further study. Nevertheless, the increase in expression of these genes in the germ-free control group indicate that post hatch maturation is in part programmed rather than a response to environmental stimulants such a bacterial colonization or feed components.

Interestingly, chicken *GH* was dramatically upregulated from hatch to 24 hours of age. Chicken GH has a critical role in controlling growth and metabolism (Nie et al., 2005) in the bird gastrointestinal tract and this increase is consistent with the dramatic increase in small intestinal length observed here in the first 24 hours and by others (Scanes et al., 1984). *Bacillus* inoculation tended to increase *GH* expression in the ileum although this was not associated with an increase in the ileum length. In contrast, ileal *IGF-1* expression, another important regulator of cell proliferation (Laron, 2001), tended to be reduced by BS inoculation. Whether these localized changes in intestinal expression of digestive enzymes, nutrient transporters and tissue growth regulators could be a direct mechanism contributing to body weight gain or FCR improvement by *Bacillus* probiotic supplementation (EFSA, 2006b; Fritts et al., 2000) requires confirmation.

3.6   Conclusion

We employed *in ovo* inoculation and developed a system for isolation of developing embryos in individual containers to study the direct effect of *Bacillus* spores on gut development. Individual climate-controlled containers reduced the risk of microbial contamination on experimental outcomes and allowed for maintenance and study of mono-association during the early postnatal period. *In ovo* administration of *Bacillus* spores resulted in germination and colonization of the chick gastrointestinal tract as assessed at hatch confirming previous work demonstrating germination in conventional birds. The mechanisms of action of *Bacillus* probiotics in the gut may therefore include metabolic activities associated with germination and vegetative metabolism. *Bacillus subtilis* C-3102 directly and differentially modified intestinal gene expression associated with immune response and nutrient assimilation. Although there was evidence of advanced maturity of digestive and absorptive function, the effect of *in ovo Bacillus* inoculation was largely unfavourable to bird development resulting in reduced body and organ

weights and elevated expression of pro-inflammatory genes. Although host response to microbial colonization may differ during embryonic development relative to post hatch, *in ovo* bacterial inoculation may be a simple and useful approach to clarify host response pathways directly modulated by probiotic administration.

4 EFFECT OF *IN OVO* MONO-ASSOCIATION WITH BACTERIAL ISOLATES REPRESENTING MAJOR GUT TAXA ON EMBRYONIC DEVELOPMENT IN CHICKEN.

4.1 Abstract

Bacterial strains representing major taxa found in chicken intestinal tract were isolated to investigate the effect on *in ovo* mono-associated colonization and development of a chicken embryo (White Leghorn, ISA Bovans White x Lohmann LSL-Lite). Amniotic fluid was inoculated with sterile saline (Germ-Free; GF) or a bacterial isolate between day 17 and 21 of incubation (E-17-E21) in a series of experiments. No contamination of intestinal contents with viable non-inoculated bacteria was recorded at hatch including among GF control embryos. Colonization was confirmed in all embryos for *Enterococcus faecalis* ENT03, *Lactobacillus salivarius* LCT01 and *Escherichia coli* ECL01 and *E. coli* ECL02, whereas *Bacteroides fragilis* BCT06 was recovered from 5 of 8 birds and *Clostridium butyricum* CLS01 was recovered from 1 of 8 birds at hatch. Colonization density (cfu/g) was variable among strains and may have contributed to variation in response including expression of ileal genes associated with inflammation and barrier function. Hatchability, body and relative organ weight were unaffected by strain except for embryos inoculated with ECL01 or ECL02 where injection of live, but not heat killed, *E. coli* from E17 to E19, but not E20, was lethal (pipping without successful hatch). Among non-lethal inoculants, ENT03 induced the greatest inflammatory response in the ileum followed by BCT06. The LCT01 strain induced expression of barrier-associated genes without induction of inflammatory genes. For ECL01 inoculated at E17, a marked ileal inflammatory response observed in the ileum when live embryos were harvested at the time of pipping. In conclusion, a sterile chick intestine at hatch was confirmed. Several commensal bacterial strains taken from adult chicken intestine were well tolerated following *in ovo* administration with *L. fermentum* LCT01 inducing enhanced barrier function markers without inflammation. Non-pathogenic *E. coli* was lethal when inoculated before E20, associated with marked ileal inflammation, and suggesting that day 20 incubation represents an acute time point for programmed maturation of gut immunity.

4.2 Introduction

In avian species, the egg has multiple defenses against microbial infestation such as the cuticle cover (Gole et al., 2014) and albumen containing lysozymes and antibodies (Dearborn et al., 2017). These protections support the traditional consideration that embryos laid by healthy hens are microbiologically sterile (Furuse and Okumura, 1994). Nevertheless, although eggs can be hatched under sterile conditions to produce germ-free birds (Cheled-Shoval et al., 2014), there is current debate that gut microbial colonization may begin *in ovo* (Akinyemi et al., 2020; Roto et al., 2016)

Regardless of origin, the post hatch development of gut microbiota begins with Enterobacteriaceae and Streptococci (Benno and Mitsuoka, 1986) whereas Lactobacillaceae become dominant around four days of age (Schokker et al., 2015) followed by increasing diversity leading to a highly complex microbiota. A number of studies have established the major taxonomic groups colonizing the chicken gastrointestinal tract using molecular tools (Dumonceaux et al., 2006; Gong et al., 2007; Stanley et al., 2013b; Wei et al., 2013). At the family level, major taxa include Lactobacillaceae, Bacteroidaceae, Clostridiaceae, Enterococcaceae and Enterobacteriaceae (Lu et al., 2003).

Implications of the intestinal microbiota for health and performance have been the subject of significant study. For example, Guardia et al. (2011) reported associations between stocking density and the cecal microbial profile suggesting a link between the microbiota and daily body weight gain in broilers. Similarly, Stanley et al. (2012) and Singh et al. (2014) have reported associations between cecal bacterial composition and feed conversion efficiency. The concept of functional interactions between members of the gut microbiota and host metabolism is now widely accepted (Pedroso and Lee, 2015; Tremaroli and Backhed, 2012).

Despite accumulation of these studies, little progress has been made in understanding the role of the individual bacterial species or strains that contribute to beneficial or harmful metabolic outcomes. Only a few trials have examined the influence of a single gut bacterial species or strain, limited primarily to (zoonotic) pathogens, and probiotic candidate strains. This lack of knowledge is one of the obstacles to developing effective nutritional and management strategies designed to modify the gut microbiota to improve health and performance. Therefore, identifying how individual gut bacterial species or strains differentially modulate host metabolism could contribute

to establishing which members of the microbial community should be encouraged and which should be limited to effect positive outcomes.

The opportunity to modulate embryonic development in the chick embryo to improve development, health and performance post hatch, has been well established in the case of *in ovo* vaccine (Sarma et al., 1995) and nutrient (Dong et al., 2013; Kadam et al., 2013; Yair et al., 2015) delivery. *In ovo* delivery of putatively probiotic bacteria has also been investigated as a mechanism to enhance body weight (Pender et al., 2017), gut development (Edens et al., 1997; Roto et al., 2016), immune related gene expression (Pender et al., 2017), and barrier function (Edens et al., 1997) in the hatched chick. However, there have been no systematic studies comparing the effect of *in ovo* delivery of members of the major taxonomic groups present in the intestine on embryonic and early post hatch gut development.

Previously, we reported a method of inoculating the chick with bacteria *in ovo*, followed by hatching in individual small aseptic containers to preserve peri-hatch microbial status. In the current experiment, we took advantage of the combination of individual germ-free pre-hatch isolation and *in ovo* inoculation to study the effect of representatives of major taxa in the chicken gut. We hypothesized that different bacterial species will differentially affect development of the digestive tract and these differences will be informative in identifying beneficial microbial profiles.

4.3   Materials and Methods

4.3.1   Test Bacterial Strains

Representatives of five major taxa present in chicken gastrointestinal tract including Lactobacillaceae, Bacteroidaceae, Clostridiaceae, Enterococcaceae and Enterobacteriaceae were isolated by culture of ileal and cecal contents of 14-d-old chickens (Ross308) using one of six selective conditions and agars including: DHL agar "Nissui" (NISSUI Pharmaceutical co., ltd. Tokyo, Japan) for Enterobacteriaceae and BD™ Enterococcosel™ Agar (E agar, Becton, Dickinson and Co. Sparks, MD, USA) for enterococci cultured at 37 °C for 24 hours in aerobic conditions, LBS agar (NISSUI Pharmaceutical co., ltd. Tokyo, Japan) for lactic acid bacteria, NN agar (Mitsuoka, 1971) without neomycin for clostridia*,* and BBE agar (Becton, Dickinson and Co. Sparks, MD, USA) for Bacteroides at 37 °C for 48 hours in anaerobic conditions (Anaeropack System, Mitsubishi Gas Chemical Company, INC., Tokyo, Japan). Approximately 100 colonies

from each culture were further isolated by subculture and identified by sequencing of the 16SrRNA gene after amplification with universal primers (H1476: 5'-GAGTTTGATCCTGGCTCAG-3', H1478: 5'-GWATTACCGCGGCKGCTG-3') (Dorsch and Stackebrandt, 1992). Sequence analysis including quality assessment, primer removal, alignment and taxonomic assignment were conducted using MEGA6 software (Tamura et al., 2013).

A total of 6 bacterial strains were selected for study including, *Enterococcus faecalis* ENT03 (ENT03), *Bacteroides fragilis* BCT06 (BCT06), *Clostridium butyricum* CLS01 (CLS01), *Lactobacillus salivarius* LCT01 (LCT01), *Escherichia coli* ECL01 (ECL01), and *Escherichia coli* ECL02 (ECL02. Multiple aliquots of isolates were retained by overnight culture in Gifu Anaerobic Broth (GAM broth, HiMedia Laboratories Pvt. Ltd., LBS Marg, India), followed by mixing with 30% glycerol and storage at -80 °C until usage. The *E. coli*, isolates ECL01 and ECL02, were further characterized for virulence determinants by Prairie Diagnostic Services Laboratory (Saskatoon, SK, Canada). The ECL01 isolate was characterized as non-hemolytic with a 08 O serotype and negative PCR test for pyelonephritis-associated pili (P. fimbriae), aerobactin, temperature-sensitive haemagglutinin and cytotoxic necrotizing factor. The ECL02 isolate was non-hemolytic with an 069 O serotype and positive for P. fimbriae only.

4.3.2   Embryo Inoculation and Sample Collection

All experiments were conducted with the approval of the University of Saskatchewan Animal Research Ethics Board (Protocol # 20150017) according to the guidelines of the Canadian Council on Animal Care.

Fertilized eggs (White Leghorn, ISA Bovans White x Lohmann LSL-Lite) free of cracks and other defects were obtained from the University of Saskatchewan Poultry Centre (Saskatoon, SK, Canada) flock, sanitized for 30 min at 30 °C in 0.5% of sodium hypochlorite and transferred to a HEPA-filtered sterilized incubator at 37.8 °C. Prior to *in ovo* inoculation, a small hole was made in the shell at the top of each air cell using a Dremel® rotary tool (Racine, WI, U.S.). Inoculations (100 µl) were made into amniotic fluid using a sterile pipette and the shell was sealed using a food industry grade 100% silicone sealant (Kitchen grade 100% silicone, DAP®, Canada). The eggs were then placed into sterile clear individual hatching jars, sealed and maintained at 38 °C and 50% relative humidity by circulation of humidified air through a 0.45 µm filter (Nalgene

Syringe Filter 0.45μm SFCA, Thermo Scientific) using an air pump (Whisper® Aquarium Air Pump, Tetra, Blacksburg, VA, U.S.A.). In some experiments eggs were returned to a sterilized standard hatcher with HEPA filtered air supply as indicated below.

For each experiment, an aliquot of frozen isolate was thawed on ice and approximately 10 μL of contents were taken and streaked on non-selective agars including BL agar (Eiken Chemical Co., Ltd., Japan) for *B. fragilis* BCT06, *L. salivarius* LCT01 and *C. butyricum* CLS01, BBLTM TrypticacseTM soy broth with 2% agar (TS agar, Becton Dickinson and Co. Sparks, MD, USA) for *E. faecalis* ENT03, *Escherichia coli* ECL01, and *Escherichia coli* ECL02. The TS agar was incubated aerobically at 37 °C for 24 hours and the BL agar was incubated anaerobically at 37 °C for 48 hours (Anaeropack System, Mitsubishi Gas Chemical Company, INC., Tokyo, Japan). Fifteen minutes before *in ovo* inoculation, 10 typical colonies of each strain were harvested and dissolved in 10 mL sterile saline. Where experiments required assessment of inoculant dose, 10-fold dilutions in sterile saline were prepared. The inoculants were warmed to 37 °C before *in ovo* inoculation to avoid giving cold shock to the embryo. Remaining inoculant was placed on ice immediately following inoculation. An aliquot of inoculant remaining after inoculation was applied to the appropriate selective agar for culture to confirm viability and for enumeration.

4.3.3   Experiment 4-1 *In ovo* inoculation with *B. fragilis* BCT06, *E. faecalis* ENT03, or *L. salivarius* LCT01

Embryos (n=40) at day 17 of incubation (E17) were randomly assigned to 4 treatment groups balanced for egg weight at E0 (10 eggs per group). At the end of day 17 of incubation, embryos in one treatment group were inoculated with 100μL of sterile saline by *in ovo* inoculation and designated as the germ-free (GF1) group. The embryos in remaining three groups were inoculated with 100 μL saline containing fresh isolated colonies of *B. fragilis* BCT06 (BCT06), *E. faecalis* ENT03 (ENT03) or *L. salivarius* LCT01 (LCT01) at 7.26, 7.11 and 5.35 log CFU/mL, respectively, as confirmed by culture of the remaining inoculant. Eggs were placed in clear sterilized individual hatching jars until hatch. At time of hatch, eight birds in each group were killed by cervical dislocation. Body weight, yolk sac weight, organ weight (gizzard, bursa and liver), and length of small intestinal segments were recorded. Cecum contents and gizzard contents were collected for culture and bacterial enumeration using agar and conditions as described in

section 4.3.1 above. Tissue samples were collected from ileum and snap frozen in liquid nitrogen followed by storage at -80 °C for gene expression analysis.

4.3.4   Experiment 4-2 *In ovo* inoculation of *C. butyricum* CLS01

A second experiment was conducted identical to experiment 4-1 to assess *in ovo* inoculation of *C. butyricum* CLS01. Embryos (n=20) at E17 were randomly assigned to 2 treatment groups balanced for egg weight at E0 (10 eggs per group). At the end of day 17 of incubation, embryos in one treatment group were inoculated with 100µL of sterile saline by *in ovo* inoculation and designated as the germ-free (GF2) group. The embryos in the remaining group were inoculated with 100µL of saline containing freshly harvested *C. butyricum* CLS01 cells at 5.16 log CFU/mL as confirmed by culture of remaining inoculant post injection. Incubation in sterile jars, euthanasia and sampling were performed as in Experiment 4-1.

4.3.5   Experiment 4-3: *In ovo* inoculation of live and heat killed *E. coli*

A preliminary experiment (results not shown) demonstrated that less than 5% of embryos inoculated with live ECL01 at E17 hatched. To further investigate this, fresh cultures of *E. coli* ECL01 and *E. coli* ECL02 were prepared for inoculation by method described in 4.3.2 above and stored on ice. An aliquot of *E. coli* ECL01 was heat treated at 80 °C for 30 min in water bath and confirmed non-viable by culture. The inoculants were warmed to 37 °C before *in ovo* inoculation to avoid giving cold shock to the embryo. A total of 76 chicken embryos at E17 were randomly assigned to one of four treatments balanced for weight at E0 (19 eggs per treatment). Sterile saline (100µl) was inoculated to Control (CON) group whereas the remaining groups were inoculated 100µl of 1.1×10^8 CFU of live ECL01, heat killed ECL01 (hkECL01) or 1.2×10^8 CFU of live ECL02. After the inoculation, all eggs were incubated in HEPA-filtered sterilized hatcher until the end of 22 days of incubation. Body weight of hatched birds was measured.

4.3.6 Experiment 4-4: *E. coli* ECL01 inoculation dose

A total of 70 chicken embryos at E17 were randomly assigned to five treatments balanced by weight at E0 (14 eggs per treatment). Sterile saline (100µl) was inoculated into the Control group (CON) amniotic fluid. The four remaining treatment groups were inoculated with 100µl saline containing *E. coli* ECL01 such that each embryo was inoculated with 1.0×10^2 (10E2), 1.0×10^4 (10E4), 1.0×10^6 (10E6), or 1.0×10^8 (10E8) CFU. After the *in ovo* inoculation, all eggs were incubated in a HEPA-filtered sterilized hatcher until the end of 22 days of incubation. Body weight of hatched birds was measured at the end of 21 days of incubation and at the end of 22 days of incubation.

4.3.7 Experiment 4-5: Timing of *E. coli* ECL01 *in ovo* inoculation

A total of 70 chicken embryos at E17 were randomly assigned to seven treatments balanced by weight at E0 (10 eggs per treatment). All treatment groups were placed in HEPA filtered sterilized hatcher until *in ovo* inoculation was performed at E17, E18, E19 and E20. At each inoculation day, with the exception of E20, a control group (CON) was inoculated with sterile saline (100µl). Treatment embryos received 100µl of *E. coli* ECL01 (1.0×10^8 CFU per bird) at each incubation time point. After the inoculation, all eggs were immediately returned to the HEPA filtered hatcher until the end of 22 days of incubation. Body weight of hatched birds was measured at the end of 21 days of incubation and at the end of 22 days of incubation.

4.3.8 Experiment 4-6: Effect of *E. coli* ECL01 *in ovo* inoculation at E17 on chick physiology at pipping.

Chicken embryos (n=40) at E17 were randomly assigned to four experimental groups balanced by weight at E0 (10 eggs per treatment). Sterile saline (100 µL) was inoculated *in ovo* in two of four experimental groups at the end of day 17 of incubation whereas the other two groups received 1×10^2 CFU/bird of *E. coli* ECL01 at E17. After the inoculation, all eggs were placed in environmentally controlled individual HEPA-filtered sterile jars until start of pipping was confirmed by observation of a crack or small hole on the shell surface of egg. Time of pipping was

recorded for each egg and the embryo was killed by cervical dislocation. Euthanasia and sample collection was as in Experiment 4-1.

4.3.9   Contamination check and bacterial enumeration

To confirm the microbial status in intestine of pipping or newly-hatched chicks, 50 µL of fresh gizzard and cecal contents were directly spread on TS agar and incubated at 37 °C for 24 hours under aerobic conditions and on BL agar incubated at 37 °C for 48 hours under anaerobic conditions (Anaeropack System, Mitsubishi Gas Chemical Company, INC., Tokyo, Japan). To enumerate inoculated strains, fresh contents were diluted (1:10 w/v) in 0.1% peptone water and 50 µL spread on selective media and cultures as described at 4.3.1 followed by enumeration of colonies normalized for weight of contents applied to the plate. All cultures were incubated at 37 ºC for 24-48 hours.

4.3.10   RNA extraction from ileum tissue and quantitative PCR gene expression analysis

Total RNA was extracted by RNAeasy Mini Kit (Qiagen, Mississauga, ON) from 30 mg of mixed frozen ileal tissue, ground by mortar and pestle under liquid nitrogen incorporating RNase-Free DNase (Qiagen, Mississauga, ON) to remove genomic DNA from the sample. After RNA concentration was quantified by NanoDrop spectrophotometer (Thermo Scientific, Wilmington DE) as described at Chapter 3, the total RNA (5 µg) was reverse transcribed with random primers using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA, USA). Quantitative real-time PCR (qPCR) was conducted using CFX96 real-time PCR detection system on a C1000 thermal cycler (Bio-Rad Laboratories, Inc., California, U.S.A.). The reaction included 2.0 µL of a 1/100 dilution of cDNA mixed with 1.0 µL of 10 µM forward and reverse primer (Table 3.1), 10.0 µL of SsoFastTM EvaGreen® Supermix (Bio-Rad Laboratories, Inc., California, USA) and 6.0 µL of nuclease-free water. Reaction conditions were 95 °C for 2 min followed by 40 cycles at 95 °C for 5 seconds and annealing at 53-61 °C for 5 seconds (see Table 3.1). A melting curve analysis was conducted at the completion of amplification cycles by increasing temperature from 65 °C to 95 °C in 0.5° C increments for 5 seconds each. To prepare standard curves, 1.0 µL of a 5-fold dilution series of pooled cDNA in triplicate was generated and assigned an arbitrary concentrations value consistent with the dilution.

All standard curves demonstrated a PCR efficiency between 98.8% and 117%. Samples were analyzed in duplicate and duplicates with greater than 10% coefficient of variation were repeated. Four housekeeping genes, *GAPDH, RPL30, SDHA* and *TFRC* (Table 3.2) were used to normalize expression of genes of interest for Experiment 4-1 and Experiment 4-6. In Experiment 4-2, *GAPDH* and *PRL30* were used to normalize expression of genes of interest as abundance of *SDHA* and *TFRC* transcripts were significantly affected by CLS01 inoculation.

4.3.11 Statistical analysis

All results were expressed as the mean ± standard error. Statistical analysis was performed using SAS for Windows version 9.4 (SAS Institute Inc., Cary, NC, USA). Percent hatched, mortality and pipped data were compared with appropriate control group using Proc FREQ procedure with FISHER option. For all other continuous variables, the Proc Mixed procedure with treatment (inoculant) as the source of variation and using bird as the experimental unit.

Where the effect of inoculant was significant, Dunnett's post hoc means separation test was performed to differentiate means. Normal distribution of each data set was confirmed using Proc UNIVARIATE with NORMAL option prior for testing main effects. For all tests, a level of 0.05 was used to determine statistical differences and a level of $0.10 \geq P \geq 0.05$ was indicated as a trend.

4.4   Results

4.4.1   Experiment 4-1 and 4-2 (*In ovo* inoculation of *B. fragilis* BCT06, *E. faecalis* ENT03, *L. salivarius* LCT01, or *C. butyricum* CLS01)

4.4.1.1   Bacterial colonization

No bacterial growth was confirmed by culture under aerobic and anaerobic conditions, of contents from gizzard and cecum of GF chicks for both experiments. No aerobic bacterial growth was confirmed on culture of contents from chicks inoculated with BCT06, LCT01 and CLS01 and no anaerobic bacterial growth was observed in gut contents from ENT03 treatment. Where bacterial growth occurred, colony morphology was uniform and consistent with expected morphology on both non-selective and selective media. Mean number of the inoculated strains in

gizzard and cecal contents at hatch are given in Table 4.1. Colonization of ENT03 and LCT01 were confirmed in the cecum and gizzard of all birds with the highest counts observed in the cecum. For the BCT06 group, *B. fragilis* was cultured in gizzard and cecum of only 5 of 8 birds sampled. Furthermore, the number of *B. fragilis* in contents from positive birds was highly variable. *Clostridium butyricum* CLS01 was cultured from a single cecum obtained from one bird.

Table 4.1 Mean (± SE) number (log CFU/g) of *B. fragilis* BCT06, *E. faecalis* ENT03, *L. salivarius* LCT01 and *C. butyricum* CLS01 in gizzard and cecum contents at hatch.

Treatment	Detected strain	Gizzard (log CFU/g)[1]		Cecum (log CFU/g)[1]	
BCT06	*B. fragilis* BCT06	5.52 ± 0.67	(5/8)	8.29 ± 1.32	(5/8)
ENT03	*E. faecalis* ENT03	6.21 ± 0.32	(8/8)	9.79 ± 0.12	(8/8)
LCT01	*L. salivarius* LCT01	5.40 ± 0.41	(8/8)	6.44 ± 0.46	(8/8)
CLS01	*C. butyricum* CLS01	ND[2]	(0/8)	2.9	(1/8)

[1]Mean ± S.E. (Number of samples: Detected / Tested),
[2] ND: Not Detected, Detection limit was 2.0 CFU log per gram of contents.

4.4.1.2   Incubation time and percent hatchability

Total hatchability was 93% in Experiment 4-1 and 100% in Experiment 4-2. Three eggs, one egg in each of GF1, ENT03 and BCT06 groups did not finish hatching by 525 hours of incubation (Figure 4.1, Figure 4.2). The unhatched egg in ENT03 treatment contained a fully developed embryo which likely died around E20. Remaining unhatched eggs in GF1 and BCT06 contained fully developed live chicks without obvious defect that were still pipping at 525 hours of incubation. Mean incubation time (not shown) was not significantly different among the treatment groups although a numerical increase in mean time to hatch compared to GF1 was observed in *E. faecalis* ENT03, *B. fragilis* BCT06 and *L. salivarius* LCT01 groups. Indeed, the first birds in the ENT03 group started hatching 19 hours later than birds in the GF1 group.
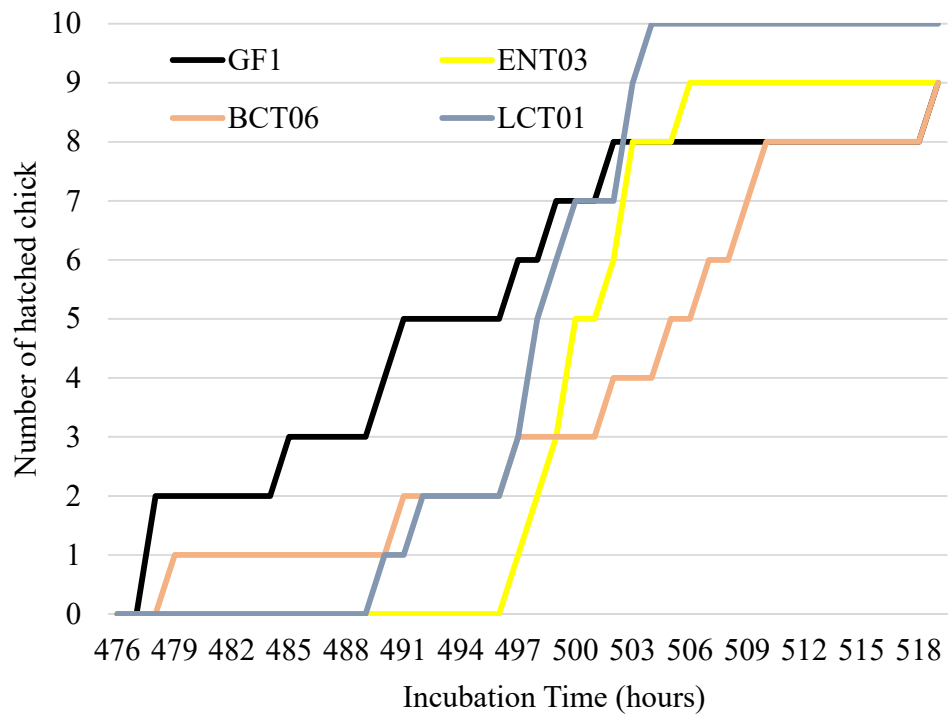
Figure 4.1 Incubation time prior to hatch for germ-free (GF) embryos and embryos inoculated with *E. faecalis* ENT03 (ENT03), *B. fragilis* BCT06 (BCT06) or *L. salivarius* LCT01 (LCT01).
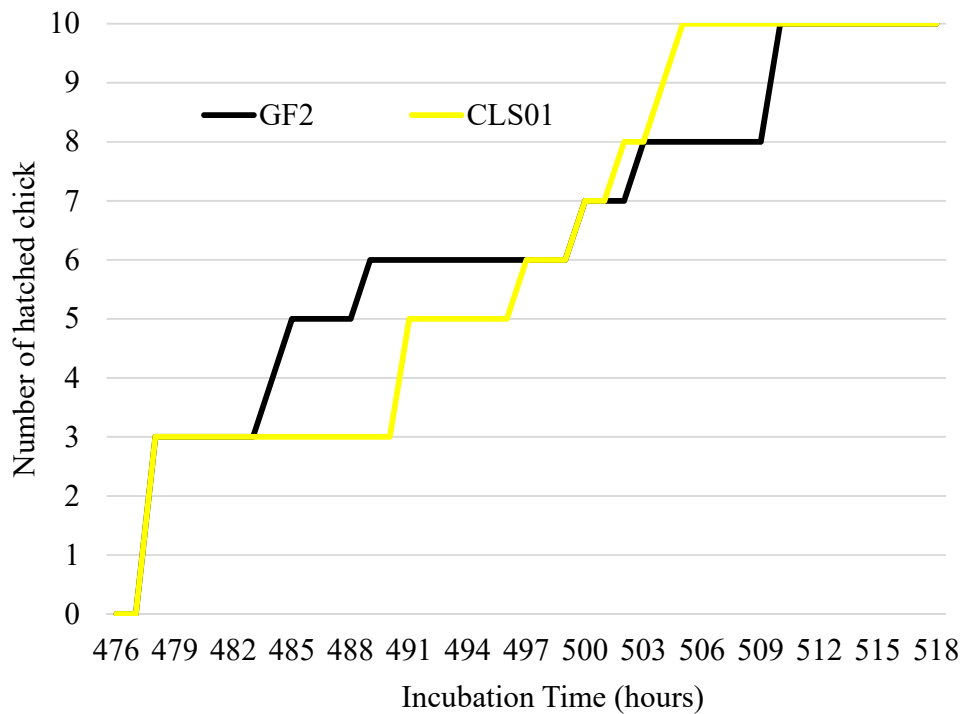
Figure 4.2 Incubation time prior to hatch for germ-free (GF) embryos and embryos inoculated with *C. butyricum* CLS01 (CLS01).

4.4.1.3 Body weight, organ mass and length

No significant effect of *B. fragilis* BCT06, *E. faecalis* ENT03, *L. salivarius* LCT01 and *C. butyricum* CLS01 *in ovo* inoculation was observed on body weight, yolk sac weight and yolk sac-free body weight among treatment groups (Table 4.2). Relative weight of gizzard, bursa and liver at hatch is shown in Table 4.3. *In ovo* inoculation of *B. fragilis* BCT06, *E. faecalis* ENT03, *L. salivarius* LCT01 and *C. butyricum* CLS01 did not affect relative organ weight. No differences were found in relative small intestinal length at hatch between germ-free (GF) birds and *B. fragilis* BCT06, *E. faecalis* ENT03, *L. salivarius* LCT01 and *C. butyricum* CLS01 inoculated birds (Table 4.4).

Table 4.2 Mean (± SE) for egg weight (EW) at the start of incubation (E0) and at day 17 of incubation (E17) and mean body weight (BW), yolk sac weight and yolk sac-free body weight for Germ-free (GF) and *B. fragilis* BCT06, *E. faecalis* ENT03, *L. salivarius* LCT01 and *C. butyricum* CLS01 inoculated birds at hatch.

| Treatment | E0 EW (g) | E17 EW (g) | At sampling | | |
			BW (g)	Yolk sac weight (g)	Yolk sac-free body weight (g)
Experiment 4-1					
GF 1	60.5 ± 0.72	53.3 ± 0.77	42.7 ± 0.77	6.09 ± 0.26	36.6 ± 0.76
ENT03	60.5 ± 0.70	52.7 ± 0.63	42.4 ± 0.52	5.89 ± 0.26	36.6 ± 0.39
BCT06	60.6 ± 0.61	53.2 ± 0.62	43.3 ± 0.75	5.68 ± 0.30	37.6 ± 0.68
LCT01	60.5 ± 0.73	52.7 ± 0.66	42.9 ± 0.55	5.70 ± 0.27	37.2 ± 0.55
p value	*0.9988*	*0.9007*	*0.8293*	*0.6972*	*0.5711*
Experiment 4-2					
GF 2	60.5 ± 0.37	55.0 ± 0.41	45.0 ± 0.68	6.57 ± 0.29	38.4 ± 0.75
CLS01	60.5 ± 0.35	55.0 ± 0.34	45.3 ± 0.56	6.58 ± 0.25	38.7 ± 0.57
p value	*0.9251*	*0.9838*	*0.7218*	*0.9629*	*0.7526*

Table 4.3 Mean (±SE) relative weight (g/100g of BW) of gizzard, bursa of Fabricius and liver in Germ-free and *B. fragilis* BCT06, *E. faecalis* ENT03, *L. salivarius* LCT01 and *C. butyricum* CLS01 inoculated birds at hatch.

Treatment	Gizzard (g/100g BW)	Bursa (g/100g BW)	Liver (g/100g BW)
Experiment 4-1			
GF 1	3.05 ± 0.13	0.14 ± 0.01	2.08 ± 0.09
ENT03	3.03 ± 0.08	0.16 ± 0.01	2.19 ± 0.06
BCT06	2.95 ± 0.15	0.18 ± 0.02	2.15 ± 0.06
LCT01	3.01 ± 0.08	0.15 ± 0.01	1.99 ± 0.04
p value	*0.9396*	*0.1979*	*0.1357*
Experiment 4-2			
GF 2	2.75 ± 0.10	0.14 ± 0.01	1.99 ± 0.06
CLS01	2.80 ± 0.08	0.14 ± 0.01	2.00 ± 0.07
p value	*0.6803*	*0.8152*	*0.9362*

Table 4.4 Mean (±SE) relative small intestinal segment length (mm / 100g BW) cum) in Germ-free and *B. fragilis* BCT06, *E. faecalis* ENT03, *L. salivarius* LCT01 and *C. butyricum* CLS01 inoculated birds at hatch.

Treatment	Duodenum (mm)	Jejunum (mm)	Ileum (mm)	Length of total intestine (mm)	Cecum (mm)
Experiment 4-1					
GF 1	127 ± 4.4	262 ± 6.1	242 ± 4.2	631 ± 11.5	69 ± 2.2
ENT03	133 ± 4.1	272 ± 9.3	249 ± 11.0	654 ± 18.3	68 ± 1.3
BCT06	122 ± 2.9	254 ± 10.6	237 ± 8.9	613 ± 18.3	66 ± 2.4
LCT01	120 ± 5.1	259 ± 9.9	264 ± 10.2	652 ± 23.6	67 ± 0.7
p value	*0.3657*	*0.5649*	*0.1850*	*0.3759*	*0.6733*
Experiment 4-2					
GF 2	112 ± 3.1	229 ± 10.1	249 ± 10.3	591 ± 20.7	67 ± 1.5
CLS01	115 ± 1.8	237 ± 6.3	241 ± 5.9	593 ± 11.5	60 ± 1.5
p value	*0.4938*	*0.5474*	*0.4899*	*0.9493*	*0.7862*

4.4.1.4   Ileal gene expression

In contrast to gross parameters, bacterial inoculation modified specific transcript abundance in ileal tissue in a manner unique to each inoculated bacterial species (Table 4.5). Inoculation with *E. faecalis* ENT03 induced marked changes on gene expression in ileal tissue including increased ($P < 0.05$) transcript abundance of *IL-8* and Toll-like receptors (*TLR2* and *TLR4*). Further, ENT03 increased ($P < 0.05$) abundance of *CDN1* in contrast to reducing ($P < 0.05$) CDN5 abundance and tended ($P < 0.10$) to increase *MUC2* expression. Expression of *PepT-1* in ENT03-inoculated birds was also significantly higher ($P < 0.05$) than in the GF treatment. Similar to ENT03, BCT06-inoculated birds demonstrated increased ($P < 0.05$) abundance of the proinflammatory cytokine *IL-6* and *TLR4*. A numerical increase in *IL-8* and *TLR2* was observed in the ileum of BCT06 birds. As for ENT03, a lower ($P < 0.05$) abundance of *CDN5* expression was observed following BCT06 treatment. Comparatively few changes in gene expression in the ileum were observed following inoculation with LCT01 saving increased *CLDN4* and a trend ($P < 0.10$) for increased *MUC2* expression. Only upregulation ($P < 0.05$) of *CDN5* was found in CLS01 compared to downregulation of this transcript observed for ENT03 and BCT06. Although only one bird was confirmed to be colonized with CLS01 at hatch, a trend towards downregulation ($P < 0.10$) of *IL-8* and upregulation ($P < 0.01$) of *CDN5* was observed.

Table 4.5 Mean (±SE) fold change in expression of genes in the ileum for Germ-Free (GF) and *B. fragilis* BCT06, *E. faecalis* ENT03, *L. salivarius* LCT01 and *C. butyricum* CLS01 inoculated birds at hatch.

Treatment	Experiment 4-1					Experiment 4-2		
	GF1	BCT06	ENT03	LCT01	*P value*	GF2	CLS01	*P value*
IL-6	1.0 ± 0.21	5.8 ± 2.10[**]	1.5 ± 0.69	1.3 ± 0.30	*0.0176*	1.0 ± 0.38	1.9 ± 1.12	*0.4720*
IL-8	1.0 ± 0.16	1.9 ± 0.53	11.6 ± 4.54[**]	1.4 ± 0.37	*0.0091*	1.0 ± 0.18	0.6 ± 0.12	*0.0879*
TLR2	1.0 ± 0.19	2.1 ± 0.56	2.9 ± 0.63[**]	0.8 ± 0.29	*0.0129*	1.0 ± 0.18	0.9 ± 0.18	*0.6963*
TLR4	1.0 ± 0.14	2.3 ± 0.60[*]	2.4 ± 0.37[**]	0.9 ± 0.14	*0.0105*	1.0 ± 0.14	1.0 ± 0.14	*0.5210*
APN	1.0 ± 0.13	0.8 ± 0.08	1.1 ± 0.18	1.1 ± 0.10	*0.2359*	1.0 ± 0.13	1.1 ± 0.10	*0.5680*
SGLT-1	1.0 ± 0.15	0.7 ± 0.11	1.3 ± 0.17	1.0 ± 0.21	*0.1081*	1.0 ± 0.12	1.3 ± 0.12	*0.1182*
PepT-1	1.0 ± 0.15	0.6 ± 0.11	1.9 ± 0.23[**]	0.9 ± 0.11	*0.0001*	1.0 ± 0.15	1.3 ± 0.15	*0.1738*
cGH	1.0 ± 0.09	1.6 ± 0.26	1.4 ± 0.20	1.4 ± 0.24	*0.1873*	1.0 ± 0.18	1.1 ± 0.15	*0.5528*
IGF-I	1.0 ± 0.05	0.7 ± 0.10	0.8 ± 0.12	0.8 ± 0.16	*0.3163*	1.0 ± 0.07	1.0 ± 0.11	*0.8257*
CDN1	1.0 ± 0.08	1.0 ± 0.13	1.8 ± 0.39[**]	1.0 ± 0.11	*0.0441*	1.0 ± 0.11	1.0 ± 0.12	*0.7774*
CDN4	1.0 ± 0.04	0.8 ± 0.09	1.0 ± 0.15	1.5 ± 0.22[**]	*0.0112*	1.0 ± 0.08	0.9 ± 0.18	*0.6541*
CDN5	1.0 ± 0.08	0.6 ± 0.07[**]	0.5 ± 0.09[**]	1.2 ± 0.12	*0.0001*	1.0 ± 0.07	1.5 ± 0.13	*0.0035*
PCNA	1.0 ± 0.09	0.8 ± 0.10	1.5 ± 0.26[**]	1.1 ± 0.41	*0.0028*	1.0 ± 0.11	1.1 ± 0.10	*0.7292*
MUC2	1.0 ± 0.11	0.9 ± 0.10	1.7 ± 0.26[*]	1.7 ± 0.41[*]	*0.0482*	1.0 ± 0.10	1.0 ± 0.07	*0.9861*

[**] Significantly ($P < 0.05$) different from GF control by two-sided Dunnett's Multiple comparison.

[*] Trend to differ ($0.05 < P < 0.10$) from GF control by two-sided Dunnett's Multiple comparison.

4.4.2 Experiment 4-3: Effect of live *E. coli* and heat killed *E. coli in ovo* inoculation on hatching ratio

For control, saline-inoculated eggs no mortality was observed and 100% of the inoculated eggs were observed pipping and fully hatched by E22 (Figure 4.3). Inoculation at E17 with live ECL01 or ECL02 significantly reduced the percent of eggs pipping ($P < 0.001$). Only 16% of ECL01 inoculated eggs hatched and 0% of eggs inoculated with ECLO2 hatched. At the end of E22, 21% of ECL01 birds, and 89% of ECL02 inoculated birds were dead. Pipping rate, hatch rate and mortality were unaffected when heat killed ECL01 was inoculated at E17.

Mean body weight at hatch is shown in Table 4.6. Due to high mortality rates, BW for eggs inoculated with live ECL02 and ECL01 were excluded for analysis. Inoculation with heat killed ECL01 did not affect body weight at hatch.
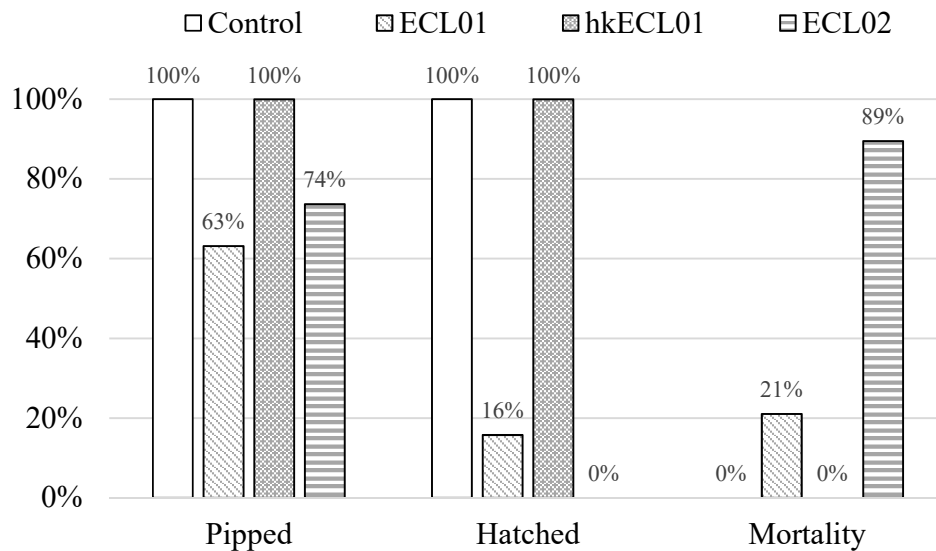
Figure 4.3 Percent of pipped eggs, hatched eggs and percent mortality following 22 d incubation when saline (Control), live *E. coli* ECL01 (ECL01), heat killed *E. coli* ECL01-(hkECL01) or live *E. coli* ECL02 (ECL02) were inoculated at E17 of incubation.

Table 4.6 Mean (± SE) egg weight (EW) at the start of incubation (E0) and at day 17 of incubation (E17), plus mean *(± SE)* body weight (BW) and number of birds alive at end of 22 d incubation for embryos inoculated at E17 with saline (Control) , *E. coli* ECL01 (ECL01), heat killed *E. coli* ECL01 (hkECL01) or *E. coli* ECL02 (ECL02).

Treatment	E0 EW (g)	E17 EW (g)	BW at Hatch[1] (g)	Mortality (Alive/Total)
Control	60.6 ± 0.61	53.6 ± 0.61	42.8 ± 0.52	(19/19)
ECL01	60.6 ± 0.60	53.7 ± 0.57	39.9 ± 1.23	(3/19)
hkECL01	60.6 ± 0.59	53.4 ± 0.57	41.9 ± 0.56	(19/19)
ECL02	60.6 ± 0.58	53.5 ± 0.55	-	(0/19)
p value	*0.9999*	*0.9908*	*0.1201*	

[1] *E. coli* ECL01 and ECL02 groups were removed from statistical analysis due to limited sample numbers.

4.4.3 Experiment 4-4: Effect of different dosage of *E. coli* ECL01 *in ovo* inoculation on hatching ratio

A summary of the dose-response effect of *E. coli* ECL01 *in ovo* inoculation at E17 on hatching is given in Figure 4.4. All birds hatched in the Control group at the end of 22 days of incubation with 0% mortality. The proportion of pipped eggs in the *E. coli* ECL01-inoculated group ranged from 71-86%, however, no birds inoculated with *E. coli* ECL01 *in ovo* hatched excepting a single embryo (7%) given 100 CFU. At the end of day 22, mortality rate was 0% for control birds and 7-21% (including hatched and birds yet to hatch) for birds given *E. coli* ECL01. The majority of *E. coli*-treated eggs started the hatching process (confirmed pipping), and although 80% or more were alive at day 22, the inner shell membrane had already become dry and no further progress in hatching was expected.

Figure 4.4 Percent of pipped eggs, hatched eggs and mortality after 22 d incubation for control (CON) bireds and birds given four different dosages of *E. coli* ECL01 inoculated embryo at E17.

4.4.4 Experiment 4-5: Effect of different timing of *E. coli* ECL01 *in ovo* inoculation on hatching ratio

A summary of the effect of timing of *E. coli* ECL01 *in ovo* inoculation on hatching is given in Figure 4.5. No difference in percent of pipped birds at the end of 22 day of incubation for embryos inoculated with saline or *E. coli* ECL01 was observed for any of the inoculation time points studied. All control birds successfully hatched at the end of 22 day of inoculation. However, an adverse effect of *E. coli* ECL01 *in ovo* inoculation on hatchability was confirmed for *in ovo* inoculation at E17 ($P < 0.0001$), E18 ($P < 0.0001$) and E19 ($P < 0.0001$). In contrast, all embryos inoculated with *E. coli* ECL01 at E20 hatched. A significantly higher ($P < 0.001$) mortality (80-100% mortality) was observed following *E. coli* inoculation between E17 and E19 compared to control birds (0% mortality). However, all birds inoculated at E20 with *E. coli* ECL01 survived.
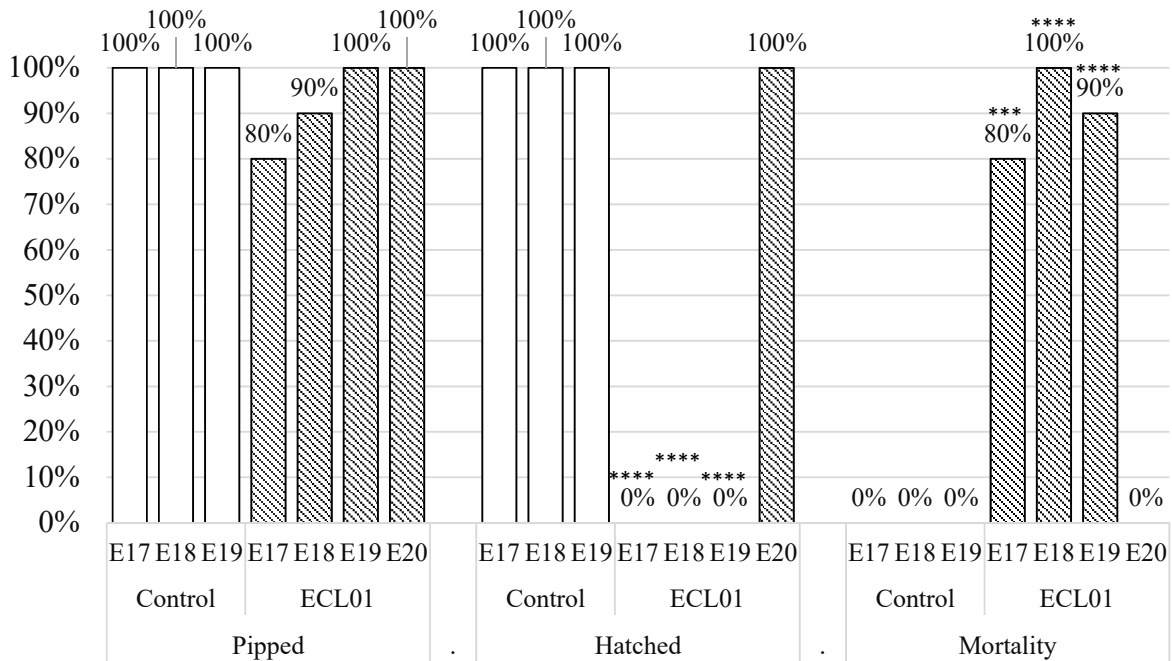
*** $P < 0.001$, **** $P < 0.0001$ Comparison with Control group in each different inoculation day.

Figure 4.5 Percent of pipped eggs, hatched eggs, and mortality at end of 22 day of incubation for Control birds (CON) and birds inoculated with *E. coli* ECL01 (ECL01) at E17, E18, E19 and E20.

4.4.5 Experiment 4-6: Effect of *E. coli* ECL01 *in ovo* inoculation at E17 on chick physiology at pipping.

No anaerobic bacterial growth was found in gizzard and cecal contents for birds in GF and *E. coli* ECL01 inoculated groups. For birds in GF group no aerobic bacterial growth was observed however *E. coli* ECL01 inoculation yielded aerobic bacteria on culture of gizzard (7.19 ± 0.18 CFU/g) and cecal contents (10.1 ± 0.16 CFU/g). Growth of *E. coli* ECL01 in intestinal contents occurred to a high density, especially in the cecum.

All birds in GF group were observed pipping and hatched alive (Figure 4.6). However, *in ovo* inoculation of *E. coli* ECL01 at E17 significantly decreased ($P < 0.05$) the percentage of pipped

eggs to 60%. Two (10%) of the *E. coli* ECL01-inoculated birds that failed to pip were dead after day 22 of incubation.

Progress of pipping is shown at Figure 4.6. The first sign of pipping was confirmed at 475 hours of incubation (19 days and 16 hours) in the *E. coli* ECL01-inoculated group and 476 hours in the germ-free group. The mean time (h:min) to start of pipping was earlier ($P < 0.01$) for the 12 of 20 eggs in the *E. coli* group that started pipping (482:19) compared to birds in GF group (492:30)
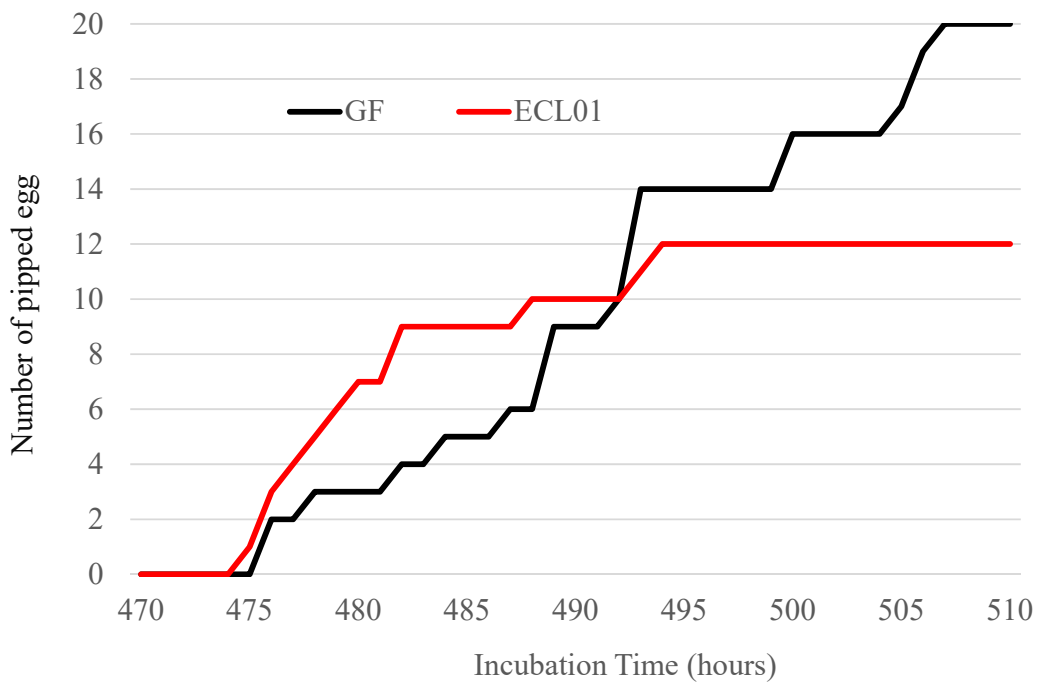
Figure 4.6 Incubation time (hours) to pipping for germ-free birds (GF) and *E. coli* ECL01 inoculated birds (ECL01) at E17.

4.4.5.1   Body weight, organ mass and length

Body weight at the start of pipping was significantly decreased ($P<0.0001$) in *E. coli* ECL01-inoculated birds compared with GF birds (Table 4.7). *E. coli* ECL01 *in ovo* inoculation significantly increased ($P < 0.001$) remaining yolk sac weight at the start of pipping and the larger yolk sac accounted for a significantly smaller ($P < 0.0001$) yolk sac-free body weight in *E. coli* ECL01 inoculated birds compared with birds in GF group.

Table 4.7 Mean (± SE) for egg weight (EW) at the start of incubation (E0) and at day 17 of incubation (E17) and mean body weight (BW), yolk sac weight and yolk sac-free body weight at the start of pipping for Germ-free birds and birds inoculated with *E.coli* ECL01 (ECL01) at E17.

Treatment	E0 EW (g)	E17 EW (g)	At sampling BW (g)	Yolk sac weight (g)	Yolk sac-free body weight (g)
GF	60.7 ± 0.29	55.0 ± 0.28	44.2 ± 0.49	7.41 ± 0.344	37.0 ± 0.60
ECL01	60.6 ± 0.26	55.0 ± 0.30	37.9 ± 0.39	9.05 ± 0.215	28.9 ± 0.46
p value	*0.9227*	*0.9118*	*0.0001*	*0.0004*	*0.0001*

In ovo inoculation with *E. coli* ECL01 tended to decrease ($P < 0.10$) relative gizzard weight, significantly increased ($P < 0.0001$) relative liver weight at the start of pipping without affect relative weight of bursa of Fabricius (Table 4.8).

Table 4.8 Mean (±SE) relative weight (g/100g of BW) of gizzard, Bursa of Fabricius and liver at the start of pipping in Germ-free (GF) and *E. coli* ECL01 (ECL01) birds inoculated at E17.

Treatment	Gizzard (g/100g BW)	Bursa (g/100g BW)	Liver (g/100g BW)
GF	2.59 ± 0.074	0.11 ± 0.007	1.69 ± 0.018
ECL01	2.40 ± 0.054	0.12 ± 0.007	2.13 ± 0.069
p value	*0.0689*	*0.2509*	*0.0001*

No effect of *E. coli* ECL01 *in ovo* inoculation was observed on relative small intestinal segment length, however, ECL01 reduced ($P < 0.05$) the length of the cecum (Table 4.9). Interestingly, development of mucosal edema was grossly observable in the ileum of *E. coli* ECL01- inoculated birds (Figure 4.7).

Table 4.9 Mean (±SE) relative small intestinal segment length (mm / 100g BW) at pipping in Germ-free (GF) and *E. coli* ECL01 (ECL01) birds inoculated at E17.

Treatment	Duodenum	Jejunum	Ileum	Length of total small intestine	Cecum
			(mm / 100g of body weight)		
GF	49 ± 1.2	96 ± 2.6	97 ± 2.8	243 ± 6.0	28 ± 0.7
ECL01	50 ± 1.5	94 ± 1.6	93 ± 1.8	237 ± 3.6	26 ± 0.7
p value	*0.7098*	*0.4703*	*0.2734*	*0.4605*	*0.0355*
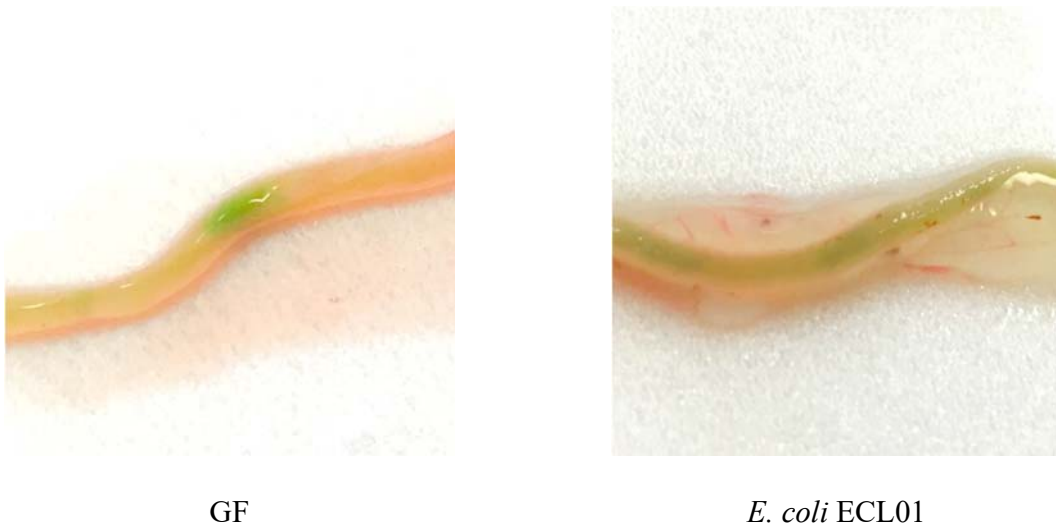
GF   *E. coli* ECL01

Figure 4.7 Picture of ileum for germ-free (GF) bird and *E. coli* ECL01 inoculated bird.

4.4.5.2   Gene expression analysis in the ileum

A summary of mean fold change in expression of genes of interest in the ileum for GF birds and *E. coli* ECL01-inoculated birds is given in Table 4.10. A marked effect of *E. coli* ECL01 *in ovo* inoculation was observed in abundance of pro-inflammatory cytokines. Significant upregulation ($P < 0.0001$) was observed for *IL-1*, *IL-6* ($P < 0.001$) and *IL-8* ($P < 0.0001$) gene expression in *E. coli* ECL01-inoculated bird compared with GF birds. Expression of *TLR2* in ECl01 was also significantly higher ($P < 0.001$) than in the GF group, however no significant effect was observed in *TLR4*. Significantly higher ($P < 0.05$) expression of *APN* in ECL01-inoculated birds was also observed. For tight junction proteins, significant upregulation ($P < 0.001$) in *CDN1* and downregulation ($P < 0.0001$) in *CDN5* transcripts was observed for the ECL01 group. Abundance of *CDN4* transcripts in *E. coli*-inoculated birds also tended to be lower ($P < 0.10$) than in GF birds. Finally, downregulation ($P < 0.0001$) in *IGF-I* and *PCNA* for *E. coli* ECL01-inoculated birds was also observed.

Table 4.10 Mean (± SE) fold change in expression of genes in the ileum at pipping for Germ-Free (GF) and *E. coli* ECL01 birds inoculated at E17.

Treatment	Germ-free	*E. coli* ECL01	*P value*
IL-1	1.0 ± 0.12	71.5 ± 15.01	*0.0001*
IL-6	1.0 ± 0.13	3.1 ± 0.73	*0.0028*
IL-8	1.0 ± 0.15	47.7 ± 5.98	*0.0001*
TLR2	1.0 ± 0.09	2.0 ± 0.24	*0.0002*
TLR4	1.0 ± 0.09	1.3 ± 0.23	*0.2346*
APN	1.0 ± 0.07	1.3 ± 0.12	*0.0272*
SGLT-1	1.0 ± 0.12	0.7 ± 0.08	*0.1121*
PepT-1	1.0 ± 0.09	1.1 ± 0.27	*0.6668*
cGH	1.0 ± 0.09	1.1 ± 0.14	*0.5007*
IGF-I	1.0 ± 0.11	0.3 ± 0.02	*0.0001*
CDN1	1.0 ± 0.08	2.4 ± 0.36	*0.0002*
CDN4	1.0 ± 0.09	0.7 ± 0.11	*0.0601*
CDN5	1.0 ± 0.05	0.6 ± 0.06	*0.0001*
PCNA	1.0 ± 0.04	0.6 ± 0.05	*0.0001*
MUC2	1.0 ± 0.11	1.0 ± 0.29	*0.1619*

4.5   Discussion

We utilized the *in ovo* inoculation model to assess and compare the independent impact on chick development, of five bacterial isolates representing major taxonomic families in the gastrointestinal tract of chickens. The approach identified markedly different colonization patterns for each species associated with variation in impact on incubation time, mortality, body weight, gross organ weights and expression of genes in the intestine associated with intestinal growth, barrier and digestive function. Importantly, when chicks were hatched in sterilized individual isolated containers, no evidence of pre-hatch microbial colonization was observed consistent with the previous work of our lab and others (Muramatsu et al., 1994). The confirmation of a germ-free intestine at hatch and the mono-association of this environment by *in ovo* inoculation of a single isolate, are important findings reaffirming the potential of the gnotobiotic animal model as recent reports present evidence that gut microbial colonization begins in the embryo (Akinyemi et al., 2020; Lee et al., 2019). Clearly, bacteria identified using molecular tools in these studies do not normally contribute to gastrointestinal microbial colonization.

An interesting finding reported here was the variation in colonization patterns observed among the five different bacterial isolates following *in ovo* inoculation. The *E. faecalis* (ENT03)

and *E. coli* (ECL01) isolates colonized all embryos reaching densities in mono-association at levels approaching that of complex communities observed in adult birds of 9-10 log CFU/g cecal contents at the time of hatch. The *L. salivarius* (LCT01) isolate also colonized all embryos but surprisingly only attained a colonization density in mono-association of about 6 log CFU/g. The *B. fragilis* (BCT06) isolate was recovered at hatch from only 5 of 8 birds and perhaps most surprising colonized to approximately 10 log CFU/g cecal contents in 3 of the 5 birds where BCT06 was recovered. The *C. butyricum* (CLS01) isolate was recovered in only a single bird at a relatively low (about 3 log CFU/g) colonization density.

Variation in colonization success and/or density following bacterial inoculation in ex-germ-free animals is not unexpected. For example, Phillips et al. (1962) reported *Alcaligenes faecalis* ATCC9220 failed to grow in the gut of germ-free chicks. Similarly, *Bacteroides melaninogenicus* was not recovered from ex-germ-free mice even after a secondary inoculation 60 days after the first inoculation (Gibbons et al., 1964). Baba et al. (1991) reported that a *Bacteroides sp*. did not always colonize when inoculated in 2-day-old ex-germ-free chicks. Popoff et al. (1985) successfully mono-associated ex-germ-free chickens at 7 d of age with two *C. butyricum* strains that reached about 8.0 log CFU/g four weeks after the inoculation. Thus, *C. butyricum* seems to have the ability to colonize adult birds in mono-association. Variations in the colonization pattern observed here, despite the isolation of bacterial inoculants from the conventional chicken gastrointestinal tract, likely reflect the unique properties of the *in ovo* environment and the unique metabolic requirements of each bacterium.

We do not believe the failed colonization observed for BCT06 and CLS01 represent technical errors as nearly 200 eggs inoculated with other isolates were successfully colonized. Furthermore, live microorganisms were confirmed in our inoculant preparations immediately following *in ovo* inoculation. Oxygen level in the embryonic gut may be one of the key factors contributing to uneven colonization results. In the case of BCT06 and CLS01, both isolates represent anaerobic species and were the only bacterial isolates that failed to colonize all chicks, indicating they may be sensitive to high oxygen levels present in the otherwise sterile gut of the chicken embryo as compared to a gut lumen largely devoid of oxygen in conventional adult birds (Albenberg et al., 2014). Low luminal gut oxygen levels in adult birds are mediated by utilization of oxygen diffusing from the host tissues by aerobic and facultatively anaerobic bacterial community members (Crank and Gupta, 1972). Indeed, *E.coli* and *Enterococcus* spp., which

colonized *in ovo* to consistently high density in the current experiment, are known to have flexibility in their utilization of multiple electron acceptor systems to maximize a growth advantage in the environment (Espey, 2013). It is likely that for BCT06 and CLS01, were disadvantaged by the high oxygen environment initially encountered in the embryonic gut. It is likely that in some cases, immediately following inoculation when bacterial number were low, oxygen levels encountered had a sterilizing effect. However, it remains unclear why in some cases BCT06 was able to colonize to high density despite the oxygen levels encountered in a mono-associated environment devoid of oxygen utilizers such as *E. coli* and *Enterococcus* species.

Nutrient availability in the embryonic chicken gut may also have affected bacterial colonization success and density. The avian egg is a closed system and sufficient or essential nutrient for bacterial growth may not be available without nutrients supplied through feed consumption. In the case where Popoff et al. (1985) reported mono-association of two *C. butyricum* strains in ex-germ-free chickens, these birds were consuming a cereal-based diet formulated to meet the birds nutrient requirement and likely also providing unique nutrients unavailable in the embryonic gut and potentially supporting clostridial growth.

Finally, the unique essential or limiting nutrient for some bacteria may be supplied by the metabolic activity of other bacteria in the complex conventional gut microbial ecosystem. Microbial cross-feeding is well recognized in complex systems (Canon et al., 2020; Das et al., 2018), such as the use of lactate produced by *Lactobacillus plantarum* as a substrate for *Acetobacter pomorum* to produce and provide amino acids that are essential to *Lactobacillus plantarum* (Henriques et al., 2019). Most *Lactobacillus* spp. exhibit characteristic requirements for a number of amino acids (Morishita et al., 1981) unlike *E.coli* which can synthesize all 20 of the standard amino acids (Price et al., 2018). Indeed, a lack of availability of specific amino acids in the embryonic gut environment could explain the relatively low abundance of *L. salivarius* observed in the current study. Interestingly, *C. butyricum* was found to contain all the genes encoding enzymes responsible for the biosynthesis of all 20 amino acids (Storari et al., 2016). However, the biotin biosynthesis pathway was incomplete for *C. butyricum* (Himmi et al., 1999). These functional differences in metabolic capacity among the inoculant strains also likely played a major role in colonization success.

With the exception of *E. coli*, the remaining four representatives of the adult commensal microbiota, when administered *in ovo*, did not markedly affect chick hatchability and development

in terms of BW at hatch or any gross parameters. This is consistent with previous reports where *Lactobacillus* spp. (Alizadeh et al., 2020; Sivalingam et al., 2017; Triplett et al., 2018; Wilson et al., 2019a) and *Enterococcus* spp. (Beck et al., 2019; Castañeda et al., 2020; Castañeda et al., 2019) were inoculated. A lower yolk weight and longer intestine were reported with *Enterococcus faecium in ovo* inoculation (Castañeda et al., 2019) a result observed only as numerical differences here with ENT03. Majidi-Mosleh et al. (2017a) also reported that *in ovo* inoculation of *Enterococcus faecium or Pediococcus acidilactici*, a lactic acid bacterium in the same family as *Lactobacillus* spp., did not affect hatchability or body weight at 1 day of age. To our knowledge there are no previous reports on inoculation with *Bacteroides* and *Clostridium* spp.

Differential effects of each of the remaining four bacterial species were observed on analysis of ileal gene expression. *Enterococcus faecium* ENT03 inoculation *in ovo* appeared to induce the greatest local gene expression responses associated with a proinflammatory response characterized by a more than 10-fold increase in *IL-8* and significant increases in both *TLR2* and *TLR4*. *Enterococcus faecalis* is one of the most common *Enterococcus* sp. in the chicken gut (Muhammad Attiq et al., 2018). However, it is known as an opportunistic species (Jørgensen et al., 2017) and frequently associated with endocarditis in broiler chickens (Chahota et al., 2001; Prasath et al., 2017). *E. faecalis* is also known as a cause of urinary-tract infection in humans (Li et al., 2020) and in chicken is considered as one of the sources of the disease (Poulsen et al., 2012). Upregulation of IL-8 by *E. faecalis* infection was also confirmed in human gastric cancer cell model (Strickertsson et al., 2013). Interestingly, in contrast to observations made here, Majidi-Mosleh et al. (2017) reported no significant difference, compared to controls, in *MUC2* expression in the ileum of 21 d-old chick embryos inoculated with *E. faecium* or *P. acidilactici* at day 17 of incubation. Majidi-Mosleh et al. (2017a) did not measure proinflammatory gene expression limiting further comparison. Nevertheless, the response to *E. faecium* is likely highly strain specific.

Interestingly, we observed increased *MUC2* and *CDN4* expression in the ileum of *L. fermentum*-inoculated embryos without evidence of an inflammatory response. Assuming that increased expression of these genes contributes to enhanced barrier function, the ability to stimulate barrier function without a marked inflammatory response may support a probiotic role in birds.

Some evidence of a proinflammatory response to Bacteroides was also observed although to a far lesser extent than ENT03. The BCT06 response was characterized by an increase in IL-6 and TLR2 without any evidence of impact on transcripts measured as markers of barrier function. As noted above BCT06 colonization density was variable and recovered in only 5 of 8 birds. Interestingly the numerically highest transcript abundance for these genes was observed for the 3 birds colonized at 9 log CFU/g (data not shown) suggesting a relationship between response and colonization density. Indeed, BCT is a Gram-negative bacterium such that the inflammatory response could be associated with mucosal exposure to cell wall endotoxin although the apical orientation of *TLR2* is likely tightly controlled (Yu and Gao, 2015).

Down regulation of *CDN5* lead to altered tight junction structure and pronounced barrier dysfunction (Zeissig et al., 2007). The pro-inflammatory responses noted for ENT03 and BCT06 were associated with a decrease in transcript abundance for *CDN5* and could signal lost barrier function in response to these bacterial strains. Whether a similar response to any of these strain would be observed in a post hatch gut may depended on the development of innate barrier mechanisms and the extent to which these organisms are able to penetrate the barrier to activate inflammation (Martina et al., 2017).

Both commensal, nonpathogenic *E. coli* strains, ECL01 and ECL02, were confirmed lethal when inoculated as live isolates before E19 even at the lowest dose of 2 log CFU/embryo in agreement with a report by Graham et al. (2019). Lethality was only confirmed with *E. coli* strains among the 5 bacterial species tested here and numerous other strains reported elsewhere (Alizadeh et al., 2020; El-Moneim et al., 2020; Pender et al., 2017; Siwek et al., 2018). To rule out a toxigenic response to endotoxin present in *E. coli* Gram negative cell wall, heat killed *E. coli* ECL01 was inoculated. Results indicated that the dramatic effect on hatchability and ultimately lethality required the live organism. This finding agrees with Graham et al. (2019), where the *in ovo* inoculation of tetracycline also prevented the lethality of *E. coli in ovo* inoculation. This lack of lethal effect of heat killed *E. coli* is also consistent with the tolerance of embryos to inoculation with Gram negative *Bacteroides sp.* in the present study.

Most interestingly, the effect of live *E. coli* on hatchability was lost when inoculated at day 20 of incubation or at the time of hatch (data not shown). Indeed *E. coli* is known as an early colonizing bacterium post hatch in the chick (Benno and Mitsuoka, 1986) without any known adverse effects. Response to live *E. coli* inoculation to 1 week old germ-free birds was not reported

as lethal and repeated oral immunization with the heat-killed *E. coli* 02 was not fatal and failed to stimulate serum or intestinal antibodies (Parry et al., 1977). Thus, we conclude that E20 most likely represents a significant and acute stage of maturation of gastrointestinal barrier function in the embryo.

On the other hand, *Escherichia coli* is also recognized as a cause of a variety of disease syndromes in poultry, including yolk sac infection, omphalitis, respiratory tract infection, swollen head syndrome, septicemia, and cellulitis (Gomis et al., 2003). In this trial, approximately 10^4 CFU/g of *E. coli* ECL01 was recovered from blood at the sampling (data not shown) indicating the bacterial translocation and sepsis was a likely cause of lethality. Even so, the *in ovo* inoculation of non-pathogenic *E. coli*, did not result in immediate mortality, indeed, independent of the day of inoculation, embryos largely survived until day 21 of incubation and the majority initiated pipping although they were unsuccessful in completing the pipping process.

Harvest of surviving chicks at the time of pipping not surprisingly found reduced yolk sac body weight, and evidence of a marked ileal inflammatory response as indicated by gross edema and marked elevation in transcript abundance of, particularly, *IL-1* and *IL-8*. Interestingly, we recorded a decline in *CDN5* consistent with a possible loss of barrier function (Zeissig et al., 2007) but an increase in *CDN1* which strongly connects intestinal absorbing epithelial cells as tight junction protein (Gharib-Naseri et al., 2020). Although our ECL01 strain did not present with markers of pathogenicity in avian species, clearly this organism interacted differently with the embryonic mucosa compared to the Gram negative BCT06, although at high colonization density this organism also induced significant inflammatory gene expression in the ileum.

4.6 Conclusion

A total of 5 bacterial strains were selected from 5 major taxa in the chicken gut and the effect of these bacterial species were evaluated in mono-associated embryos following *in ovo* inoculation. The lack of bacterial growth detected in the tract of control, non-inoculated embryos maintained under sterile and HEPA-filtered environmental conditions confirmed our ability to establish mono-associated embryos and supports the initiation of gut microbial colonization post hatch under conventional conditions. Colonization success and density was inoculant dependent and may have contributed to variation in ileal inflammatory and barrier function responses

observed. All inoculants were well tolerated by the embryo with the exception of *E. coli* which was lethal when inoculated between day 17 and 19 of incubation. Of the non-lethal inoculants *E. faecalis* induced the greatest inflammatory response in the ileum followed by *B. butyricum*. *Lactobacillus fermentum* was able to induce expression of barrier-associated genes without induction of inflammatory genes, a response which could support a probiotic role. *E. coli* lethality was associated with inoculation of the live organisms and a marked ileal inflammatory response observed at the time of pipping. Interestingly, a lethal response was not observed when *E. coli* was inoculated at day 20 incubation consistent with programmed events in maturation of gut immunity at this time.

5 THE EFFECT OF DIETARY SUPPLEMENTATION WITH *BACILLUS SUBTILIS* C-3102 SPORES ON CHICKEN GASTROINTESTINAL TRACT DEVELOPMENT IN A GNOTOBIOTIC SIMPLIFIED GUT MICROBIOTA MODEL.

5.1 Abstract

Bacillus spp. are supplemented globally in broiler chicken diets although the mechanism(s) by which *Bacillus* probiotics provide health and/or performance benefits are not well understood. Since the complexity of the gut microbiota challenges assessment of mechanisms of probiotic action, germ-free chickens were inoculated with a simplified microbiota (SM) as a model to study of bacteria-bacteria and bacteria-host interactions. Fertilized eggs (Ross308) were sanitized and hatched in four sterile gnotobiotic isolators (16 birds/isolator). At 1 day of age, all chicks were given 0.5 mL of a SM, containing 10^8 CFU per mL of each of *Bacteroides fragilis* BCT06, *Lactobacillus salivarius* LCT01, *Clostridium butyricum* CLS01, *Enterococcus faecalis* ENT03 and *Escherichia coli* ECL01. Birds in two isolators received feed supplemented with *Bacillus subtilis* C-3102 (BS) spores (3×10^5 CFU/g). Birds (8 birds/isolator) were killed at 7 and 14 d of age for measurement of body weight, organ size, ileal gene expression and microbial colonization. Culture and molecular analysis of contents identified all 5 bacterial species in all birds without evidence of contaminating species. BS reduced ($P < 0.05$) the number of *E. coli* and *E. faecalis*, relative weight of gizzard and liver and relative length of jejunum, ileum and cecum. Relative expression of toll-like receptor-4 were upregulated ($P < 0.05$) in BS group. Aminopeptidase N and peptide transporter-1 tended to be increased ($P < 0.10$) in BS group at d 7 and were significantly upregulated ($P < 0.05$) at d 14. Tight junction proteins claudin (*CDN*) 1 and *CDN5* were reduced ($P < 0.05$) by BS at d 7 only. All species represented in the SM colonized the ex-germ-free chicken gastrointestinal tract and appeared to form a stable community. BS supplementation appeared to modify relative abundance of species represented in the simplified microbiota associated with changes in ileal gene expression that could mediate performance and health benefits.

5.2 Introduction

It is well recognized that the highly complex intestinal microbial community is a major mediator of the physiology, metabolism, and energy homeostasis of their host including the chicken (Kogut, 2013; Stanley et al., 2012; Stanley et al., 2013a). Germ-free animal models have

been used extensively in many animal species including mice (Faith et al., 2014; Gibbons et al., 1964), rats (Rachel et al., 2016), chickens (Cheled-Shoval et al., 2014; Drew et al., 2003; Reyniers et al., 1950), pigs (Holman et al., 1966; Kastel et al., 2007; Willing and Van Kessel, 2007; Willing and Van Kessel, 2009), guinea pigs (Cobb et al., 1991; Samuel et al., 1963), and zebrafish (Tan et al., 2019) to confirm the physiological roles of the intestinal microbiota and to elucidate the direct effect of community members on gastrointestinal tract development and function. A major advantage of the germ-free system is that differences between germ-free animals and mono or di-associated animals can be wholly ascribable to the direct effects of the inoculated strain revealing the importance of the community member and pathways mediating the host response. While the approach can provide important insight into host:microbial interactions there are several disadvantages.

Firstly, the microbial colonization of the gastrointestinal tract provides an important signal driving gastrointestinal development dramatically affecting innate and acquired immunity (Shi and Walker, 2004; Walker, 2017) as well as digestive and absorptive function (Willing and Van Kessel, 2007). As a result, the underdeveloped tract of the germ-free animal may respond differently to an inoculated microorganism compared to a conventional animal. Furthermore, the lack of competitive environment in mono-associated tracts often leads to colonization of the inoculated organism at a high density (Schaedler et al., 1965) and with potentially different metabolic activity relative to a complex conventional environment, again potentially altering the nature of the host response (Dieleman et al., 2000; Smith et al., 2007).

Clearly, studies where inoculations are performed using conventional animals can overcome these limitations. However, conventional poultry studies are limited by a highly complex and dynamic microbial colonization pattern (Oakley et al., 2014; Shang et al., 2018) with considerable animal-to-animal variation even when performed under controlled conditions (Kers et al., 2018). Moreover, large differences in microbiota profiles have been shown between groups of birds from highly controlled replicate trials performed in the same lab (Stanley et al., 2013b). Therefore, conventional studies investigating microbial inoculants or other modifiers of microbial colonization often report variable outcomes (Franklin and Ericsson, 2017; Jin et al., 1997). Furthermore, conventional studies are challenged to demonstrate the mechanisms of action of gut modifiers given the considerable animal-to-animal variation in microbial colonization patterns, the difficulty of establishing whether consistent changes in community composition occurs and

whether changes in host physiology are mediated directly by the inoculant or indirectly due to changes in the relative abundance of other community members.

To overcome the challenges associated with establishing mechanisms of action for probiotic bacteria and other gut microbiota modifiers, we developed a gnotobiotic model where birds are maintained with a minimal "simplified microbiota" comprised of bacterial species representing five major taxonomic families present in the chicken gastrointestinal tract including Lactobacillaceae, Bacteroidaceae, Clostridiaceae, Enterococcaceae (Lu et al., 2003). We hypothesized that this simplified microbiota model would provide a consistent environment more representative of the physiology of conventional birds. This model would permit elucidation of the mechanisms of action of probiotics and other gut modifiers including a differentiation of host response pathways activated directly by the probiotic or indirectly by inducing changes in relative abundance of other community members. In the present study, the simplified microbiota model was used to probe the mechanisms of action of a *Bacillus* probiotic (*Bacillus subtilis* C-3102) on gut development in broiler chickens.

5.3   Materials and Methods

5.3.1   Animals and experimental design

All animal experiments were conducted with the approval of the University of Saskatchewan Animal Research Ethics Board (Protocol # 20120074) according to the guidelines of the Canadian Council on Animal Care. All test birds were hatched as germ-free birds and reared according to methods previously developed in our laboratory (Cheled-Shoval et al., 2014; Drew et al., 2003). A total 144 of Ross 308 fertilized eggs weighing 61.6 ± 2.4 g (ranging from 56.1-65.4 g) at E0 were used. All eggs were sterilized by immersion in 0.5% of sodium hypochlorite at 30 °C degrees for 12 minutes and incubated in HEPA filtered incubators (Robbins®, US) sanitized and sterilized using formaldehyde gas. At day 19 of incubation, all eggs were weighed and candled to permit removal of unfertilized eggs, dead embryos and eggs with a cracked shell. Furthermore, eggs which lost over 13% of E0 weight were removed.

The remaining 120 embryos were assigned to one of 4 treatment groups (30 eggs per group) balanced for E0 weight such that mean E0 weight per treatment was 62.2 ± 0.5 g. Eggs in each treatment group were placed in one of four sterilized and HEPA filtered isolator units and re-

sanitized by exposure to 2% of paracetic acid solution (35%, FMC Corporation, Philadelphia, CAS No. 79-21-0) for 12 min within the isolator entry port chamber. Isolator temperature and relative humidity (RH) were maintained between 33-36 °C and 40-70% RH, respectively, until hatch. Uniform hatchability was observed in all four isolators such that the hatching ratio ranged between 77% and 87%. After the hatching, 8 healthy male and 8 healthy females were selected from hatched bird by feather sexing resulting in 16 birds per isolator. Non-selected birds were euthanized. At 1 day of age, all chicks were given 0.5 ml of a cocktail of Simplified Microbiota (SM), containing 1.0×10^8 CFU per ml of each of 5 bacterial strains as detailed below, by oral gavage.

A corn and soybean meal-based starter diet sterilized using gamma irradiation (5 Mrads) and meeting nutrient requirements (NRC, 1994) was fed *ad libitum* during the trial. To account for radiation destruction, 0.2g per kg of vitamin A, D, and E premix (DSM, Ayr, Ontario, Canada) was supplemented to the feed in addition to standard vitamin requirements. Sterile filtered (0.22 μM; Thermo Fisher Scientific, MA USA) water was also provided *ad libitum*. Birds in two isolators received feed inoculated with *Bacillus subtilis* C-3102 spores (provided by Calpis Co., Ltd.) by hand mixing 3×10^5 CFU/g of feed.

5.3.2 Simplified microbiota preparation

Bacteroides fragilis BCT06, *Lactobacillus salivarius* LCT01, *Clostridium butyricum* CLS01, *Enterococcus faecalis* ENT03 and *Escherichia coli* ECL01, isolated from ileal and cecal contents of 14-d-old chickens, were selected as relatively abundant colonizers within each of the 5 major bacterial taxa present in the conventional chicken gastrointestinal tract for inclusion in the SM. Each bacterium was cultured using conditions described in Chapter 4. The combined SM inoculant was made by mixing each of the 5 bacterial solutions to produce a cocktail containing 1.0×10^8 CFU/mL in sterile saline. Freshly prepared SM cocktail was stored on ice until aseptically transferred into each isolator unit and used to inoculate birds by gavage 1 ml per bird of the cocktail with syringe within 1 hour. Culture of a retained subsample confirmed inoculant composition which ranged from a low of 8.2 to a high of 9.0 CFU/g of each bacterium.

5.3.3 Euthanasia and sample collection

Birds (4 males and 4 females per treatment) were killed by cervical dislocation and removed from the isolator at 7 and 14 d of age. Chick body weight and length of the small intestinal segments, duodenum (duodenal loop), jejunum (proximal end of duodenal loop to Meckel's diverticulum), and ileum (Meckel's diverticulum to ileo-cecal junction) were measured and recorded. The wet weight of gizzard, liver, spleen and bursa of Fabricius was also recorded. Intestinal contents from crop, gizzard, ileum and cecum were aseptically collected from 6 birds and immediately processed for bacterial enumeration by culture-based methods. Intestinal tissue representing the medial region of ileum was placed in 10% formalin for histochemical analysis and snap frozen by liquid nitrogen for later storage at -80 °C to permit analysis of gene expression.

5.3.4 Bacterial enumeration

The SM inoculant cocktail or fresh contents from ileum and cecum of each bird were diluted (1:10 w/v) in 0.1% peptone water and 50 µL spread on both non-selective media and selective media. Colonies were enumerated on BL agar with 5% sheep blood (Eiken Chemical Co., Ltd., Tokyo, Japan) for total anaerobes, BBL^{TM} Trypticase^{TM} soy broth with 2% agar (Becton, Dickinson and Co. Sparks, MD, USA) for total aerobes and *B. subtilis* C-3102, DHL agar "Nissui" (NISSUI Pharmaceutical co., ltd. Tokyo, Japan) for Enterobacteriaceae, LBS agar (NISSUI Pharmaceutical co., ltd. Tokyo, Japan) for *L. salivarius*, NN agar (Mitsuoka, 1971) without neomycin for *C. butyricum,* BBE agar (Becton, Dickinson and Co. Sparks, MD, USA) for *B. fragilis* and BD^{TM} Enterococcosel^{TM} Agar (E agar, Becton, Dickinson and Co. Sparks, MD, USA) for *E. faecalis*. Aerobic bacteria were enumerated following culture at 37 °C for 24 h and anaerobic bacteria were enumerated after culture in an anaerobic jar (GasPak anaerobic system, Becton Dickinson Franklin Lakes NJ) at 37 °C for 48 hours. For enumeration of *B. subtilis* C-3102 spores, the remaining diluted samples were heat treated at 65 °C for 30 min to kill vegetative cells prior to plating on selective agar, for culture and enumeration.

5.3.5 Molecular identification of cultured strains

After the enumeration procedure, a total of 16 colonies were randomly selected from each selective media and re-streaked on appropriate selective media to ensure isolation. Bacterial cells were harvested from each isolated colony using a sterilized toothpick and eluted into 100 µL of Nuclease-Free Water (Ambion, USA). DNA extraction was performed by heating to 95 °C for 5 min, followed immediately by 4.0 °C for 10 min., using a C1000 Touch PCR system (Bio-Rad Laboritories, Hercules, CA). The region corresponding to nucleotides 11 to 536 of the *E. coli* 16S rRNA gene were amplified using universal bacterial primers (H1476: 5'-GAGTTTGATCCTGGCTCAG-3'and H1478: 5'-GWATTACCGCGGCKGCTG-3') following a method previously described (Hill et al., 2010). A total of 10µl of each PCR product was purified and sequenced by Eurofins Canada (Toronto, Canada). All sequencing results were trimmed of primer sequence, aligned by MEGA6 software (http://megasoftware.net/) and assigned a microbial identity using BLAST (https://blast.ncbi.nlm.nih.gov) and the Ribosomal Database Project (RDP) Classifier provided by the RDP (http://rdp.cme.msu.edu).

5.3.6 Histochemistry

After formalin-fixation in 10% neutral buffered formalin at least for 24 hour, tissue samples were submitted to the Prairie Diagnostic Services Laboratory (Saskatoon, SK) for paraffin embedding, sectioning and staining with hematoxylin and eosin. As described in Chapter 3, the mean length of at least 10 villi and 10 crypts per cross section per bird were measured from at least four different cross sections by a blinded observer using a Axiostar plus light microscope (Carl Zeiss Canada Ltd., Toronto, ON) and AxioVision 4.1 measurement software (Carl Zeiss Canada Ltd.).

5.3.7 RNA extraction from ileum tissue and quantitative PCR gene expression analysis

Frozen ileal tissues were ground using a mortar and pestle under liquid nitrogen. Total RNA was extracted from 30 mg of ground tissue using an RNeasy Mini Kit (Qiagen, Mississauga, ON) incorporating RNase-Free DNase (Qiagen, Mississauga, ON). Optical density at 260 and 280 nm was determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington DE)

and only samples with a 260/280 ratio between 1.80 and 2.00 were retained for gene expression analysis. A High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA, USA) was used to transcribe total RNA (5 μg) by random hexamer and cDNA was stored at -80 °C until analysis. Specific transcript abundance was measured using quantitative real-time PCR (qPCR) carried out using CFX96 real-time PCR detection system on a C1000 thermal cycler (Bio-Rad Laboratories, Inc., California, U.S.A.). Reactions (20 μL) contained 2.0 μL of a 1/100 dilution of cDNA, 1.0 μL of 10 μM forward and reverse primer (Table 3.1 Chapter 3), 10.0 μL of SsoFast^{TM} EvaGreen® Supermix (Bio-Rad Laboratories, Inc., California, USA) and 6.0 μL of nuclease free water. Reaction conditions were 95 °C for 2 min followed by 40 cycles at 95 °C for 5 seconds and annealing at 53-61°C for 5 seconds (see Table 3.1). A melting curve analysis was conducted at the completion of amplification cycles by increasing temperature from 65 °C to 95 °C in 0.5 °C increments for 5 seconds each. A 5-fold dilution series of pooled cDNA was used as a standard curve and PCR efficiency of standard curves ranged between 98.8% and 117%. Mean abundance of three housing keeping genes (see Table 3.2, Chapter 3 for primers), including Ribosomal protein L30 (*RPL30*), Succinate dehydrogenase complex subunit A (*SDHA*), and Transferrin receptor protein 1 (*TFRC*), was used to normalize expression of genes of interest. The mean arbitrary value for each housekeeping gene, interpolated from the standard curve, was divided by the arbitrary value of the gene of interest to normalize expression values (Livak and Schmittgen, 2001). Fold change was calculated relative to the mean normalized arbitrary value for the control treatment at 7 d of age.

5.3.8   Whole transcriptome analysis using mRNAseq

Total RNA isolated from ileal tissues (n=24; 12 per treatment) was adjusted to 100 ng RNA/μL and assessed for quality using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA was then reverse transcribed and barcoded cDNA libraries were sequenced on a HiSeq® 2500 Sequencing System (Illumina, Inc., San Diego, CA, USA) at the National Research Council Canada (NRC: Saskatoon, SK, Canada). As detailed in Chapter 3 (Section 3.3.8), sequence results were examined for quality using FastQC (Babraham Bioinformatics) and Trim Galore! (Babraham Bioinformatics) was used to remove adapter sequences. The trimmed reads were aligned to *Gallus gallus* reference genome GRCg6a_v95

using HISAT2 v. 2.2.0 (Kim et al., 2015). The resultant bam files were imported into SeqMonk v1.47.2 (conditions: duplicate reads not removed, minimum mapping quality 20, primary alignments only, RNA-seq data, paired end), probes generated (RNASeq Quantitation Pipeline: transcript features mRNA, library type: non-strand specific, libraries are paired end, merge transcript isoforms), manual quantitation correction 0.05, and reads normalized against control samples.

Pathway analysis of genes identified as changing ≥3 fold was conducted using PANTHER (www.pantherdb.org) Overrepresentation Test (Released 2020-07-28, Reactome version 65 Released 2020-11-17) with FISHER test. Gene Ontology (GO) terms were identified using PANTHER and GO Ontology database DOI: 10.5281/zenodo.4081749 (Released 2020-10-09). Dot plots were generated using ggplot2 in R

5.3.9 Statistical analysis

All data was analyzed with SAS for Windows version 9.4 (SAS Institute Inc., Cary, NC, USA) using Proc Mixed procedure. Isolator and gender were initially inserted in the model, identified as non-significant sources of variation and subsequently removed. The final statistical model was a factorial arrangement with BS treatment, age at sample collection and their interaction as sources of variation, the individual bird was the experimental unit. Where a significant interaction between *Bacillus* supplementation and age was observed, a One-way-ANOVA was performed and Tukey HSD selected for multiple comparison among treatment groups. For all tests, a level of 0.05 was used to determine statistical differences and a level of $0.10 \geq P \geq 0.05$ was indicated as trend.

5.4 Results

5.4.1 Microbial status

All inoculated bacterial strains were recovered from ileal and cecal contents at 7 and 14 d of age based on selective culture results (Figure 5.1). Further, based on colony growth and morphology observed on non-selective agars cultured under aerobic and anaerobic conditions (Figure 5.2), no evidence of contamination was observed. For example, consistent with the culture

characteristics of the 6 inoculated strains (5 SM strains plus *B. subtilis*) only three colony morphotypes were found on non-selective TS medium cultured aerobically and only three morphotypes were confirmed on non-selective BL medium cultured anaerobically. On isolation and 16S rRNA gene sequencing of random colonies representing all morphotypes selected from non-selective media, only the 5 inoculated strains in the SM cocktail were identified except in the case of contents taken from birds in isolators supplemented with *B. subtilis,* where the corresponding 16S rRNA gene sequence for this bacterium was also recovered.
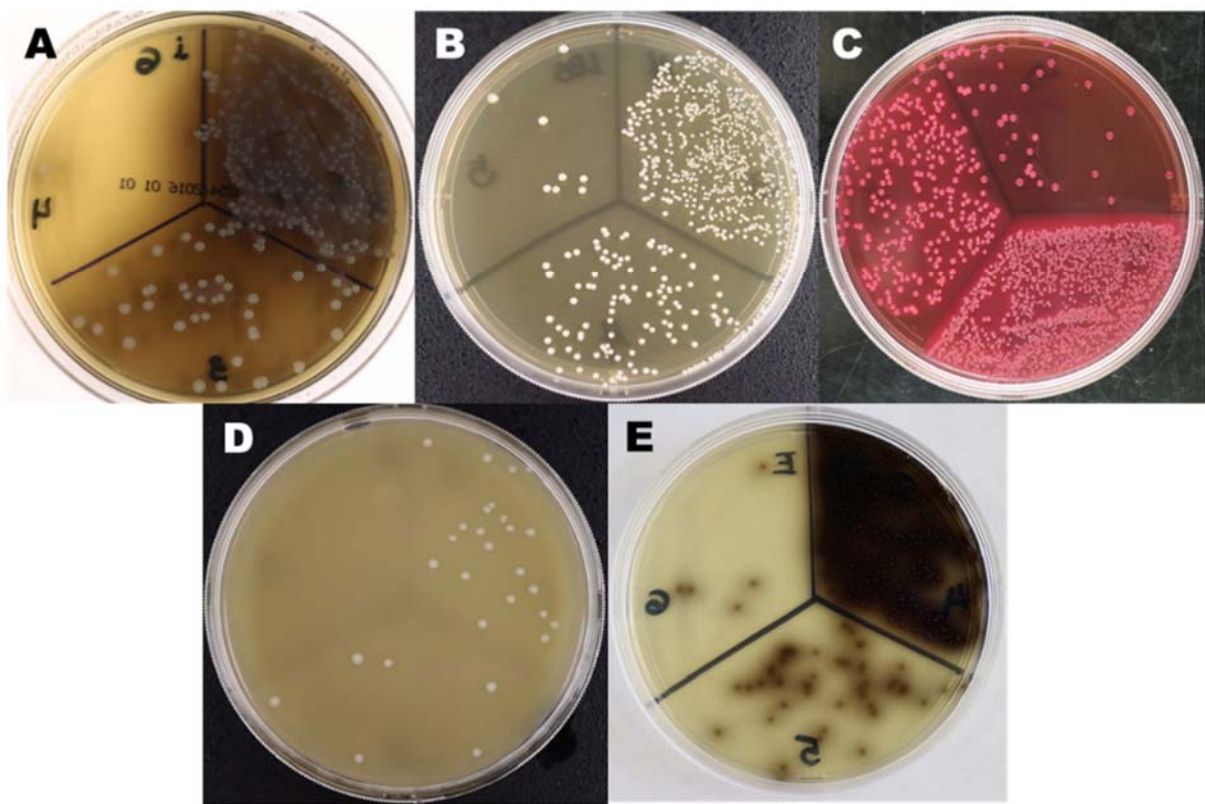
Figure 5.1 Image showing uniform colony morphology following culture on selective media of ileal contents taken at 14 d of age from birds inoculated with a SM and birds inoculated with SM supplemented with BS. Panel A, *Bacteroides fragilis* BCT06 colonies on BBE medium cultured anaerobically; Panel B, *Lactobacillus salivarius* LCT01 colonies on LBS medium cultured anaerobically; Panel C, *Escherichia coli 08* ECL01 colonies on DHL medium cultured aerobically; Panel D, *Clostridium butyricum* CLS01 colonies on NN medium cultured anaerobically; Panel E, *Enterococcus faecalis* ENT03 colonies on E medium cultured aerobically.
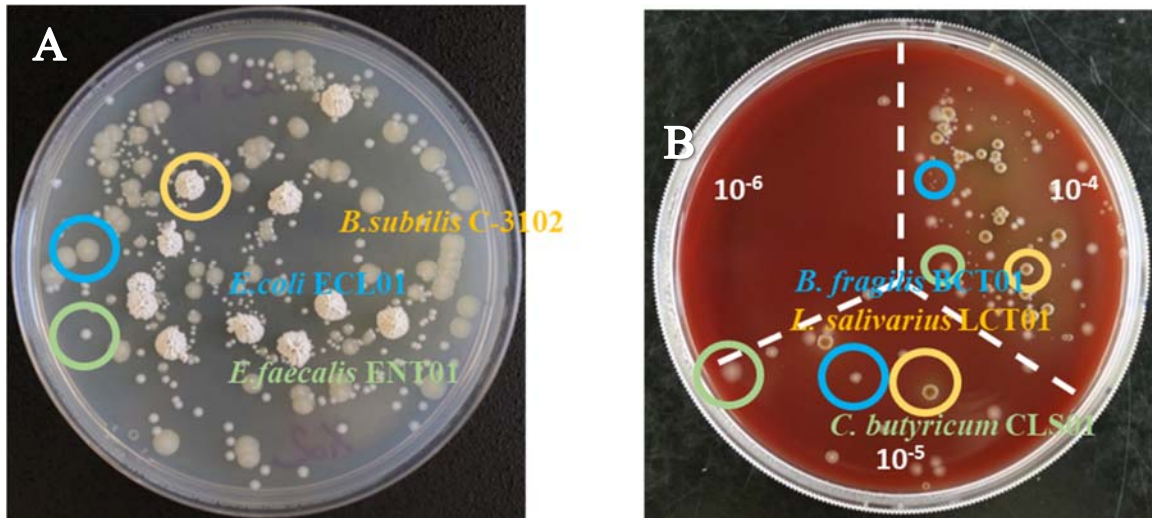
Figure 5.2 Image showing multiple colony morphologies following culture of ileal contents taken at 14 d of age from birds inoculated with a SM and birds inoculated with SM supplemented with BS. Panel A, colonies formed on aerobic culture of contents on TS medium; Panel B, colonies formed on anaerobic culture of contents using BL medium with 5% sheep blood. Colony morphologies consistent with inoculated and supplemented species are indicated.

Table 5.1 Mean (±SE) number (log CFU/g) of five different bacteria species in the ileum contents of Simplified Microbiota (SM) birds and SM birds supplemented with *B. subtilis* (BS) at 7 and 14 day of age.

Treatment	Total Anaerobe	*Bacteroides*	*Clostridium*[1]	*Enterobacteria ceae*	*Enterococcus*	*Lactobacillus*
7 days old						
SM	7.5 ± 0.11	6.2 ± 0.26	3.5 ± 0.18^b $(6/12)^2$	6.9 ± 0.18	6.9 ± 0.10	6.9 ± 0.15
SM plus BS	7.5 ± 0.13	6.4 ± 0.28	4.0 ± 0.08^{ab} $(5/12)^2$	6.8 ± 0.12	6.7 ± 0.10	7.0 ± 0.11
14 days old						
SM	7.6 ± 0.15	6.4 ± 0.23	4.2 ± 0.14^a $(10/12)^2$	6.9 ± 0.16	7.3 ± 0.14	6.7 ± 0.24
SM plus BS	7.4 ± 0.13	5.9 ± 0.16	3.9 ± 0.19^{ab} $(7/12)^2$	6.3 ± 0.10	7.0 ± 0.12	7.0 ± 0.17
Bacillus	*0.3566*	*0.3461*	*0.7694*	*0.0149*	*0.0136*	*0.2132*
Day	*0.6977*	*0.4752*	*0.1091*	*0.0809*	*0.0026*	*0.7364*
Bacillus x Day	*0.5414*	*0.1245*	*0.0232*	*0.0992*	*0.9442*	*0.7315*

[1] Means of positive sample recorded.

[2] Detected / Total Samples

[ab] Values in same column with different superscripts are significantly different ($P < 0.05$)

Table 5.1 provides results for selective culture-based enumeration of the 5 species in the SM cocktail for ileal contents collected at 7 and 14 d of age. Culture results indicated three inoculated species, namely *E. coli* ECL01, *E. faecalis* ENT03 and *L. salivarius* LCT01 colonized the ileum at similar densities around 7 log CFU/mL. The colonization density of *B. fragilis* BCT06 was somewhat lower at approximately 6 log CFU/mL. Ileal colonization with *C. butyricum* CLT01 was detected in 5 to10 of the 12 birds sampled at 3.5-4.2 log CFU/mL and markedly lower than the other inoculants. Age increased ($P < 0.05$) colonization by *E. faecalis* ENT03 whereas age tended ($P < 0.10$) to decrease colonization by *E. coli* ECL01. Supplementation with *B. subtilis* C-3102 significantly reduced ($P < 0.05$) the number of *E. coli* ECL01 and *E. faecalis* ENT03 in ileal contents although the effect on *E. coli* ECL01 was greater at 14 d of age as indicated by a trend ($P < 0.10$) for an Age x BS interaction. A significant ($P < 0.05$) Age by BS interaction was observed for *C. butyricum* CLT01 reflecting an increase in *Clostridium* spp. colonization between 7 and 14 d of age for control birds whereas BS appeared to have a positive effect on *Clostridium* colonization in 7-d-old birds and a negative impact on 14-d-old birds such that no age effect was apparent for BS supplemented birds. Colonization of the SM strains in the cecum at day 7 and day 14 are shown in Table 5.2.

Colonization density in the cecum was higher than in the ileum for all bacteria and again colonization density of *E. coli* ECL01, *E. faecalis* ENT03 and *L. salivarius* LCT01, based on selective culture, were similar at approximately 9 log CFU/mL. The colonization density of *B. fragilis* BCT06 increased dramatically compared to ileum reaching the highest colonization density compared to all other species at 10 log CFU/mL. Cecal colonization with *C. butyricum* CLT01 also increased relative to ileum ranging from 5.1-6.3 log CFU/mL but remained markedly lower than other members of the SM. *C. butyricum* CLT01 was not recovered from cecum of all birds at 7 d of age but was found in all birds at 14 d of age. Surprisingly, age decreased ($P < 0.05$) colonization by *B. fragilis* BCT06. Also, an Age by BS interaction ($P < 0.01$) indicated a decrease in *Clostridium* colonization in control birds positive for this genus between 7 and 14 d of age, however, all birds (12/12) were positive for *Clostridium* at 14 d of age. In contrast to ileum, BS did not affect *Clostridium* in the cecum at 7 days of age and increased *Clostridium* colonization at 14 d of age. An Age by BS interaction ($P < 0.01$) was also observed for *E. coli* ECL01 colonization such that *B. subtilis* C-3102 supplementation lowered *E. coli* ECL01 at 14 d of age only.

Table 5.2 Mean (±SE) number (log CFU/g) of five different bacteria species in the cecum contents of Simplified Microbiota (SM) birds and SM birds supplemented with *B. subtilis* (BS) at 7 and 14 day of age.

Treatment	Total Anaerobe	*Bacteroides*	*Clostridium*[1]	*Enterobacteriaceae*	*Enterococcus*	*Lactobacillus*
7 days old						
SM	10.3 ± 0.04	10.1 ± 0.50	6.3 ± 0.19a (9/12)[2]	9.1 ± 0.11ab	9.1 ± 0.04	9.4 ± 0.08
SM plus BS	10.4 ± 0.08	10.2 ± 0.08	6.1 ± 0.39ab (3/12)[2]	9.2 ± 0.11ab	9.0 ± 0.09	9.4 ± 0.14
14 days old						
SM	10.3 ± 0.12	10.0 ± 0.17	5.13 ± 0.21b (12/12)[2]	9.5 ± 0.09a	9.2 ± 0.06	9.2 ± 0.08
SM plus BS	10.0 ± 0.13	9.6 ± 0.25	6.3 ± 0.12a (12/12)[2]	8.9 ± 0.15b	9.1 ± 0.07	9.3 ± 0.15
Bacillus	*0.6462*	*0.4044*	*0.0431*	*0.0595*	*0.2154*	*0.3437*
Day	*0.0390*	*0.0384*	*0.0483*	*0.7913*	*0.1066*	*0.1671*
Bacillus x Day	*0.0716*	*0.1877*	*0.0084*	*0.0057*	*0.3985*	*0.4394*

[1] Means of positive sample recorded.
[2] Detected / Total Samples
[ab] Values in same column with different superscripts are significantly different ($P<0.05$)

5.4.2 Enumeration of *B. subtilis* C-3102 total viable cells and spores

Culture-based enumeration of *B. subtilis* total viable cells and spores (Figure 5.3) in crop, gizzard, ileum and cecum contents at 7 and 14 d of age, for birds supplemented with this probiotic are given in Table 5.3. The number of viable *B. subtilis* C-3102 was significantly lower ($P < 0.05$) in gizzard compared with other regions at 7 and 14 d of age. From 40% to 50% of *B. subtilis* C-3102 was germinated in the crop, gizzard and ileum. A significantly ($P < 0.05$) higher percent of spores were found in the cecum at both ages such that the proportion of vegetative cells was only 10% to 20% in the cecum contents. Furthermore, a significantly lower number of total *Bacillus* spp. in contents was confirmed at 14 d of age in all four regions. No age effect was observed on spore percentage at any location.

Table 5.3 Total number of *B. subtilis* C-3102 (log CFU/g); number of *B. subtilis* spores (log CFU/g) and percentage of spores in different gut regions at 7 and 14 d of age in gnotobiotic chickens colonized with a simplified microbiota (SM) plus *B. subtilis* (BS).

	Crop[1]	Gizzard	Ileum	Cecum	Region effect
	(log CFU/g contents)				*(P value)*
7 days old					
Total *B. subtilis*	5.9 ± 0.52^a	4.6 ± 0.14^b	5.4 ± 0.06^a	5.5 ± 0.11^a	*0.0001*
B. subtilis spores	5.6 ± 0.44^a	4.1 ± 0.23^b	5.0 ± 0.11^a	5.4 ± 0.11^a	*0.0001*
Spores (%)	54 ± 10.3^{ab}	50 ± 8.6^b	54 ± 9.4^{ab}	83 ± 6.2^a	*0.0370*
14 days old					
Total *B. subtilis*	4.7 ± 0.09^b	4.2 ± 0.04^c	5.1 ± 0.04^a	5.2 ± 0.07^a	*0.0001*
B. subtilis spores	4.4 ± 0.05^c	4.0 ± 0.06^d	4.8 ± 0.05^b	5.2 ± 0.07^a	*0.0001*
Spores (%)	59 ± 8.8^b	66 ± 4.8^b	58 ± 6.5^b	91 ± 2.3^a	*0.0008*
Age Effect					
Total *B. subtilis*	*0.0039*	*0.0156*	*0.0016*	*0.0437*	
B. subtilis spores	*0.0007*	*0.4900*	*0.2438*	*0.1010*	
Spores (%)	*0.7291*	*0.1166*	*0.7874*	*0.2216*	

[1] Number of samples for crop was 8 except at 7 days of age when sample number equaled 4.
[abc] Values in same row with different superscripts are significantly different ($P < 0.05$)

5.4.3 Body weight, organ mass and length

Body weight gain over the 14 d period met the performance objective for commercially reared ROSS308 birds (Aviagen, 2014). Body weight was not significantly affected by *B. subtilis* C-3102 supplementation of SM birds (Table 5.4). The relative weight of gizzard and liver were significantly smaller ($P < 0.01$) and the bursa of Fabricius greater ($P < 0.01$) at 14 compared with 7 d of age (Table 5.5). The relative weight of gizzard and liver were significantly decreased ($P < 0.05$) by *B. subtilis* C-3102 supplementation whereas the relative weight of bursa and spleen were unaffected (Table 5.5).

As anticipated, the relative length of each segment of the small intestine and the cecum were reduced between 7 and 14 d of age. Total intestinal length was significantly decreased ($P < 0.01$) by *B. subtilis* C-3102 supplementation primarily reflecting a significant reduction of jejunum ($P < 0.001$) and ileum ($P < 0.05$) length (Table 5.6). An Age by BS interaction for total length ($P < 0.10$) and for jejunal length ($P < 0.05$) suggested this effect was more pronounced at 14 than at 7 d of age. The relative length of the cecum was also reduced ($P < 0.01$) by *B. subtilis* C-3102 supplementation.

Table 5.4 Mean (± SE) of body weight for Simplified Microbiota birds (SM) and SM plus *B.subtilis* C-3102 inoculated bird at 7 days old and 14 days old.

Treatment	Body Weight (g)
7 days old	
SM	173 ± 5.5
SM plus BS	183 ± 4.4
14 days old	
SM	485 ± 15.6
SM plus BS	490 ± 12.9
Bacillus	*0.4649*
Age	*0.0001*
Bacillus x Age	*0.8469*

5.4.4 Small Intestinal Histology

Mean villus height, crypt depth, villus height to crypt depth ratio (V:C) and thickness of *muscularis mucosa* in duodenum is given in Table 5.7. Increased villus height was observed from 7 to 14 d of age ($P < 0.01$) and for BS-supplemented birds ($P < 0.05$). An interaction (P < 0.01)

for crypt depth indicated that BS increased crypt depth at 7 but not at 14 d of age. An interaction observed for the V:C ratio indicated BS decreased V:C at 7 and increased V:C at 14 d of age. However, means separation detected only a significant age effect where V:C increased from 7 to 14 d of age. The thickness of the *musuclaris mucosa* was not affected by age or BS supplementation. In jejunum, villus height and V:C increased ($P < 0.001$), crypt depth decreased ($P < 0.01$) and the *musuclaris mucosa* thickness increased ($P < 0.05$) between 7 and 14 d of age (Table 5.8). *Bacillus* supplementation tended to decrease ($P < 0.10$) crypt depth and increased ($P < 0.001$) the V:C ratio but did not affect other parameters. In the ileum, age increased ($P < 0.001$) villus height and V:C ratio. *Bacillus* supplemented birds demonstrated significantly ($P < 0.001$) deeper crypts and increased V:C ratio. An Age by BS interaction indicated *Bacillus* supplementation increased ($P < 0.05$) thickness of the *muscularis mucosa* at 14 days of age only (Table 5.9).

Table 5.5 Mean (±SE) relative weight (g/100g of BW) of gizzard, bursa of Fabricius, liver and spleen in Simplified Microbiota bird (SM) and SM plus *B.subtilis* C-3102 supplemented bird at 7 and 14 days old.

Treatment	Gizzard (g/100g BW)	Bursa (g/100g BW)	Liver (g/100g BW)	Spleen (g/100g BW)
7 days old				
SM	3.62 ± 0.08	0.15 ± 0.01	3.94 ± 0.13	0.08 ± 0.00
SM plus BS	3.36 ± 0.11	0.17 ± 0.01	3.67 ± 0.10	0.07 ± 0.00
14 days old				
SM	2.25 ± 0.08	0.20 ± 0.01	2.94 ± 0.08	0.08 ± 0.00
SM plus BS	2.15 ± 0.05	0.18 ± 0.01	2.77 ± 0.08	0.08 ± 0.00
Bacillus	*0.0374*	*0.8636*	*0.0381*	*0.5511*
Age	*0.0001*	*0.0068*	*0.0001*	*0.4017*
Bacillus x Age	*0.3643*	*0.1215*	*0.6494*	*0.7277*

Table 5.6 Mean (±SE) relative length (mm /100g of BW) of small intestinal segment and cecum in Simplified Microbiota bird (SM) and SM plus *B. subtilis* C-3102 supplemented bird at 7 and 14 days old.

Treatment	Duodenum	Jejunum	Ileum	Total small intestine	Cecum
	(mm / 100g BW)				
7 days old					
SM	91.1 ± 2.43	197.8 ± 6.78^{a}	181.6 ± 6.89	470.5 ± 13.95	37.3 ± 1.15
SM plus BS	84.6 ± 2.96	167.5 ± 7.10^{b}	165.5 ± 6.01	417.6 ± 12.74	32.3 ± 2.57
14 days old					
SM	41.6 ± 1.39	86.3 ± 4.73^{c}	82.2 ± 5.10	210.1 ± 10.71	17.1 ± 0.75
SM plus BS	40.9 ± 1.54	83.6 ± 2.94^{c}	74.5 ± 2.55	199.0 ± 6.35	15.4 ± 0.68
Bacillus	*0.1016*	*0.0048*	*0.0309*	*0.0064*	*0.0274*
Age	*0.0001*	*0.0001*	*0.0001*	*0.0001*	*0.0001*
Bacillus x Age	*0.1828*	*0.0168*	*0.4436*	*0.0692*	*0.2673*

[ab] Values in same column with different superscripts are significantly different (P < 0.05)

Table 5.7 Mean (±SE) villus height, crypt depth, villus height to crypt depth ratio (V:C) and thickness of muscularis mucosa in duodenum for in Simplified Microbiota bird (SM) and SM plus *B.subtilis* C-3102 supplemented bird at 7 and 14 days old.

Treatment	Villus height (μm)	Crypt depth (μm)	V:C	Muscularis mucosa (μm)
7 days old				
SM	1131 ± 22.5	105 ± 3.7[b]	10.9 ± 0.39[b]	110 ± 4.0
SM plus BS	1201 ± 28.8	117 ± 5.7[a]	10.3 ± 0.28[b]	116 ± 4.7
14 days old				
SM	1492 ± 37.5	117 ± 2.8[a]	12.8 ± 0.35[a]	114 ± 2.6
SM plus BS	1546 ± 28.8	111 ± 1.6[ab]	13.9 ± 0.19[a]	120 ± 3.0
Bacillus	*0.0398*	*0.3381*	*0.3918*	*0.1202*
Age	*0.0001*	*0.3359*	*0.0001*	*0.2916*
Bacillus x Age	*0.7845*	*0.0037*	*0.0074*	*0.8558*

[ab] Values in same column with different superscripts are significantly different ($P < 0.05$)

Table 5.8 Mean (±SE) villus height, crypt depth, villus to crypt ratio (V:C) and thickness of muscularis mucosa in jejunum for in Simplified Microbiota bird (SM) and SM plus *B.subtilis* C-3102 supplemented bird at 7 and 14 days old.

Treatment	Villus height (μm)	Crypt depth (μm)	V:C	Muscularis mucosa (μm)
7 days old				
SM	656 ± 33.2	77 ± 3.5	8.6 ± 0.38	84 ± 3.3
SM plus BS	686 ± 36.9	73 ± 3.4	9.4 ± 0.33	88 ± 3.8
14 days old				
SM	846 ± 43.7	69 ± 2.8	12.3 ± 0.57	93 ± 2.1
SM plus BS	892 ± 36.8	62 ± 3.3	14.7 ± 0.47	93 ± 3.4
Bacillus	*0.3223*	*0.0826*	*0.0007*	*0.4383*
Age	*0.0001*	*0.0045*	*0.0001*	*0.0345*
Bacillus x Age	*0.8272*	*0.6181*	*0.0863*	*0.5674*

Table 5.9 Mean (±SE) villus height, crypt depth, villus to crypt ratio (V:C) and thickness of muscularis mucosa in the ileum for in Simplified Microbiota bird (SM) and SM plus *B. subtilis* C-3102 supplemented bird at 7 and 14 days old.

Treatment	Villus height (μm)	Crypt depth (μm)	V:C	Muscularis mucosa (μm)
7 days old				
SM	370 ± 15.0	47 ± 1.3	7.8 ± 0.22	86 ± 2.8[ab]
SM plus BS	366 ± 13.4	50 ± 1.1	7.3 ± 0.17	84 ± 3.5[ab]
14 days old				
SM	448 ± 17.0	45 ± 1.4	10.0 ± 0.33	82 ± 3.1[b]
SM plus BS	489 ± 20.0	52 ± 1.5	9.5 ± 0.30	93 ± 2.4[a]
Bacillus	*0.2799*	*0.0007*	*0.0467*	*0.1192*
Age	*0.0001*	*0.7661*	*0.0001*	*0.2771*
Bacillus x Age	*0.1915*	*0.1711*	*0.9690*	*0.0309*

[ab] Values in same column with different superscripts are significantly different ($P < 0.05$)

5.4.5 Ileal gene expression

Fold change in expression of selected genes relative to the SM control birds at 7 d of age, is given in Table 5.10. Pro-inflammatory *IL-6* was not affected by age or treatment whereas as for *IL-8*, a trend ($P < 0.10$) for an interaction indicated BS increased expression at day 14 only. Expression of *TLR4* increased ($P < 0.05$) with age. Among nutrient digestion and absorption-related genes, *PepT1* increased ($P < 0.0001$) with age and both *APN* ($P < 0.01$) and *PepT1* ($P < 0.001$) increased with BS supplementation. The expression of the tissue growth promoting gene, *IGF-1* increased with age ($P < 0.001$) and with BS supplementation ($P < 0.01$). Among the tight junction and barrier function related genes, *CDN1* was increased (P $<$ 0.05) whereas, *CDN4*, and *CDN5* decreased ($P < 0.05$) with age. Interestingly, BS supplementation reduced expression of *CDN5* at 7 but not 14 days of age as indicated by an age by BS interaction ($P < 0.01$). An interaction ($P < 0.05$) for *PCNA* expression, indicated BS supplementation lowered expression of this gene at 7 d of age only. Mucin 2 expression (*MUC2*), tended to be increased ($P < 0.10$) by BS supplementation.

Table 5.10 Mean (±SE) fold change in expression of genes in the ileum for Simplified Microbiota bird (SM) and SM plus *B. subtilis* C-3102 supplemented bird at 7 and 14 days old.

Treatment	IL-6	IL-8	TLR2	TLR4	APN	SGLT-1	PepT-1
7 days old							
SM	1.00 ± 0.41	1.00 ± 0.33	1.00 ± 0.06	1.00 ± 0.09	1.00 ± 0.04	1.00 ± 0.06	1.00 ± 0.03
SM plus BS	0.87 ± 0.24	0.95 ± 0.19	1.44 ± 0.12	1.10 ± 0.07	1.14 ± 0.03	1.18 ± 0.08	1.24 ± 0.08
14 days old							
SM	0.94 ± 0.34	0.98 ± 0.15	1.67 ± 0.41	1.33 ± 0.29	1.03 ± 0.04	1.18 ± 0.04	1.27 ± 0.06
SM plus BS	0.96 ± 0.42	1.85 ± 0.15	1.99 ± 0.40	2.19 ± 0.46	1.32 ± 0.09	1.26 ± 0.09	1.84 ± 0.11
Bacillus	*0.8458*	*0.0967*	*0.3595*	*0.1659*	*0.0080*	*0.1142*	*0.0004*
Age	*0.9601*	*0.0764*	*0.1392*	*0.0403*	*0.1796*	*0.1337*	*0.0002*
Bacillus x Age	*0.7964*	*0.0635*	*0.8782*	*0.2693*	*0.3283*	*0.5522*	*0.1290*

Treatment	cGH	IGF-I	CDN1	CDN4	CDN5	PCNA	MUC2
7 days old							
SM	1.00 ± 0.10	1.00 ± 0.05	1.00 ± 0.07	1.00 ± 0.03	1.00 ± 0.10[a]	1.00 ± 0.26[ab]	1.00 ± 0.06
SM plus BS	0.95 ± 0.07	1.38 ± 0.05	0.73 ± 0.07	1.05 ± 0.04	0.76 ± 0.08[b]	0.74 ± 0.11 [c]	1.14 ± 0.10
14 days old							
SM	1.30 ± 0.32	1.46 ± 0.04	1.11 ± 0.25	0.94 ± 0.04	0.38 ± 0.03[c]	0.99 ± 0.05[ab]	0.93 ± 0.07
SM plus BS	1.27 ± 0.30	1.58 ± 0.07	1.49 ± 0.41	0.88 ± 0.04	0.40 ± 0.04[c]	1.36 ± 0.14 [a]	1.17 ± 0.08
Bacillus	*0.8938*	*0.0055*	*0.8180*	*0.9419*	*0.0277*	*0.7115*	*0.0524*
Age	*0.2674*	*0.0003*	*0.0470*	*0.0418*	*0.0001*	*0.0449*	*0.8382*
Bacillus x Age	*0.9736*	*0.1236*	*0.1395*	*0.3264*	*0.0082*	*0.0414*	*0.5929*

[abc] Values in same column with different superscripts are significantly different ($P<0.05$)

5.4.6 Whole transcriptome analysis using mRNAseq

Total 95 genes were found to be ≥3-fold different between SM and SMB, 50 genes were up-regulated, 45 down-regulated (Figure 5.4). Although PANTHER did not identify specifically enriched pathways, even after lowering the threshold for fold change to 1.5-fold comparison of SM vs SM plus BS Gene Ontology terms indicate that regulated genes were predominately associated with innate and acquired immune response terms.
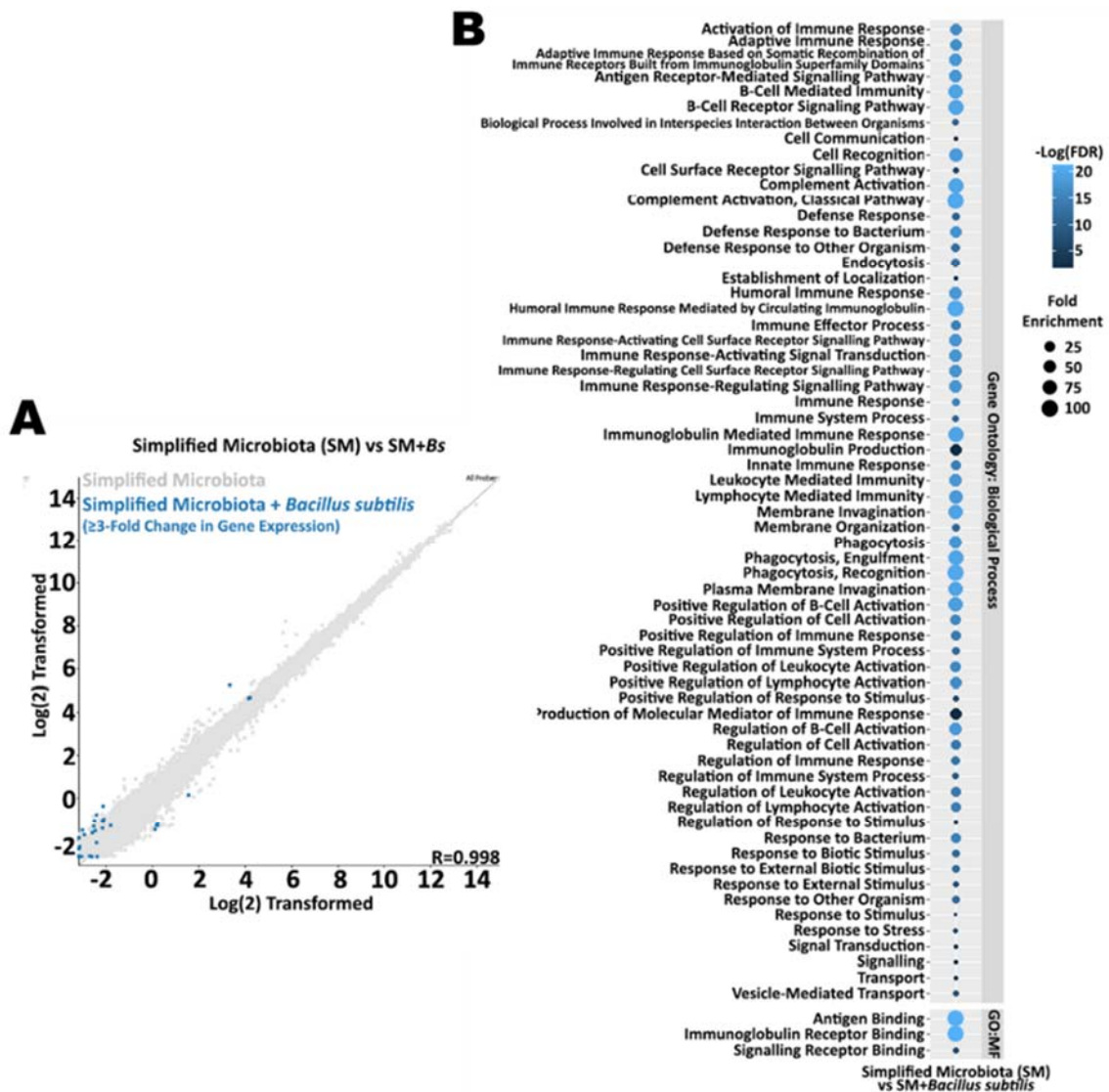
Figure 5.3 Scatterplots comparing the expression of individual genes (A) and showing enrichment of Gene Ontology terms for biological processes (B) in the ileum for Simplified Microbiota bird (SM) and SM plus *B.subtilis* C-3102 supplemented bird at 14 days old.

5.5   Discussion

The composition of the chicken intestinal microbial community is highly diverse and influenced by a number of environmental factors (Rehman et al., 2007) contributing to observations of large variations in microbiota composition when comparing individuals from different populations and from different environments (Stanley et al., 2013b). In the present study, we established a highly simplified microbial population representing the major taxa identified in chicken gastrointestinal tract using a highly controlled environment. We aimed to minimize animal-to-animal variation and simplify the complex microbiome to better study mechanisms of action of gut-active products. A similar simplified microbiota approach to study the host microbial interface has been reported by others in rodents (Faith et al., 2014; Hansen et al., 2015a; Raibaud et al., 1980; Schaedler et al., 1965), pigs (Miniats and Jol, 1978) and poultry (Morishita et al., 1971; Watkins et al., 1982).

All 5 bacterial species in the SM cocktail inoculated into germ-free isolator-reared birds on the day of hatch were recovered from intestinal contents at 7 and 14 days of age. The number of total anaerobes enumerated in the cecum of SM birds was higher than in the ileum and consistent with observations in conventional birds aged 7 to 23 d of age (Alzueta et al., 2003; Fukata et al., 1999; Guo et al., 2004; Jin et al., 1998; Spring et al., 2000). Furthermore, *E. faecalis* ENT03 and *E. coli* ECL01 were enumerated in the ileum and cecum of gnotobiotic SM birds at densities (CFU/g) similar to total Enterobacteriaceae, and *Enterococcus* spp., enumerated using selective culture methods, in conventional birds of similar age (Fukata et al., 1999; Guo et al., 2004; Jin et al., 1998; Spring et al., 2000).

Enumeration of *B. fragilis* BCT06 in the ileum of SM birds indicated colonization density similar to that reported by Maruta et al. (1996) in conventional birds at 14 d of age using selective media. Interestingly, the colonization density of *B. fragilis* BCT06 in the cecum of gnotobiotic SM birds was markedly (2-3 log CFU/g) higher than previously observed for selective culture of Bacteroides in the cecum of birds of similar age (Fukata et al., 1999; Guo et al., 2004). This suggests the Bacteroides colonization in the cecum of conventional birds is limited by competition for resources by bacteria from a taxonomic group(s) not represented in the SM.

Enumeration of *L. salivarius* LCT01 in SM birds indicated colonization densities in the cecum similar to observations in conventional birds. However, *L. salivarius* LCT01 colonization in the ileum of gnotobiotic SM birds was markedly (1-2 log CFU/g) less than observed by selective

culture of lactobacilli in conventional birds of similar age (Jin et al., 1998; Jin et al., 1996c; Maruta et al., 1996a). This probably reflects the imperfect selectivity of culture media for the *Lactobacillus* genus in conventional studies as well as the extreme diversity of this genus which comprises 261 species (Zheng et al., 2020). Although *L. salivarius* LCT01 was selected as a dominant and representative bacterial strain for the Lactobacillaceae family, this single species may not possess the genetic and metabolic diversity to colonize the ileum at a density commonly reported for selective culture enumeration of lactobacilli.

Colonization of gnotobiotic SM birds by *C. butyricum* CLS01 was confirmed in the cecum of all birds at 14 d of age, however, for cecum at 7 d of age and for ileum at both ages, *C. butyricum* CLS01 was not detected by culture in all birds. As expected, based on studies in conventional birds, colonization density was higher in the cecum compared with ileum, and although colonization density of positive birds did not markedly increase with age, the number of birds from which *C. butyricum* CLS01 could be cultured increased in both locations from 7 to 14 d of age. The colonization pattern of *C. butyricum* CLS01 in the cecum is in agreement with Clostridia colonization patterns reported in growing birds where Clostridia abundance increases around 10 to 16 days of age (Jurburg et al., 2019; Schokker et al., 2015). Also in agreement, we were unable to recover *C. butyricum* CLS01 from the chicken gut at hatch after *in ovo* inoculation at embryonic d 17 (Chapter 4). Clostridia may require essential nutrients or a highly reduced environment provided by active metabolism of other members of the gut microbial consortium. The simplified microbiota established here appeared to be able to support the growth of Clostridia in a manner similar to the conventional microbial succession pattern.

In general, the colonization density of members of the SM, by location and with age, reflected a pattern consistent with the colonization pattern of the 5 major taxa these selected species represent. Further, the growth rate of gnotobiotic SM birds, met the performance objectives for the rapidly growing Ross 308 commercial broiler (Aviagen, 2014) indicating the SM birds reared in a gnotobiotic environment were not metabolically compromised. Gross measure of organ weights, histomorphology of the small intestine and gene expression patterns all support a model system reflective of the conventional bird. Finally, statistical analysis of culture-based enumeration of the 5 members of the SM, indicated no significant effect of isolator suggesting uniformity in colonization pattern. We believe the current SM microbiota model therefore meets a number of

important criteria as a model facilitating the study of mechanisms of action of oral products functioning at the host:microbial interface.

The SM model was used here to investigate the mechanism of action of a commercially available probiotic, *Bacillus subtilis* C-3102. Germination rate of inoculated spores in the intestine is an important consideration as germination to vegetative cells is required to generate metabolically active bacilli. *Bacillus* spp. were traditionally considered obligate aerobes, and their germination in the gut unlikely. However, recent studies indicate *Bacillus* spp. are facultative anaerobes capable of reducing nitrate (Clements et al., 2002). Indeed, vegetative *B. subtilis* cells have been reported throughout the digestive tract of birds (Cartman et al., 2008; Latorre et al., 2014) when given *Bacillus* spores in feed, consistent with our findings. The degree of germination, however, remains unclear, complicated by the potential for completion of a full life cycle (germination and re-sporulation) in the gut (Bernardeau et al., 2017). Latorre et al. (2014) reported 90% germination rate based on the recovery of spores in crop compared to ileum followed by re-sporulation in the cecum. In agreement, and although *Bacillus* spore counts were surprisingly low in crop at 14 d of age relative to feed content, we recorded the lowest spore counts in gizzard and ileum consistent with Latorre et al. (2014) and with germination and re-sporulation occurring in upper gut and ceca respectively (Bernardeau et al., 2017). Also in agreement, the ratio of total *Bacillus* counts to heat resistant spores reported here, suggested a high germination rate of 55-60% in upper intestine compared to 9-17% in ceca. However, spores, as a percent of total bacillus counts reported here was much lower than our previous findings of 98% in commercial conventional birds (Hamaoka et al., 2010) and could reflect reduced nutrient composition and/or increase oxygen availability in the SM model. This interpretation would be consistent with our previous findings of a relatively low spore percent (high vegetative cell percent) in mono-associated birds at 1-2 d of age (Chapter 3) and at 14 d of age (Hamaoka et al., 2010; Hamaoka et al., 2011). It is interesting, however, that *E. coli* represented in the simplified microbiota, did not appear to be markedly advantaged by an increased oxygen supply compared with conventional birds.

In SM birds, *Bacillus subtilis* C-3102 supplementation significantly reduced *E. coli* colonization density compared with non-supplemented birds. This is consistent with a reduction in Enterobacteriaceae previously reported by several others in conventional birds supplemented with *Bacillus* spores (Fritts et al., 2000; Jin et al., 1996a; La Ragione et al., 2001; Maruta et al.,

1996a). A reduction of Enterobacteriaceae has previously been associated with improved bird performance (Johnson et al., 2018) and the reduction in Enterobacteriaceae observed here and by others in conventional birds may represent a major mechanisms of action of *Bacillus* probiotics. Although a number of mechanisms may account for the antagonism with Enterobacteriaceae, including secretion of antimicrobial compounds and competition for limited essential nutrients (Bernardeau et al., 2017), it is tempting to speculate that competition for oxygen between these two facultative anaerobes limits colonization.

Bacillus spore supplementation also appeared to limit colonization by *E. faecalis* ENT03. To our knowledge, a reduction in *Enterococcus* spp, following *Bacillus* spore supplementation has not been reported in poultry. The antagonism between these two species could be more pronounced in the simplified microbiota environment maintained here relative to conventional birds. Again, the mechanisms contributing to antagonism could be diverse, but *E. faecalis* is also a facultative anaerobe and competition for oxygen could play a role.

Bacillus supplementation in SM birds significantly reduced relative organ size for gizzard, liver, small intestine and cecum compared with non-supplemented SM birds. Reduced relative liver weight following *Bacillus* probiotic supplementation was also recorded by others (Khajeh Bami et al., 2019; Molnar et al., 2011). Despite the reduction in relative organ size, and presumably reduction in maintenance energy requirements, no difference in body weight was observed. The number of birds in the present study is almost certainly too small to detect a change in body weight, however, reduced organ weight may have increased nutrient flow to body growth consistent with previous reports of increased carcass weight (Novak et al., 2011), improved feed conversion efficiency (Fritts et al., 2000; Jeong and Kim, 2014; Nunes et al., 2012) and body weight gain (EFSA, 2006b; Fritts et al., 2000) following *Bacillus* supplementation in conventional birds.

On the other hand, compared to germ-free birds we observed an increase in relative liver weight at 1 d of age when *Bacillus subtilis* was inoculated in chick embryos at E17 (Chapter 3) and at 14-d- of age when ex-germ-free birds were fed *Bacillus* spores beginning at 1 day of age (Hamaoka et al., 2011). We hypothesize that the liver weight increase in *Bacillus* mono-associated birds may reflect an immune reaction against *Bacillus subtilis* C-3102 compared to the naïve germ-free state. In the present study, *B. subtilis* could have mitigated an increase in liver size and intestinal size associated with immuno-stimulation by the simplified microbiota either directly by

enhancing innate barriers (see below) or indirectly by limiting colonization by immuno-stimulatory simplified microbiota members such as *E. coli* and *E. faecalis*.

Bacillus spp. have been shown to promote immune response in pigs (Scharek-Tedin et al., 2013) and induce increased expression of *TLR2* and *TLR4* in macrophages (Bernardeau et al., 2017; Huang et al., 2008), a response consistent with the increase in *TLR4* in ileal tissue observed here. An increase in innate barrier function mediated by *B. subtilis* is indicated by the significant increase in *MUC2* expression in the ileum observed at 7 and 14 d of age. Others have observed an increase in *MUC2* expression in chicks following *in ovo* administration of *Bacillus* spp. (Majidi-Mosleh et al., 2017a) in support of observations here. Increased crypt depth and a lower ileal V:C at day 7 and 14 suggested BS increased epithelial turnover as a component of the innate barrier, however, PCNA expression, which could reflect either epithelial or myeloid cell proliferation in lamina propria was lower on day 7 and increased on day 14 for BS supplemented birds. Finally, *B. subtilis* supplementation also reduced expression of *CLDN5* a tight junction protein, a response that is not consistent with an increased epithelial barrier.

Mono-association of chicken embryos at day 17 of incubation with BS activated a number of genes associated wtih ontology biological processes related to immune function and specifically enriched a chemokine pathway in the ileum at both hatch and 24 hours after hatch (Chapter 3). In the present study we also observed enrichment of gene ontology terms associated with innate and acquired defense although the chemokine pathway was not specially enriched. It is noteworthy, that in the present study, BS demonstrated modulation of host immune-related genes in the presence of 5 other species of commensal organisms. The pathways identified here appear to be sensitive to BS and may not reflect a generalized response to bacterial colonization. This could contribute to enhanced protection against bacterial challenge and improved growth performance in conventional birds supplemented with *Bacillus* spores (Knap et al., 2011; Oh et al., 2017; Park and Kim, 2014).

Interestingly, analysis of gene expression identified an increase in abundance of transcripts encoding genes contributing to nutrient digestion (*APN*) and absorption (*PepT-1*) in *Bacillus* supplemented birds. This is consistent with previous similar observations in *Bacillus* mono-associated birds at 0 and 1 day of age (Chapter 3) and may indicate a direct response to *Bacillus* supplementation. This upregulation in the nutrient transporters and the local expression of tissue growth promoting *IGF-I* may also contribute improvement of feed efficiency (Fritts et al., 2000;

Jeong and Kim, 2014; Nunes et al., 2012) or body weight gain (EFSA, 2006b; Fritts et al., 2000) reported for *Bacillus* supplementation in conventional birds.

5.6   Conclusion

Five bacterial species representing the major taxa colonizing the chicken gastrointestinal tract were introduced to germ-free birds on the day of hatch and reared under gnotobiotic conditions until 14 d of age. All inoculated species colonized and produced a stable community with a similar succession profile and a relative colonization density to comparable taxonomic groups enumerated in conventional birds with high consistency among birds. This simplified microbiota model was used to investigate the mechanisms of action of a *Bacillus* probiotic that could contribute to positive health and performance outcomes. Observations indicate that *Bacillus* probiotic mechanisms of action may be indirect through inhibition of colonization by *E. coli* and *E. faecalis*. This and evidence of *Bacillus*-induced activation of immune response genes may have contributed to lower organ weights and improved efficiency of nutrient use for growth. Finally, increased expression of nutrient transporters may be mediated directly by *Bacillus* spp. and also improve growth efficiency. The Simplified Microbiota model could fill the gaps between a germ-free environment and the conventional environment and would permit elucidation of the mechanisms of action of probiotics and other gut modifiers including a differentiation of host response pathways activated directly by the probiotic or indirectly by inducing changes in relative abundance of other community members.

6 GENERAL DISCUSSION AND CONCLUSION

After the founding concept of probiotics was expressed by Metchnikoff (Metchnikoff, 1907), no clear conclusion has been made for mode of action of probiotics. We hypothesized that two major pathways could contribute as the mechanisms of action of probiotics; a direct pathway mediated by the probiotic organism and an indirect pathway mediated via a modified host intestinal microbiota. However, specific experimental systems are needed to test these hypotheses because both effects simply appear without distinction in conventional experiments while the complexity, variability and dynamic nature of the conventional microbial environment challenges the assessment of changes in microbial composition. Our challenge in this series of trials was to develop a new gnotobiotic experimental system which allowed us to test the direct effect and indirect effect of probiotics separately. The advantages of gnotobiotic experiments is a highly controlled repeatable experimental design which reduces interindividual variation (Wang and Donovan, 2015). Our original idea was to detect direct effects in response to test bacteria in a mono-associated condition established in ex-germ-free test birds, and to test whether these specific direct effects would also be observed in a simplified gut microbiota model where the indirect effects of a probiotic mediated by changes in community composition could be easily observed. We expected the accumulation of repeatable results from these two gnotobiotic experimental systems may give us valuable knowledge on mode of action of the probiotics.

6.1 Potential of chicken embryo as a model system

To take advantage of the germ-free status of chicken embryo as simpler and cheaper germ-free test animal compared with germ-free bird in traditional germ-free isolators, a first step was to establish a HEPA-filtered individual canister model. For example, the actual *in ovo* trial setting takes a net of five days from E17 to E22, and the trials can be repeated weekly by preparing the next batch of germ-free embryos while other tests are being conducted. The ease of repetition, amenability to relative high throughput could be a major advantage in probiotic evaluation.

One of other benefits of the model developed here, is the high success ratio and the minimized impact of a potential microbial contamination event to an individual bird. A total of 140 E17 germ-free embryos were used in this series of trials and monitored for microbial status at

the time of sampling. No unplanned contamination was recorded. This is a far greater success ratio than we have experienced in our germ-free chicken model which may also indicate that contamination in traditional germ-free chicken trials is acquired by bacteria present in the feed or water, as opposed to surviving exterior sterilization of eggshells or as recently suggested via vertical transmission *in ovo*. By using recent 16S rRNA sequencing technologies, it has been reported that complex microbiotas are already formed in the chicken gut during the embryonic stage in some cases (Ding et al., 2017; Lee et al., 2019). Our data show that a vertical contribution to the intestinal microbiome is at minimum, not a universal event occurring in all eggs. With respect to survival of contaminating bacteria on eggshells, it may be that our harvesting of chicks within 24 hours of birth was too earlier to detect such a contamination source. I believe that the gap between new discoveries and past knowledge must be bridged through a scientific approach. At least, the following three points should be considered: detection of bacteria by culture methods is incomplete and not all bacteria can be detected, bacterial detection by NGS does not necessarily mean the presence of live bacteria, and aseptic sampling requires skilled techniques.

Another strength of the system is its robustness as a test system. Even if case contamination occurs in the system, it will not affect the entire trial design due to independence of individual containers unlike the traditional germ-free isolator model. The use of clear individual containers also allowed flexible trial design due to the possibility of individual sampling without contaminating the environment of yet to hatch eggs. In Chapter 3 and Chapter 4, sampling was performed exactly at the timing of hatching or the start of the pipping for each individual egg. Then, additional sampling 24 hours after the pipping was linked to those precise times on an individual basis. Flexible sampling is also possible with traditional germ-free isolator by using a transfer hatch, but there is always chance of introducing a contaminant, limiting this approach. On the other hand, several defects need to be recognized as the disadvantages of the system, such as required skills for *in ovo* inoculation to amniotic fluid, immature host organs and digestive system, and no flow of feed in the intestinal tract. In particular, *in ovo* injection into amniotic fluid requires skill, and performing trials with inexperienced *in ovo* techniques may lead to erroneous conclusions.

In this trial, we hypothesized that amniotic fluid could be one of the best test bacterium delivery location to observe effect of inoculants on embryo's intestinal development because it is well established that amniotic fluid is orally absorbed by the embryo during incubation period

(Guyot et al., 2016). We confirmed this by performing *in ovo* injection of a food dye into amniotic fluid using a plastic needle (Figure 6.1, Panel A) to avoid embryo damage. The dye was easily observable in the cecum and small intestine of chicks recovered from injected eggs (Figure 6.1, Panel B and C). Also, when dye was administered in amniotic fluid chicks were recovered at hatch with green outer coloring; administration of the dye into the air sac did not result in colored birds or the observation of dye in the gastrointestinal tract (Figure 6.2, Panel A and B). In the worst case, the wrong injection location caused embryonic mortality occasionally with colored organs indicating direct injection into the embryo.

In pre-test studies, 20% mortality was recorded in *Enterococcus faecalis* ENT03 injected embryos, whereas 0% mortality was recorded at the trial reported in Chapter 4 even with over 9.0 log CFU/g of ENT03 colonization in the cecum. Therefore, the mortality in ENT03 group at the pre-trial is likely to be due to inadequacy of *in ovo* injection technique. For non-lethal bacteria other than *E. coli* in this series of trials, mortality after the *in ovo* treatment was 3.0 % in total. Basically, death cases from *in ovo* injection itself are unlikely to occur if the technique is properly mastered and the inoculant is delivered into the amniotic fluid of a healthy embryo.

As mentioned above, another disadvantage of the *in ovo* model is bias in test results due to the immaturity of the host organ's biological function. Development of intestinal epithelium begins around E8 from a flat surface and villi start to be formed at E16 (Huycke and Tabin, 2018). As shown in Chapter 3 in this thesis, villi development is still in progress after the hatch. From E17 to the time of hatching the intestinal tract is still developing and is not considered to be fully mature.

In addition, it is known that functionality of the chicken digestive tract is affected by diet and feeding system (Svihus, 2014). The absence of feed flow in the gut in this model may limit expression of the digestive or absorption function of the host. And it may also affect the colonization or proliferation results of the bacteria in the gut of chicken embryo because available nutrition in the egg is limited until hatch. Lower or uneven colonization results observed on LCT01, CLS01, and BCT06 at Chapter 4 may be supporting this hypothesis. In the future, an additional nutritional injection may be investigated to assess effect on stable colonization of the test bacteria in the gut and to better investigate host-bacteria interaction in detail.
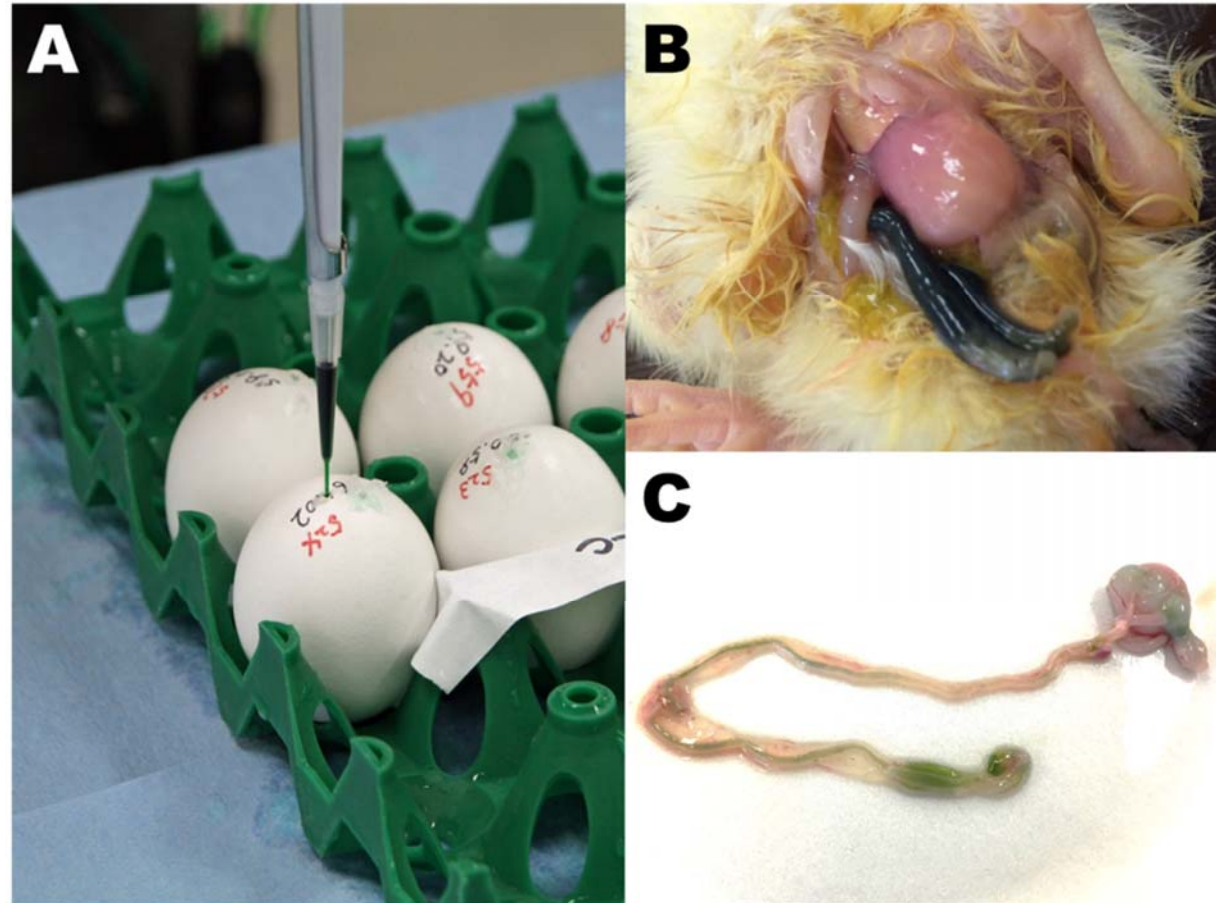
Figure 6.1 Photograph is showing *in ovo* injection of food dye to confirm injection into the amniotic fluid and flow into gastrointestinal tract. Plastic pipette tips were used to inject 100 μL of food dye into amniotic fluid (Panel A). The injected dye was readily observed in the intestinal tract of birds at hatch (Panel B and Panel C) confirming *in ovo* ingestion of amniotic fluid.
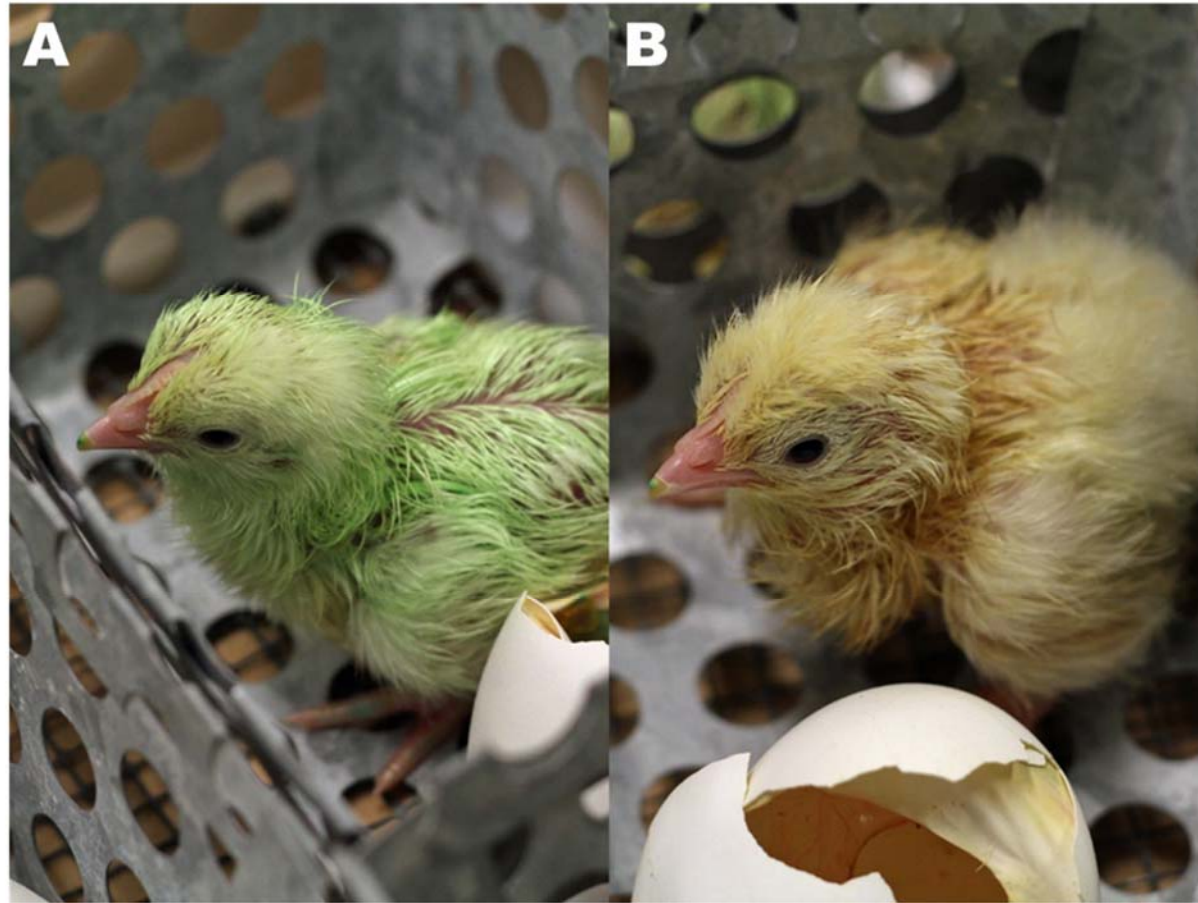
Figure 6.2 Photograph of chicks after hatching in pre-trial *in ovo* injection of a green food die. Panel A shows a chick after hatch following *in ovo* injection with 100 µL of food dye into the amniotic fluid at E17. Panel B shows a chick following injection of food dye into the air sac at E17.

The first innate immune system cells appear between E1 and E4, and development of immune system continues until 4 to 6 weeks of age (Alkie et al., 2019). Immune functions and barrier functions of E17 embryo are considered to be functionally immature. This is highlighted in our work as shown in *E. coli*-induced mortality when injection occurred before E19 (Chapter 4). Translocation of *in ovo* injected *E. coli* ECL01 and *Lactobacillus reuteri* R32 were indicated by recovery from the blood of test birds following *in ovo* injection before E19 (data not shown) in pre-trial pilot studies. Bacterial translocation shows immatureness of gut barrier functions and bacteria recovery from the blood may suggest that the antagonistic innate immune system in chicken embryo before E19 is still not strong enough to kill the invaders faster than they proliferate. Bacterial species tested in this series of trials are not commonly recovered from the blood of healthy chicken by culture-based method and therefore suggests that test commensal or putatively probiotic bacteria enter different immune compartments when exposure occurs *in ovo* and result in different immune responses compared to post hatch exposure. Therefore, it is natural to consider that not all results observed following *in ovo* embryonic exposure to test bacteria could be extrapolated to understand relationships between the host and test bacteria in a conventional environment.

6.2 Benefits and limitations of the Simplified Microbiota Model

The defined microbiota model, the gnotobiotic model, when made through inoculation with selected strains, can yield valuable knowledge on the impact of gut microbiota (Hansen et al., 2015a). The reproducibility of the defined microbiota could be an advantage to have uniformity in the results particularly when involvement of gut microbiota balance is hypothesized, especially for trials seeking mode of action of probiotics like this thesis. One of the methodologies to have defined microbiota in a chicken trial is making conventionalized chicken from ex germ-free chickens. The idea is to eliminate differences in the microbiota balance/composition between trials by giving the same seed inoculation of microbiota, glycerol stock of fecal material collected from healthy chicken, via drinking water after the hatch of germ-free chicken (Drew et al., 2003). Since 2010, conventionalized birds were made a total of four times at the University of Saskatchewan with the same fecal glycerol stock seeder and uniformity was recorded in microbial profiling results by culture-based method (Table 6.1). Among these four trials, similarity was confirmed between microbial profiling results and advantage of the conventionalized chicken method has

been demonstrated. However, the conventionalized bird method is still not free from three problems, including limits on the repeatable attempts due to seed fecal inoculant depletion, unknown and uncontrolled bacterial composition whose character has not been fully investigated, and complexity is still too high to clarify all connections among bacterial species. The simplified microbiota bird is meant to solve these defects.

As a source of microbiota for our Simplified Microbiota, we chose intestinal microbiota samples taken from conventionalized isolator-reared birds which had been generated at the University of Saskatchewan in 2010. This microbial community had three desirable characters as a source of test microbiota. Firstly, it was already confirmed that the test conventionalized broiler chickens with this microbiota would grow in line with the performance objectives. Secondly, it was confirmed that the *Bacillus* probiotics supplementation causes changes in the bacterial balance in this conventional microbiota, such as a decrease in *E. coli* and a decrease in the total anaerobe population as has been reported as an effect of the probiotic (Maruta et al., 1996a). Thirdly, the population did not contain specific pathogens which causes disease or food poisonings as dominant bacteria, such as *Salmonella* spp. and *Clostridium perfringens*. Therefore, the Simplified Microbiota from this conventional microbiota could be utilized as a base microbiota when specific pathogen challenge is needed in the future. Representatives of the 5 major taxa present in chicken gastrointestinal tracts are Lactobacillaceae, Bacteroidaceae, Clostridiaceae, Enterococcaceae and Enterobacteriaceae (Lu et al., 2003). In 2014, total 627 bacterial strains in these 5 family taxa were isolated from 14 day old, conventionalized birds made from ex-germ-free birds. Once the typical phenotype of the target bacterial family was confirmed on selective media, described in Chapter 4, and a Gram stain confirmed cell wall type, the isolated colony was dissolved into 0.1% peptone water with 30% glycerol and stored at -80 ºC for 2 months to check freezing storage resistance. The DNA was extracted from recovered bacterial strains for DNA sequencing of the region corresponding to nucleotides 11 to 536 of the *Escherichia coli* 16S rRNA gene (Hill et al., 2010). Results of DNA sequencing were put into analysis software (MEGA6) and then molecular phylogenetic trees were made by the Neighbor Joining method (Tamura et al., 2013). The evolutionary distance were computed by the Maximum Composite Likelihood method (Tamura et al., 2004) and each cluster was considered one bacterial strain and the phenotype of all strains were compared again on non-selective medium to confirm uniformity. Then, a total 6 strains of *Enterobacteriaceae*, 7 strains of *Lactobacillus*, 5 strains of *Enterococcus*, 5 strains of *Bacteroides*

and 6 strains of *Clostridium* were harvested. Compatibility between strains was examined using a "round-robin" exclusion zone method. Strains showing good compatibility were frequently observed among cultured isolates and those strains representing dominant taxa based on 16S rRNA profiling studies in birds (Lu et al., 2003) were selected for the Simplified Microbiota cocktail.

Our objectives in developing a Simplified Microbiota model were primarily two-fold. Firstly, we wanted to design an artificial microbiota consortia with a limited number of bacterial species where all members were capable of colonizing the gastrointestinal tract and formed a stable community with relative abundance consistent with a conventional environment. Conventional microbiota of chickens fed with corn-soybean based diet measured by culture-based method in the past reports were summarized in Table 6.1 for ileum and Table 6.2 for cecum. Since all these results were not analyzed with the same analytical method, direct comparison among trial results were difficult. However, the data indicated that typical dynamics and balance of gut microbiota in conventional birds were also observed in the ileum and cecum of SM birds. The number of total anaerobes were observed around 8 log CFU/g in the ileum and 10 log CFU/g in the cecum. Abundance of Enterobacteriaceae and *Enterococcus* are confirmed to be around 6 to 7 log CFU/g in the ileum and 8 to 9 log CFU/g in the cecum. Similarity was also observed in *Lactobacillus* levels between SM birds and conventional birds. Gaps observed in *Bacteroides* and *Lactobacillus* levels in the SM and original microbiota shows rooms for improvement on the selection of the constituent bacterial species for SM. However, it is considered that a stable bacterial community was established in the gut of SM birds at day 7 and the balance was kept until day 14 as we hypothesized.

Table 6.1 Mean number (log CFU/g) of five different bacteria species in small intestine contents in Simplified microbiota bird and conventional control birds enumerated by the culture-based method in the past studies with corn-soybean based diet.

Reference	Age (Days)	Total anaerobes	*Bacteroides*	*Clostridium*	*Enterobacteriaceae*	*Enterococcus*	*Lactobacillus*
Simplified Microbiota	7	7.51	6.20	3.50	6.90	6.90	6.90
Simplified Microbiota	14	7.64	6.40	4.20	6.90	7.30	6.70
Conventionalized chicken 2011A[*1]	14	7.86	NT	NT	5.58	NT	7.77
Conventionalized chicken 2011B[*2]	14	7.69	NT	NT	5.48	NT	7.40
Conventionalized chicken 2012[*3]	14	8.00	NT	NT	5.78	NT	7.31
Conventionalized chicken 2014[*4]	14	7.94	4.71	NT	5.73	6.34	7.69
Maruta et al. (1996a)	14	NT	6.97	NT	7.89	NT	8.79
Jin et al. (1996b)	14	7.75	NT	NT	7.28	NT	7.61
Jin et al. (1998)	20	8.30	NT	NT	6.81	NT	8.66
Alzueta et al. (2003)	23	NT	NT	NT	8.08	7.50	6.23
Xu et al. (2003)	49	8.47	NT	NT	7.03	NT	7.46
Xia et al. (2004)	49	8.92	NT	5.95	6.94	NT	7.52

NT:Not tested in the original report. ND: Not Detected

*1 Hamaoka et al. (2011), *2 Unpublished, *3 Hamaoka et al (2012), *4 Unpublished – Origin of Simplified Microbiota

Table 6.2 Mean number (log CFU/g) of five different bacteria species in the cecum contents in Simplified microbiota bird and conventional control birds enumerated by the culture-based method in the past studies with corn-soybean based diet.

Reference	Age (Days)	Total anaerobes	*Bacteroides*	*Clostridium*	*Enterobacteriaceae*	*Enterococcus*	*Lactobacillus*
Simplified Microbiota	7	10.30	10.10	6.30	9.10	9.10	9.40
Simplified Microbiota	14	10.27	10.00	5.13	9.50	9.20	9.20
Conventionalized chicken 2014[*2]	14	10.34	7.04	NT	7.92	7.22	8.74
Impey et al. (1982)[*1]	1	10.57	ND	7.58	8.20	9.40	4.36
Baba et al. (1991)	2	9.97	< 3.0	NT	9.87	NT	3.60
Barnes (1979)[*1]	3	NT	NT	7.66	9.06	9.10	9.38
Fukata et al. (1999)	7	10.6	7.42	NT	8.86	NT	9.94
Spring et al. (2000)	10	9.26	NT	NT	8.71	8.13	ND
Jin et al. (1998)	20	10.80	NT	NT	8.60	NT	9.48
Fukata et al. (1999)	21	10.64	8.79	NT	9.10	NT	9.82
Guo et al. (2004)	21	9.52	6.70	NT	6.93	7.36	7.54
Alzueta et al. (2003)	23	NT	NT	NT	9.27	8.48	7.30
Xu et al. (2003)	49	9.55	NT	NT	7.03	NT	7.46
Xia et al. (2004)	49	9.71	NT	6.62	7.54	NT	8.40

NT:Not tested in the original report. ND: Not Detected

*1 Means calculated from results from control groups shown in the paper.

*2 Unpublished – Origin of Simplified Microbiota

Secondly, we wanted a simplified consortia capable of mimicking specific functions of the original microbiota including competitive exclusion and stimulation of intestinal development and mucosal immunity. Although we did not perform a pathogen challenge study using the Simplified Microbiota model we did observe evidence of competitive exclusion properties similar to a conventional environment. In Chapter 5, the simplified microbiota was observed to suppress the activation of *Bacillus* spores and proliferation of vegetative cells in a manner consistent with what is normally observed in the conventional chicken gut. Indeed, when inoculated in mono association, the *Bacillus* spore to vegetative cell ratio was 59.2% and the level of colonization was 7.16 log CFU/g (Hamaoka et al., 2010), markedly different from the conventional environment and SM. Furthermore, *Bacillus* supplementation was confirmed to affect a significant reduction of Enterobacteriaceae and *Enterocccus* in SM birds similar to observations in conventional birds (Hamaoka et al., 2011).

We have not completed a direct comparative study investigating the impact of our Simplified Microbiota on intestinal functional development (digestion and absorption/immunity) compared with germ-free or conventional birds it remains as our future challenge. The five bacterial strains utilized to make the Simplified Microbiota may be insufficient to fully reproduce the function of the original microbiota. In the future, it may be possible to select bacteria strains to add functionality rather than bacterial species they belong to.

6.3 Approach to the direct effect and indirect effect of the *Bacillus* probiotics

The finding of vegetative cells of the inoculated *Bacillus subtilis* strain here supports the potential of the *in ovo* model system for evaluation of the direct effect of *Bacillus* probiotics on intestinal development and function. In Chapter 3, a longer incubation time to hatch, increased yolk sac weight and relative liver weight, reduction of yolk sac free body weight at hatch and 24 hours after hatch, and several changes in gene expression were shown as possible direct effects of the *Bacillus* probiotic. Some of these effects may be indicative of the mode of action of *Bacillus* probiotics. As discussed above, it is also considered that there are some caveats to evaluate the effect of a single bacteria strain in a mono-associated condition due to a lack of bacterial competition and immatureness of the gut, other internal organs and metabolic systems in chicken embryos (Hamburger and Hamilton, 1992; Hincke et al., 2019). Interestingly, almost all effects of mono-association shown in Chapter 3 and 4 are considered as negative impact for the host embryo.

The significant weight loss shown in *E. coli* mono-associated embryo and *Bacillus* mono-associated embryos would be the best example. However, *E. coli* is known as one of the dominant bacteria in the gut and no drastic changes on the body weight is observed with *E. coli* colonized conventional birds (Parry et al., 1977). Body weight reduction with *Bacillus in ovo* injection is also contrary to formerly reported weight gain results reported as the effect of *Bacillus* supplementation (Fritts et al., 2000; Sen et al., 2012a) or *Bacillus subtilis in ovo* inoculation (Castañeda et al., 2021). The upregulation of pro-inflammatory genes and delay of hatching may not be the preferred response to probiotic usage in this context. Thus, it is questionable whether all results observed in the mono-associated environment are expressed with equal intensity in complex gut system with conventional microbiota. The test bacteria may be showing only one side of their characters.

As an example, in Chapter 3, a significant increase of relative liver weight was observed in *Bacillus* mono-associated embryos in contrast to a significant reduction of relative liver weight in the *Bacillus* supplemented group in the Simplified Microbiota study. Clearly, relative liver weight increase at mono-association in the embryonic study is considered as results of direct effect of the *Bacillus* strain to the host embryo. While we cannot rule out other causes, the increase in liver weight likely reflects sequelae in response to a systemic inflammatory response (Dapito et al., 2012; Iseri and Klasing, 2013). On the other hand, liver weight reduction in *Bacillus* supplemented Simplified Microbiota birds could suggest a reduced systemic inflammatory response. This opposite response to the embryonic mono-association model may be mediated by reduction in *E. coli* abundance observed in the *Bacillus* group. Indeed, *E. coli* increased liver and spleen weight (Iseri and Klasing, 2013) and a liver weight increase was also confirmed with *E. coli* ECL01 inoculated embryos in Chapter 4. If *E. coli* ECL01 in the Simplified Microbiota contributed to increased relative liver weight, reduction of ECL01 by the *Bacillus* treatment may have resulted in liver weight reduction. This combination of *E. coli* reduction and liver weight reduction was also confirmed in *Bacillus* supplemented conventional birds whereas liver weight gain was observed in *Bacillus* mono-association birds at 14 day (Hamaoka et al., 2010).

Thus, there is a possibility that liver weight reduction in *Bacillus* supplemented birds may be an indirect effect mediated via *E. coli* reduction. Indirect effects expressed through other bacterial species/strains are likely to be influenced by composition of the background microbiota. For example, *E. coli* counts in chicken ileum have been reported to vary widely, from 2.0 log

CFU/g to 8.1 log CFU/g (Rehman et al., 2007). It is possible that the liver weight reduction and potential systemic inflammatory response reduction effect by *Bacillus* is more likely to be observed in *E. coli* rich environment, and less likely with low level *E. coli* in the gut.

Johnson et al. (2018) reported an inverse relationship between relative percent abundance of *Escherichia* in the gut and body weight of broiler chicken. Thus, less *Escherichia* abundance was observed in heavier birds. Effect of *Bacillus* supplementation appears as reduction of Enterobacteriacea in the SM birds in agreement with original conventional birds and it is also reported by several scientist as summarized at Table 1.2 in the literature review of this thesis. Body weight gain reported as an effect of *Bacillus* probiotics may also be an indirect effect in result of *E. coli* reduction in the gut. If this hypothesis is correct, then the expression of the weight gain effect by *Bacillus* probiotic would depend on the number of *E. coli* in the gut, and the strength of the effect would also depend on the number of *E. coli* in the test environment. The negative effects of *E. coli* on body weight gain is also supported by results at Chapter 4 and may be explained as result of increased energy cost to immunity.

6.4 Future study

The ultimate goal of our work was to elucidate the mode of action of *Bacillus* probiotics in poultry production. We hypothesize two different types of mode of action, a direct pathway and an indirect pathway via complex gut microbiota, and we are challenged to separately observe the direct effect and indirect effect by controlling gut microbiota using a germ-free environment.

Therefore, creating a simple and effective experimental system that maintains a germ-free environment without contamination is a key outcome of this thesis. The disadvantage of the current individual container system is that quantities of water and feed supply cannot be easily provided without breaking the gnotobiotic environment. It is inferred that feed/water intake is an important factor in the bacterial colonization in the gut. By developing current individual container system to enable feeding and watering on an individual basis for at least several days after hatching, other gross parameters such as body weight gain, intestinal organ development, and immune functions may also be observed. To investigate more about the mode of action of *Bacillus* probiotics on poultry production, longer term trials will be required. If it is possible to establish an aseptic chick transferring method from the individual container to traditional germ-free isolators capable of accommodating a number of birds, it will allow us to have longer term trials after the sterility of

the hatched test chicks are confirmed. Contamination has always been the biggest issue of the germ-free chicken model and the main sources of the contaminant are the environment, water, feed, and the embryo or egg shell. If the shell and embryo are key sources of contaminating bacteria, then using chicks hatched in our individual containers and confirmed germ-free at 2-3 days of age as a source of chicks for post-hatch trials in larger isolators equipped with sterile feed and water could reduce the risk of contamination in these studies

In this series of trials, the test eggs were carefully selected from fresh laid eggs by weight, candling and physical observation. In most trials, a total of 200 to 300 eggs were needed to select 40 uniform test eggs/embryos without any defect. The quality of the parent birds is thought to be deeply involved in the contamination of the eggs. Therefore, this process of selection may need to be extended to controlling the quality of parent birds to have less variation in the results of germ-free chickens.

For further understanding on relationships between complex gut microbiota and *Bacillus* probiotics, more investigation about the gut microbiota itself is necessary. The studies of six different bacterial strains in this thesis are insufficient to understand the entire gut microbiota functionality and too small in number compared to the estimated number of the gut microbial species/strains. Thus, an accumulation of more knowledge through further mono-association studies using different bacterial species is clearly needed. However, it is unrealistic to investigate all bacterial strains in the gut by mono-association studies. Therefore, an effective selection process for the test organisms would be a key factor of future trial strategies. A computational approach has already been tested by Faith et al. (2014) to identify effector strains from their bacterial libraries. Since, predominant bacterial species in the gut may not always be a strong influencer of target host phenotypes, systematic computational and experimental approaches will be important for further probiotic mode of action studies. In this series of studies, we made a quick leap from mono-associated study to SM bird study with a combination of 5 different bacterial strains. However, further accumulation of knowledge on the symbiotic relationship between bacterial species/strains can be expected by conducting combination of two or three species step by step. In particular, combination studies would provide new insights into the colonization process of bacterial species which could not establish stable colonization in mono-associated condition, such as *Bacteroides fragilis* BCT06, *Clostridium butyricum* CLS01, and *Lactobacillus salivarius* LCT01.

Ultimately, these combination studies could be developed into Simplified Microbiota formation in young chickens and it could be developed into a simple pathogen challenge model in the future. The symbiotic ability of gut microbiota to inhibit pathogen colonization in the gut is considered to be the results of direct killing, nutritional competition, and enhance immune development and response (Pickard et al., 2017). By changing the specific bacterial species in the Simplified Microbiota, direct killing process or nutritional competition processes will be revealed more clearly and simply in the challenge model. This challenge model can be used to examine not only the mechanisms of disease manifestation, but also the countermeasures against it, such as probiotics, prebiotics, essential oils, or combinations of them. For both purposes, there will be a high demand for this repeatable and simple short term challenge models.

In addition to these future improvements on the trial system, further validation is necessary for the results obtained in this series of study. Firstly, the direct effect of *Bacillus* probiotics confirmed in this study needs to be carefully re-confirmed by comparison of mono-associated studies and conventional (SM) studies. The direct effects observed here in mono-association, may not occur in the presence of other bacteria, or it could be a general reaction of the host against any bacterium. Alternatively, other direct effects could be expressed only in symbiotic relationships with other bacteria. If the direct effect is confirmed in the conventional environment and mono-associated environment both in same direction, the effect can be considered as part of the *Bacillus* probiotic mode of action pathway.

As discussed at former sections, germination and sporulation processes are likely to play an important role in the direct and indirect mode of action of *Bacillus* probiotics both. Germination of *Bacillus* occurs in the gut of chicken embryos even without water and feed. Simple germination studies could be conducted in the gut of the embryo to investigate the effect of germination on the host or metabolisms of other bacterial species by controlling the specific bacterial species present. Knocking out specific functions of the *Bacillus* probiotic strain will also be effective to drive the results.

The results of this study also suggested the existence of an indirect effect of *Bacillus* probiotics on liver weight and further research around liver function would be beneficial. The liver is known as a key frontline immune tissue (Kubes and Jenne, 2018) and a beneficial role of the microbiota in maintaining liver homeostasis is starting to be recognized (Mazagova et al., 2015). The opposite observation on liver weight changes in *Bacillus* treatment in mono-association and

in conventional microbiomes may also appear in immune system related indices with a similar trend. The increase of liver weight in *Bacillus* mono-association was the results of immune activities against the *Bacillus*, and the decrease of the liver weight in *Bacillus* supplemented SM birds appears via *E. coli* reduction by *Bacillus* strain. This hypothesis may be reinforced or rejected by conducting further exploratory research into this indirect effect. Of course, additional experiments should be conducted with SM birds in terms of reproducibility and clarity of understanding of the results. From this point of view, efforts to improve the Simplified Microbiota composition are also required to select the best constituent bacterial species for adding suitable complexity in accordance with the objectives as discussed above.

Although it is not the original purpose of this study, it would be desirable to investigate the mechanism and countermeasures for the lethality of *E. coli* infection before E19, which was confirmed in this study. The analysis of the defense mechanism of the chicken embryo around E19 would be an interesting topic separate from the mode of action of *Bacillus* probiotics. It may give us suggestions for the best timing of bacterial inoculation or stimulating the immune system of chickens for better productivity. Further investigation is expected on the egg yolk enlargement confirmed with *E. coli in ovo* inoculation. Removing the yolk at hatch has been shown to cause growth retardation (Noy and Sklan, 1997). In other words, the residual yolk at hatching contributes to subsequent growth, and the health of the embryo during incubation may affect subsequent growth through the quality of the residual yolk. The first step would be nutritional comparison of residual egg yolk between healthy neonatal chicks and *E. coli* infected chicks.

Lastly, future developments in analytical methods will allow us to investigate mode detail of current samples or current data sets. RNAseq will allow pathway analysis beyond the qPCR mRNA expression we performed in this series of trials. Even for microbiological assay, the latest molecular technology may bring changes to the current traditional germ-free model. The germ-free status has been confirmed by culture-based methods and current studies focusing on live bacteria in the trial system. Technically, the current germ-free system is not free from the effect of dead bacteria in the environment or sterile feed or water. Remaining cell wall or DNA fragments in the system may have a role or may cause unexpected variation in the results. Beyond that, there might be new pathways which connect parents and eggs we have overlooked using culture-based methods. The latest or future molecular technologies may reveal new aspects of the germ-free environment models.

6.5 Conclusions

There are many unknowns about the effects and mechanisms of action of *Bacillus* probiotics. One of the major reasons for this is the instability and low reproducibility of the results. Especially, instability of the trial results has made it difficult to reach a clear conclusion on both efficacy and mechanism of action. To answer this question, we hypothesized that two different mechanisms of actions might be available for the *Bacillus* probiotics, such as the direct effect of the probiotic on the biological function and physiology of the host animal, and the indirect effect via shifted microbiota by the probiotic supplementation. According to this hypothesis, the strength of the indirect effect will be influenced by the balance and composition of the intestinal microbiota of the test animals. Thus, test animals with different microbiota balances are expected to respond differently to the same probiotics, which may explain the instability of probiotic efficacy test results that has long troubled scientists. However, in the conventional environment, it is not possible to observe the direct and indirect effects separately. Therefore, we planned to construct a test system that could verify the two kinds of effects by taking advantage of a germ-free environment and mono-association status with the newly developed HEPA filtered individual canister model. In addition, we tried to simplify and speed up the trial by using not only ex germ free chickens but also germ-free embryos as test animals.

Through this series of study, I think the usefulness of the concept of using a germ-free environment to access the direct and indirect effects of *Bacillus* probiotics on development of chicken, which was the main objective of this paper, was demonstrated. Multiple effects were observed as a direct effect in *Bacillus* mono-associated embryo, such as significant increase of incubation time to hatch, yolk sac weight at hatch, and decrease of yolk sac free body weight. Modification of ileal gene expression was also confirmed with *Bacillus* mono-association and it may be suggesting the involvement of *Bacillus* supplementation in host immune response and nutrient transporters. It is still unclear that direct effects identified in mono-associated environment are occurring in a conventional environment. It is thought that some effects may only appear when other bacteria are not present, and others may appear even in the presence of a complex community. However, by using mono-associated status, the direct effect of *Bacillus* probiotics is clearly and separately shown.

In addition, comparison of the effect of *Bacillus* in a mono-associated condition with the effect in Simplified Microbiota trial suggested the existence of an indirect effect of *Bacillus* probiotic based on changes of liver weight. *Bacillus* supplementation significantly increased liver weight in mono-association but decreased liver weight under the Simplified Microbiota. This contradictory result could be explained by the effect of *Bacillus* probiotics on *E. coli* reduction confirmed in SM birds and liver weight increase in *E. coli* mono-associated birds. Thus, *Bacillus* probiotics itself increase liver weight, but also decrease liver weight by reducing another factor that increased liver weight, then *Bacillus* appears to reduction of liver weight in conventional environment. This hypothesis can be derived only by conducting trials in mono-association and conventional environments simultaneously, so this is also supporting usefulness of our concept in this series of study.

The clear mechanism of action of *Bacillus* probiotics has not been elucidated. The intestinal environment is very complex, and the mode of action of *Bacillus* probiotics may not be a single pathway but is highly likely to be expressed by a combination of direct and indirect pathways. Therefore, further difficulties are expected in fully understanding the mode of action of *Bacillus* probiotics in chicken production. However, with the development of these trial models in the germ-free environment and new analytical methods in the future, it must be possible to accumulate knowledge regarding the mode of action of *Bacillus* probiotics.

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8   APPENDIX

8.1   Trial egg (embryo) selection
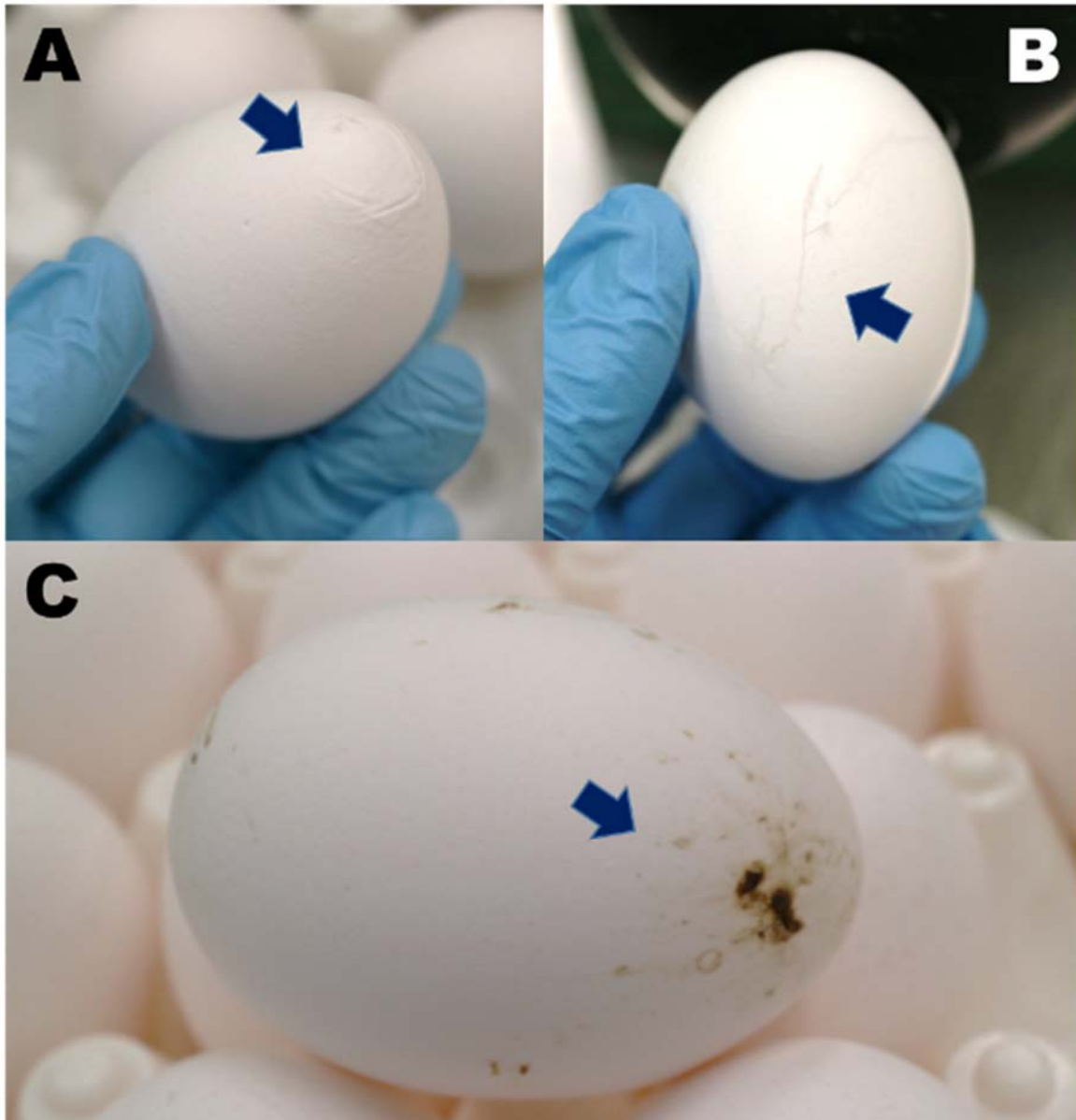
Figure 8.1 At the beginning of the trials, all fresh fertilized eggs were visually checked before candling and weighing. Photograph is showing example of eggs unsuitable for germ-free trials. Uneven surface (Panel A), cracking (Panel B), or eggs with fecal contamination (Panel C).
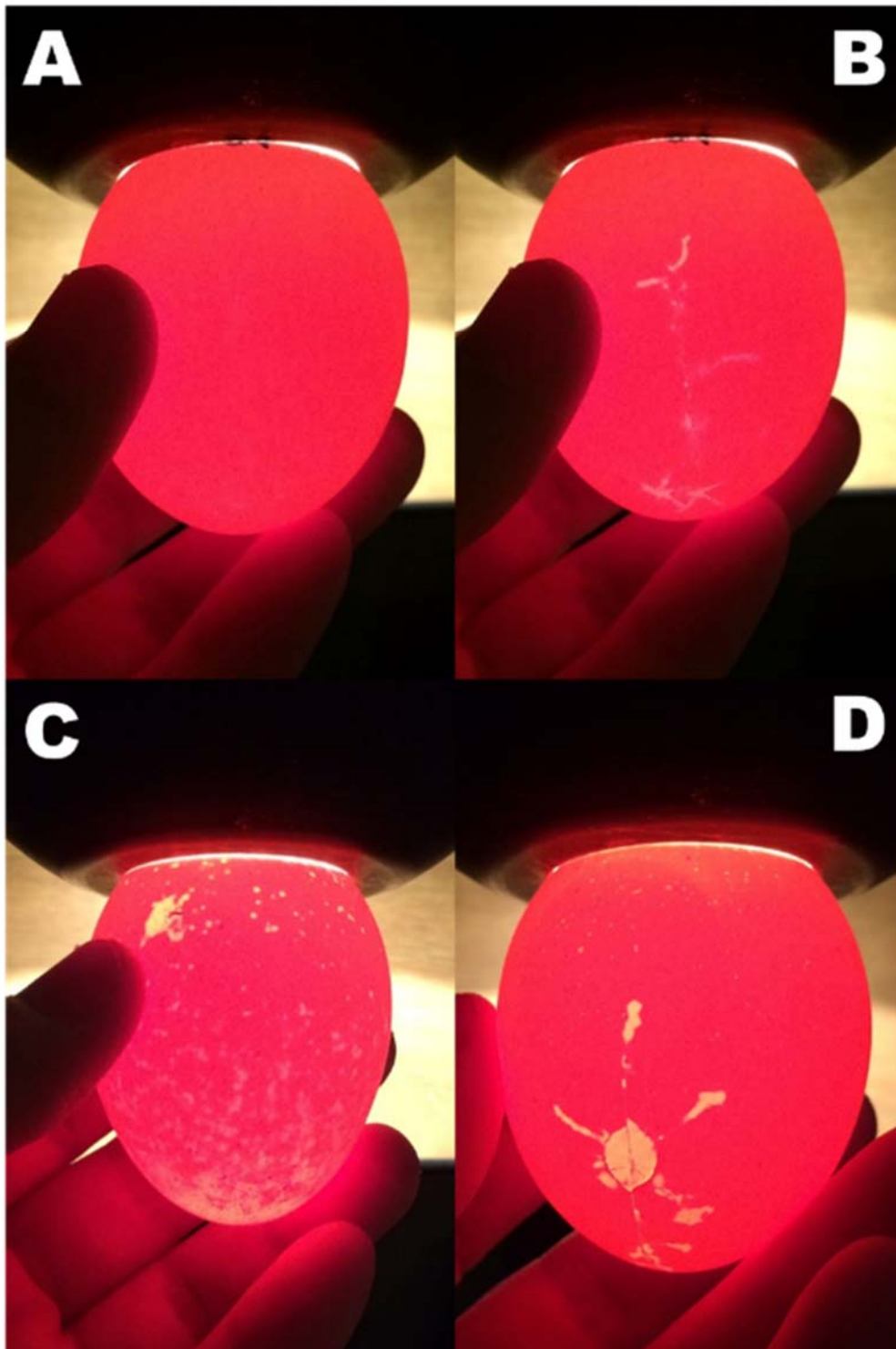
Figure 8.2 After visual checking, all eggs were candled to remove eggs with defect. Photograph is showing example of candling results of E0 eggs for germ-free trials. Healthy egg (Panel A), cracked (Panel B), spotted (Panel C), and damaged (Panel D).
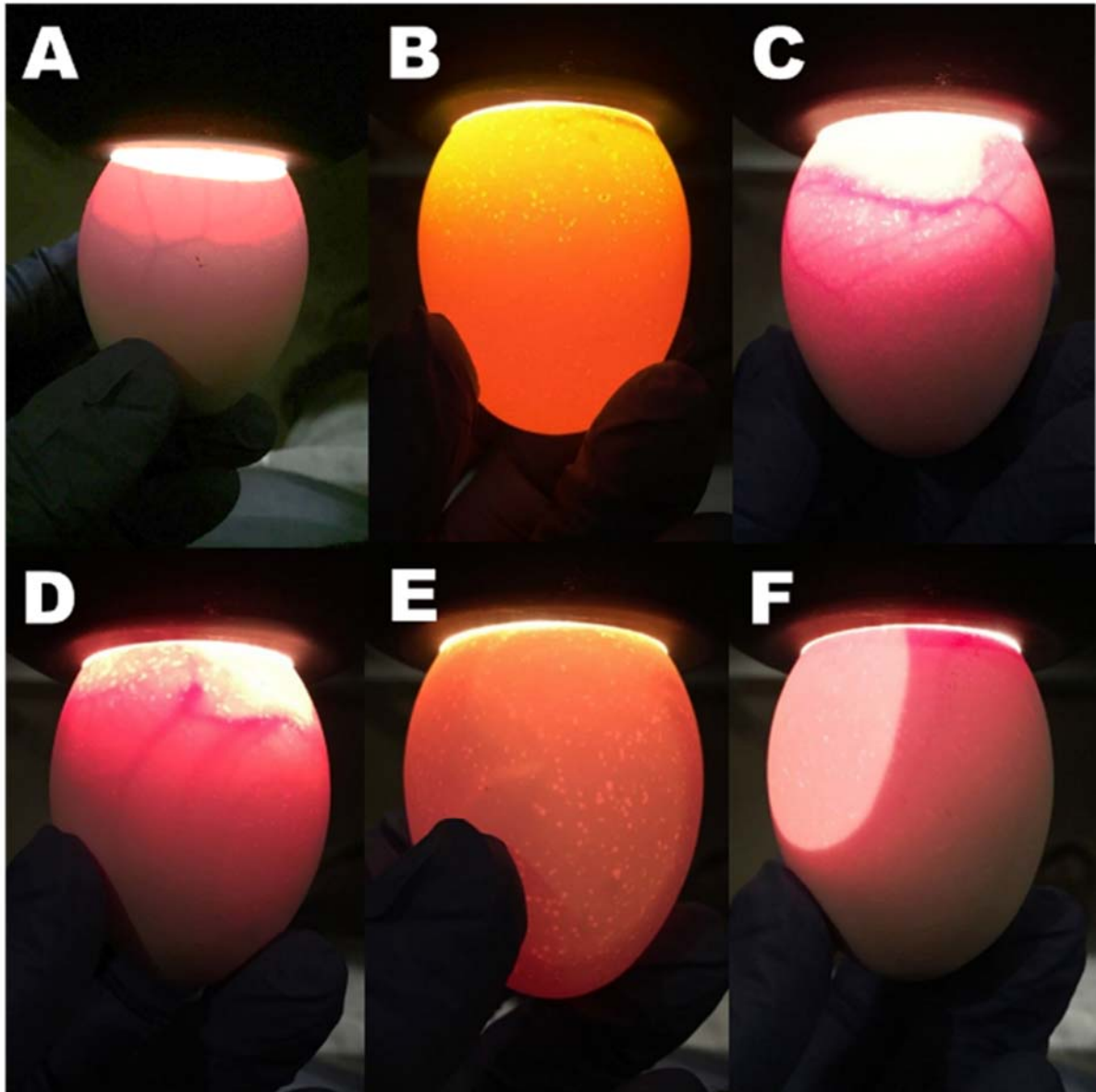
Figure 8.3 At the end of day 17 (E17), all incubated eggs were candled again to discard eggs containing undeveloped chicken embryos and with weight lose greater than the mean weight loss for all incubated eggs plus 1 standard deviation. Photograph is showing example of E17 eggs candling results, healthy E17 egg (Panel A), unfertilized or early dead (Panel B), abnormal air cell (Panel C and D), rotten due to in egg contamination (Panel E), and bad air cell location (Panel F).

8.2   Germ-free isolator (Chapter 5)
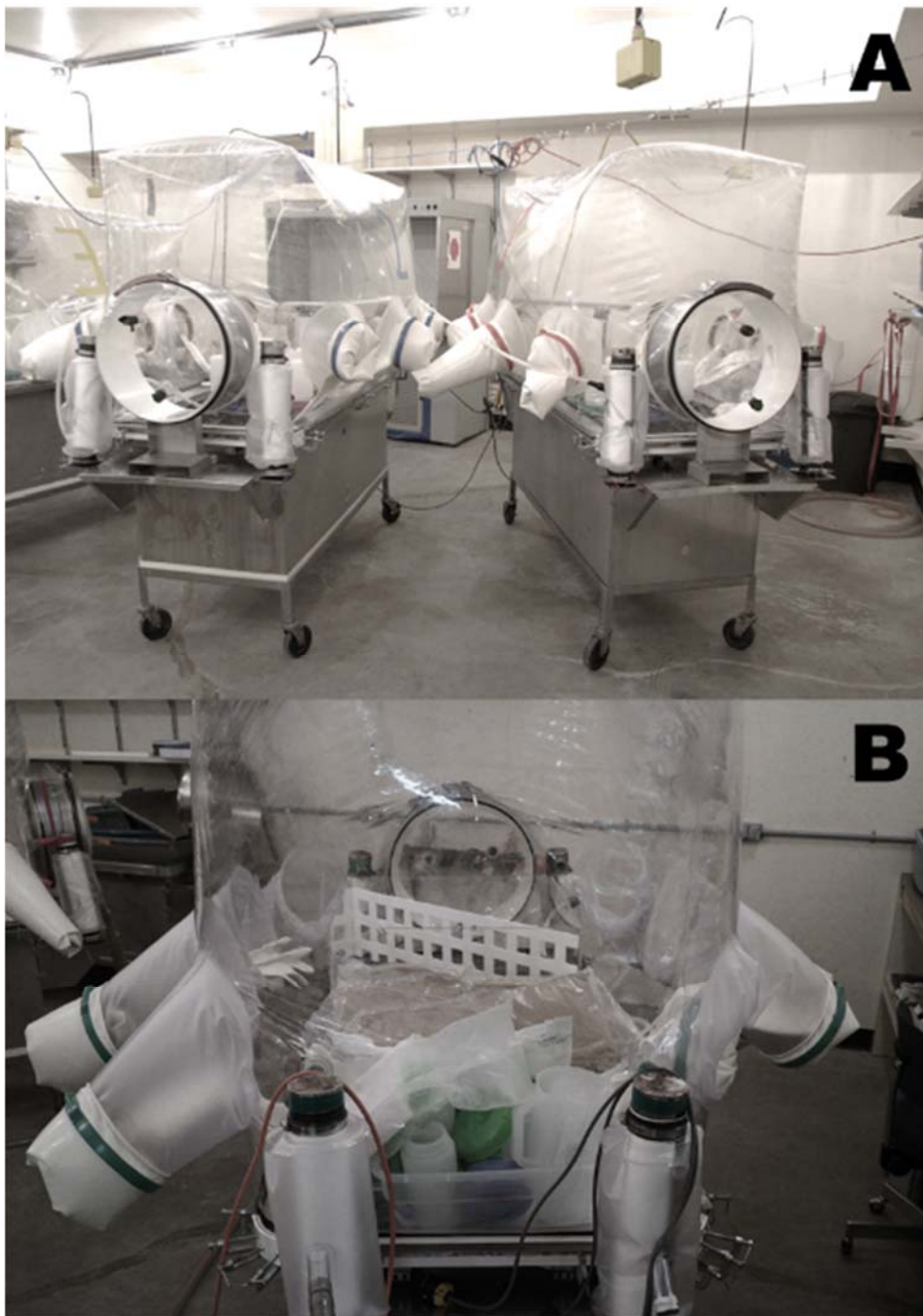
Figure 8.4 Photograph is showing germ-free isolator utilized for Simplified Microbiota trial at Chapter 5, inflated germ-free isolators from entry port side (Panel A), isolator from bottom side (Panel B).
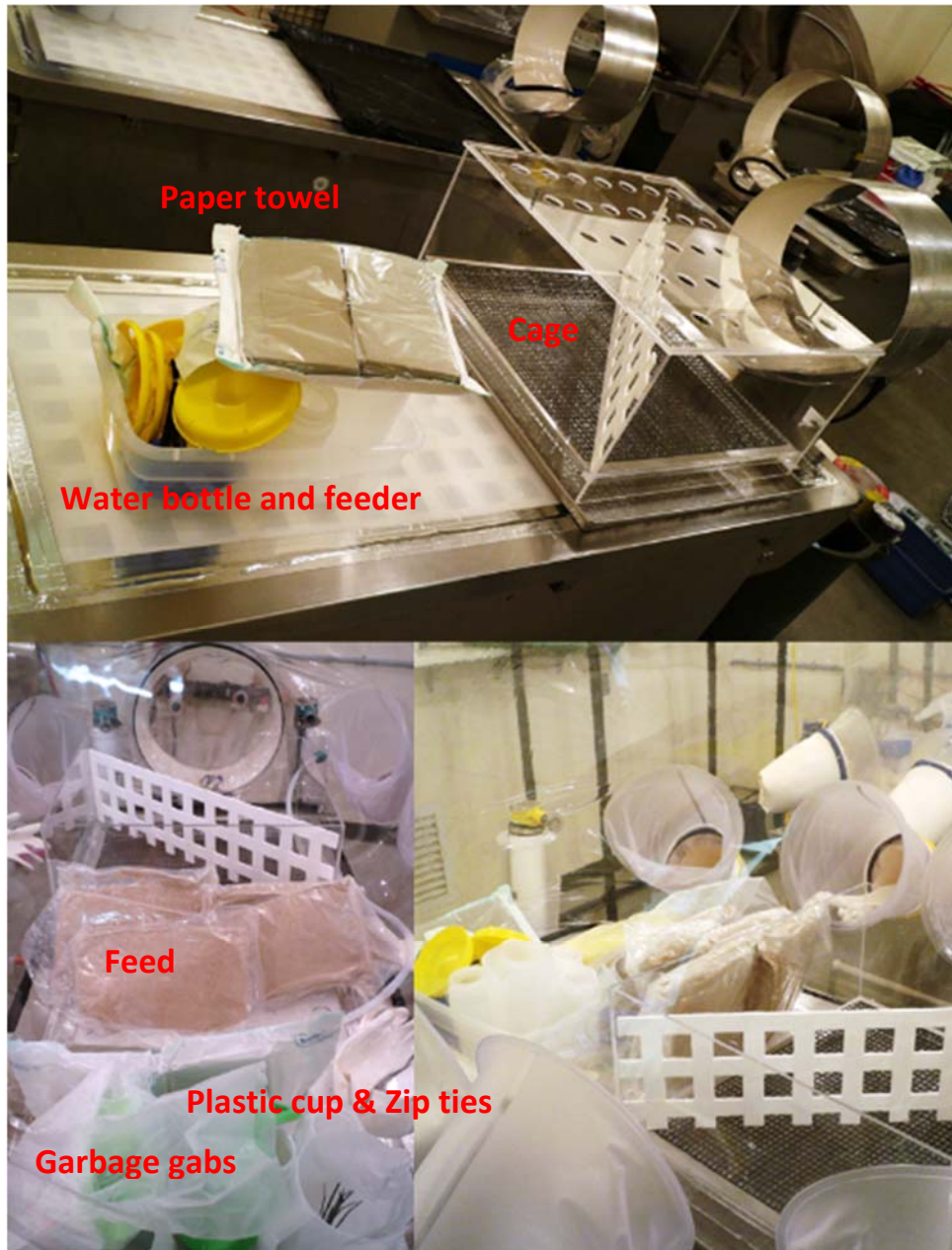
Figure 8.5 Photograph is showing items in the germ-free isolator utilized for Simplified Microbiota trial at Chapter 5.
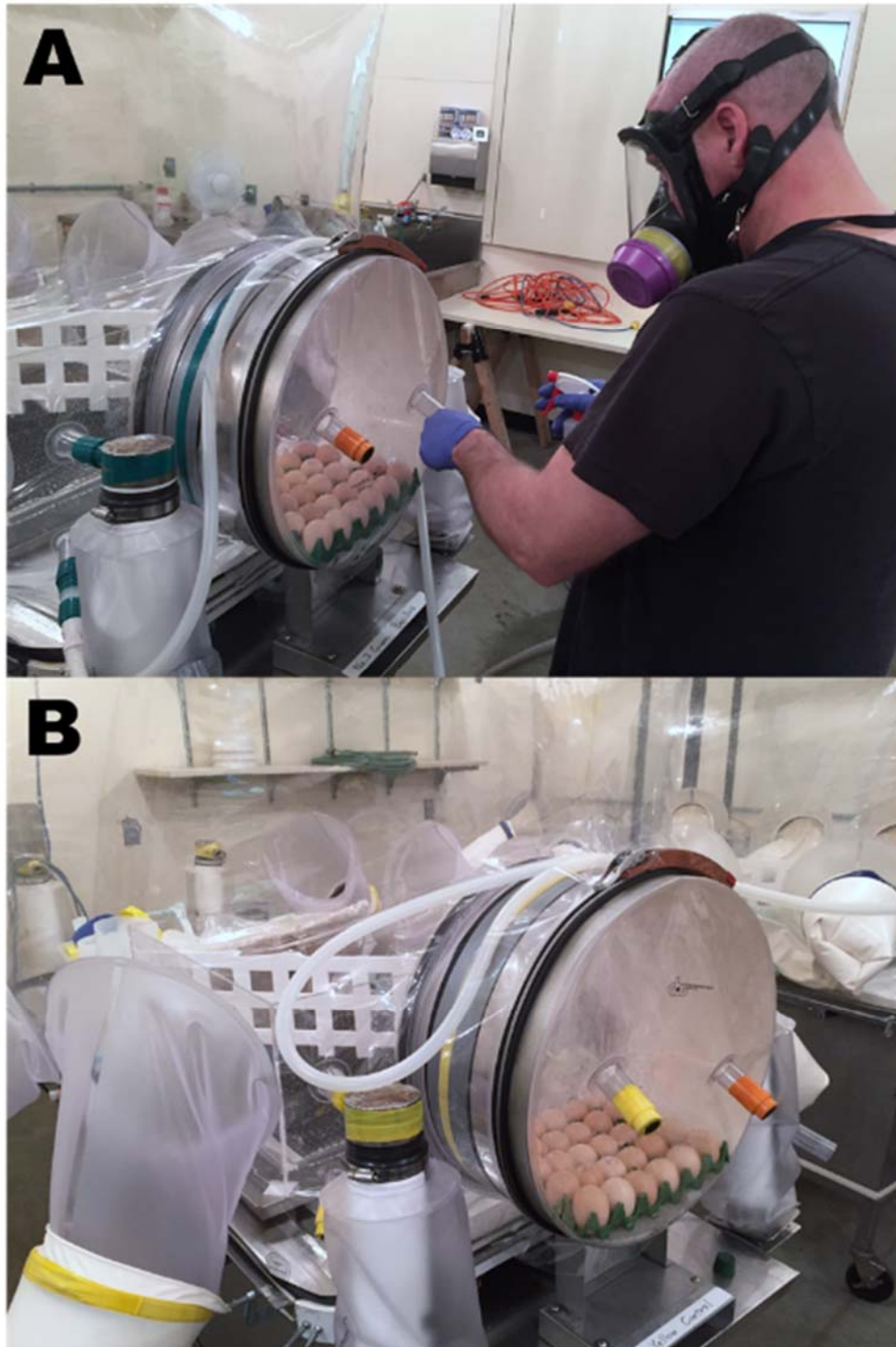
Figure 8.6 At day 19 of incubation, test eggs were transferred to HEPA filtered isolator from entry port. Eggs were re-sanitized by exposure to 2% of paracetic acid solution for 12 min within the isolator entry port chamber before introducing to inside (Panel A and Panel B).

Figure 8.7 For Simplified Microbiota trial, 8 healthy male and 8 healthy females were selected from healthy hatched birds by feather sexing resulting in 16 birds per isolator.

Figure 8.8 At 7 d of age, birds (4 male and 4 female per treatment) were removed from entry port for the were sterilized again after the sampling and the entry port was kept closed until next sampling at 14 d of age.

8.3   Sterile individual hatching jars (Chapter 3 and 4)
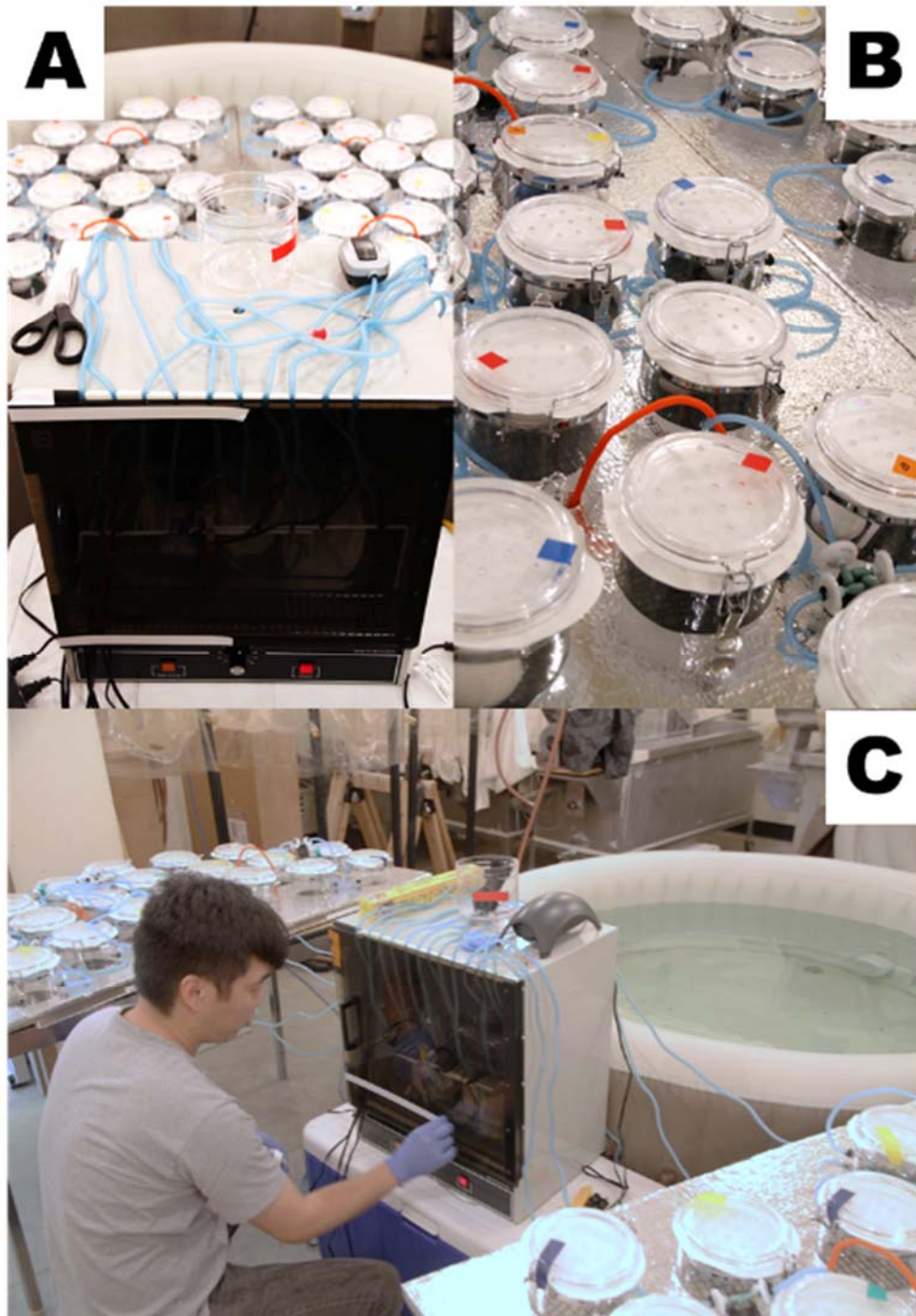
Figure 8.9 Photograph is showing sterile individual hatching jar system utilized for *in ovo* injection mono-association trials at Chapter 3 and 4. Sterile humidified air was provided from air pumps in sterile incubator (Panel A). All jars were floated in hot tub (Jacuzzi) (Pane B). All jars were connected to the system before introducing sterilized eggs (Panel C)
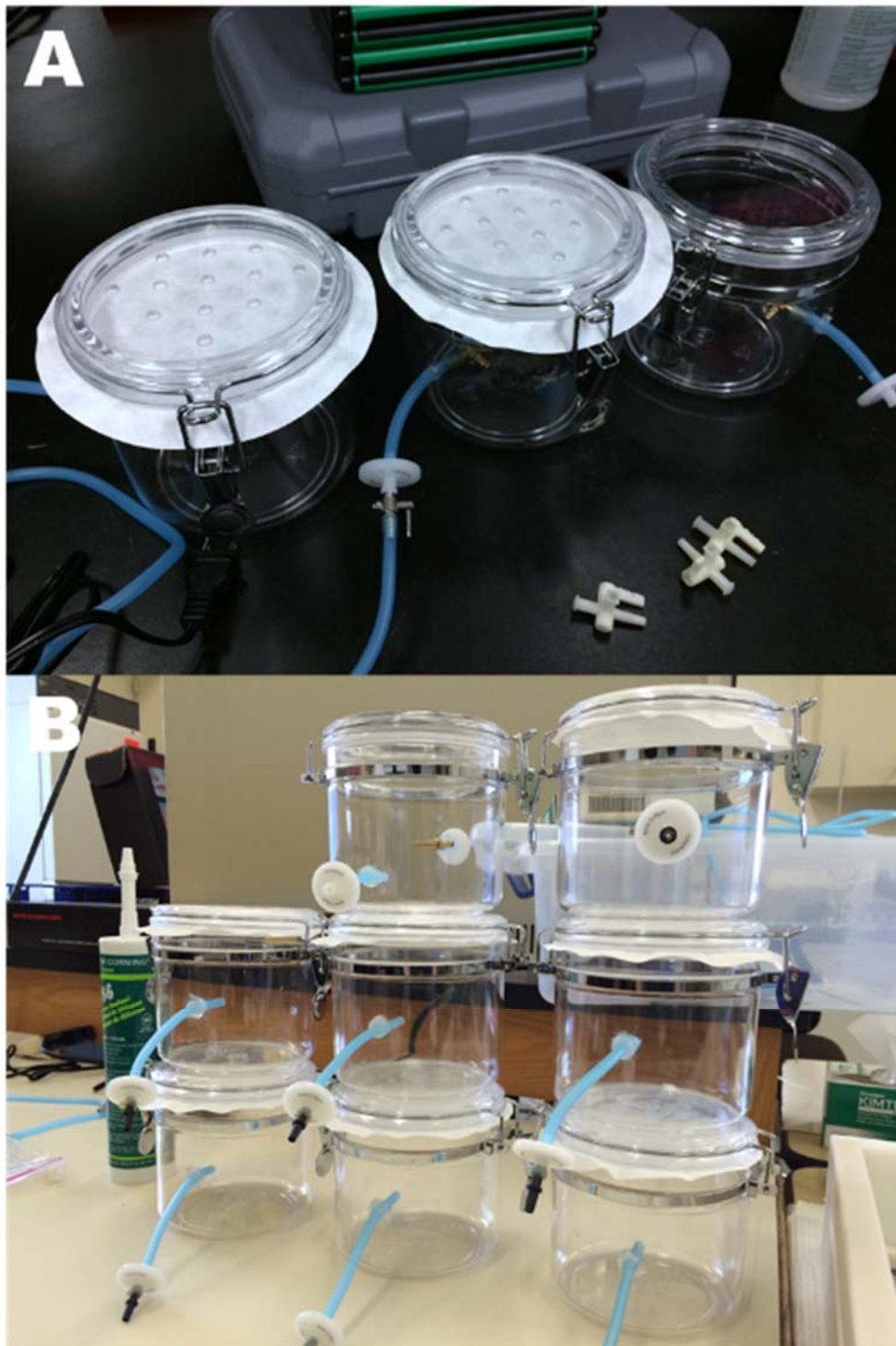
Figure 8.10 Photograph is showing sterile individual hatching jar for *in ovo* injection mono-association trials at Chapter 3 and 4. Several prototype were made (Panel A). In the final form, sterile humidified air is introduced from bottom silicone tube on the side wall and air goes out from top after filtration (Panel B)
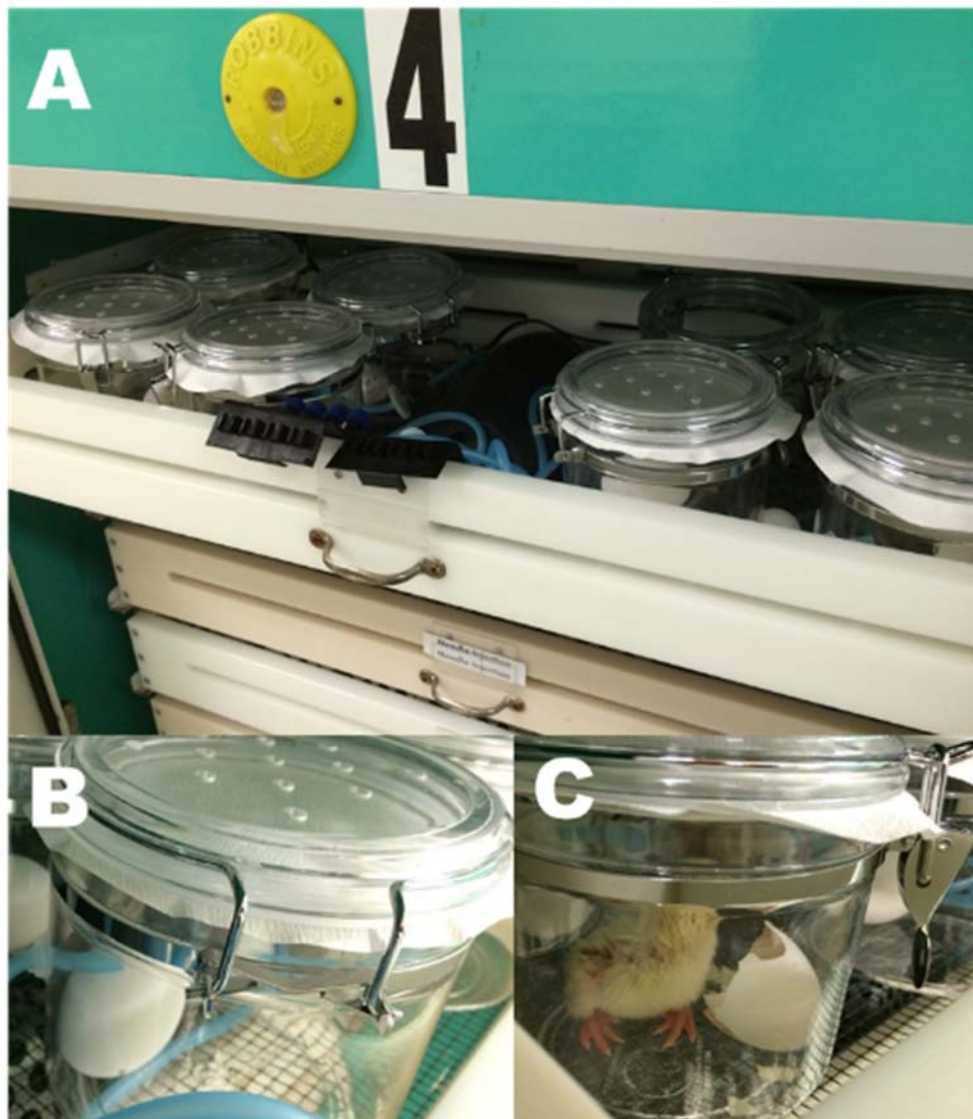
Figure 8.11 The sterile individual hatching jar system was firstly tested in traditional hatcher (Panel A and Panel B). No defect was confirmed on hatchability and germ-free status of all hatched birds were confirmed (Panel C).

Figure 8.12 To improve flexibility on observation and sampling, the sterile individual hatching jar system was run with water bath instead of hatcher (Panel A and Panel B). *In ovo* inoculation was also tested with sterilized food dye and no defect was confirmed on both hatchability and sterility of test chicks (Panel C).
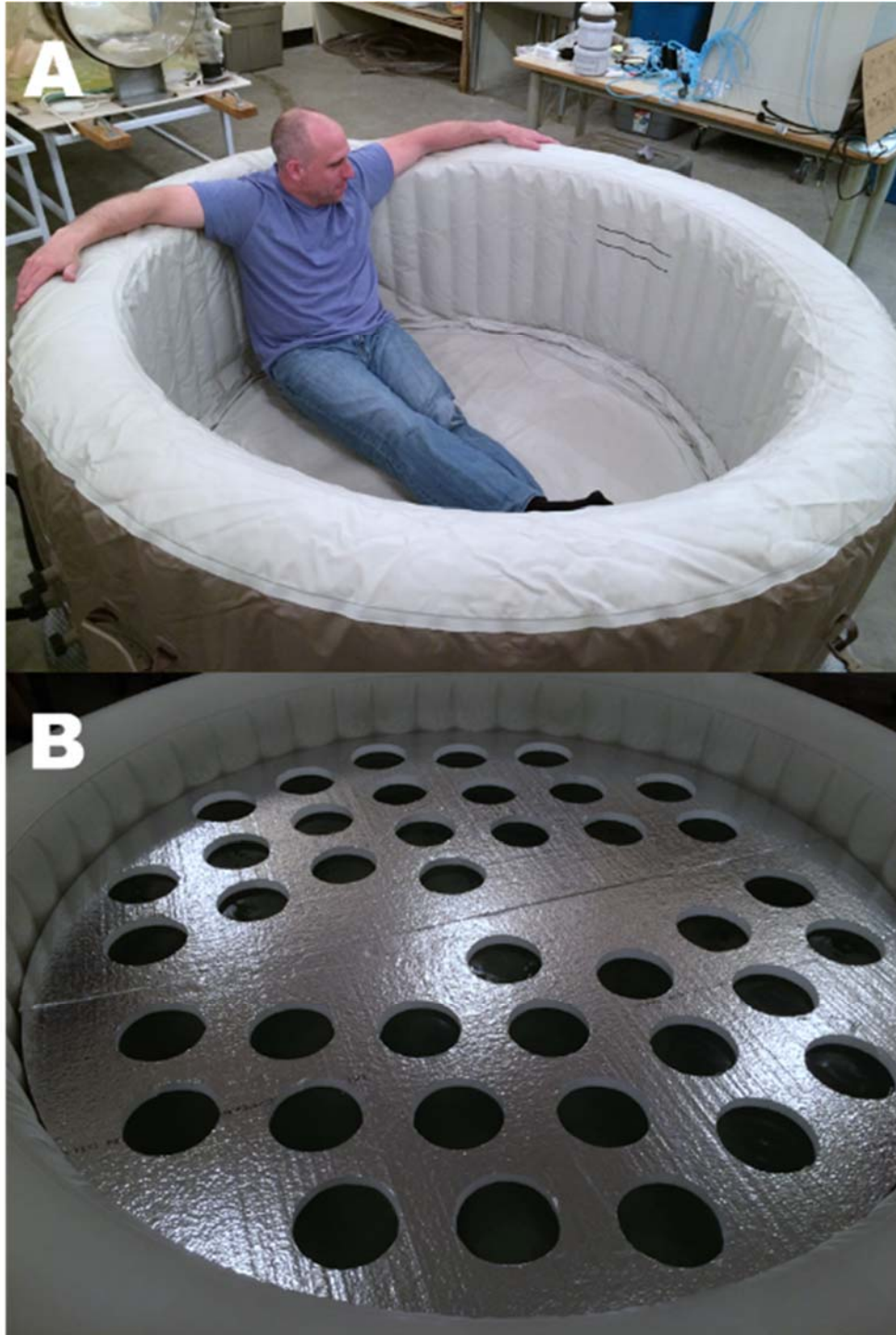
Figure 8.13 To increase capacity of the sterile individual hatching jar system, hot tub (Jacuzzi) was introduced to the lab (Panel A and Panel B). With the hot tub, the system can carry total 40 individual jars at a same time.
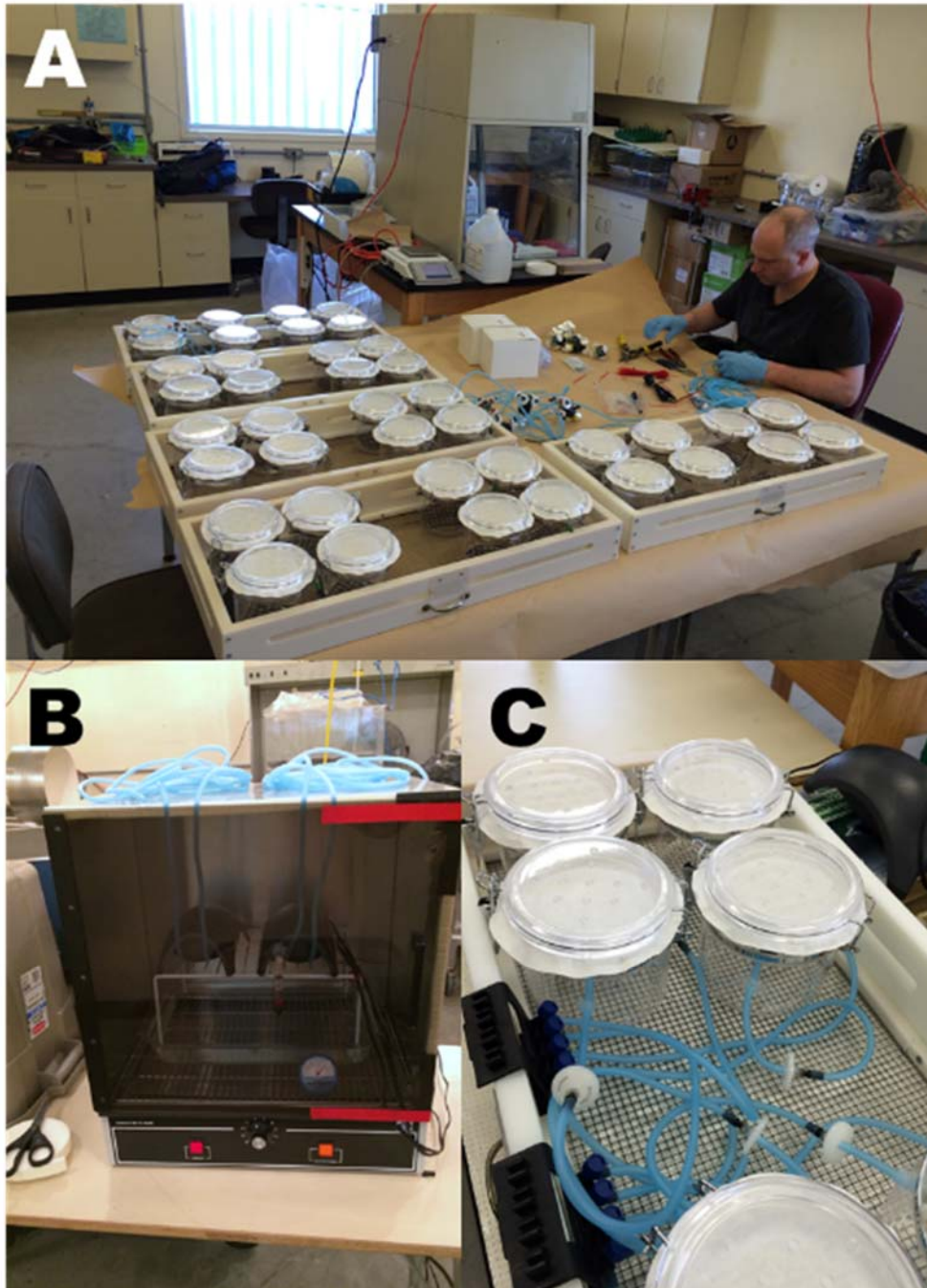
Figure 8.14 All piece of the sterile individual hatching jar system were re-assembled after careful cleaning. Once it's assembled, all system were fumigated to sterile inside and outside both (Panel A, B, C)
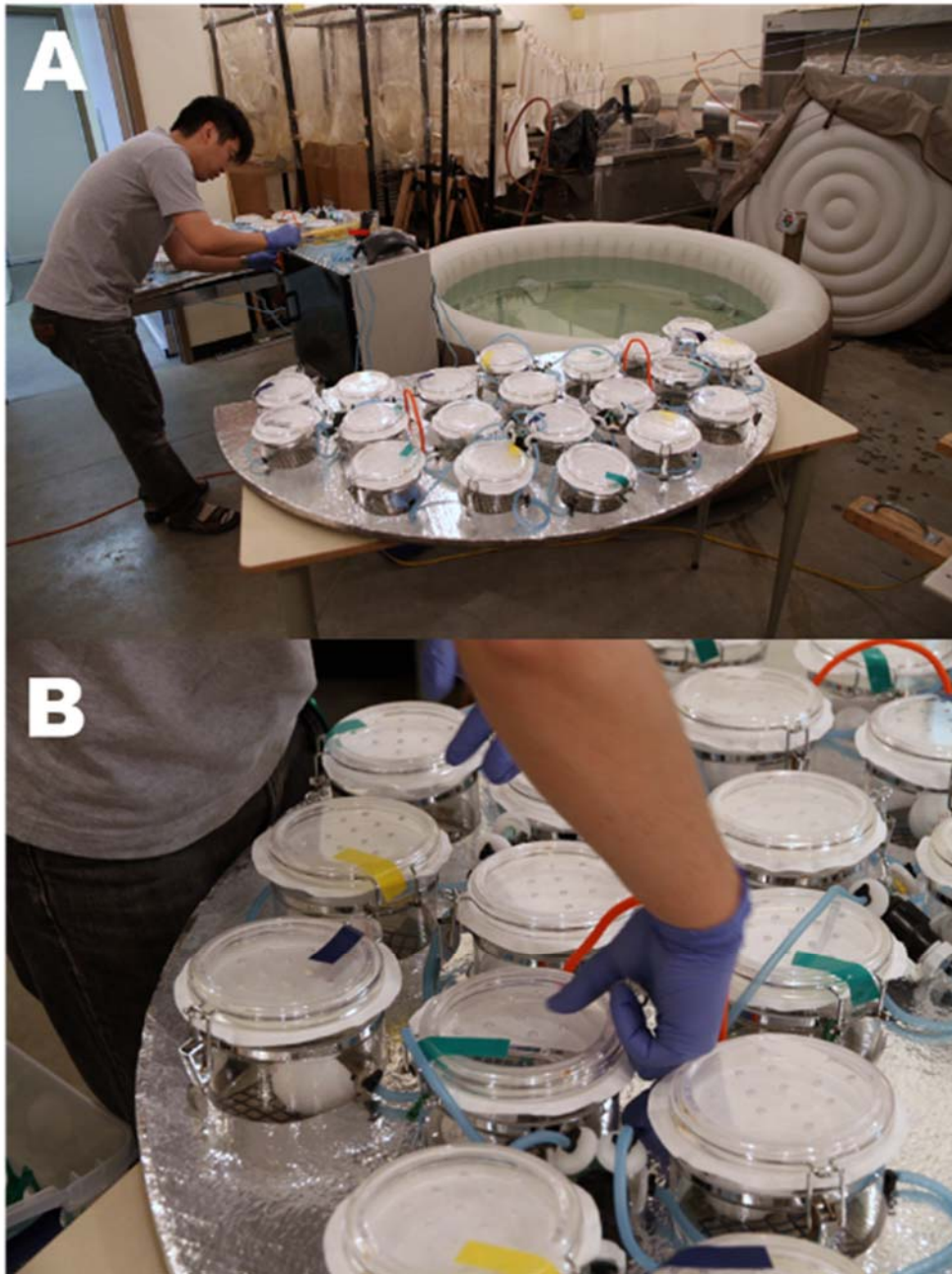
Figure 8.15 At day 17 of incubation, the eggs were placed into the sterilize individual hatching jars from top side of each jar (Panel A and Panel B).
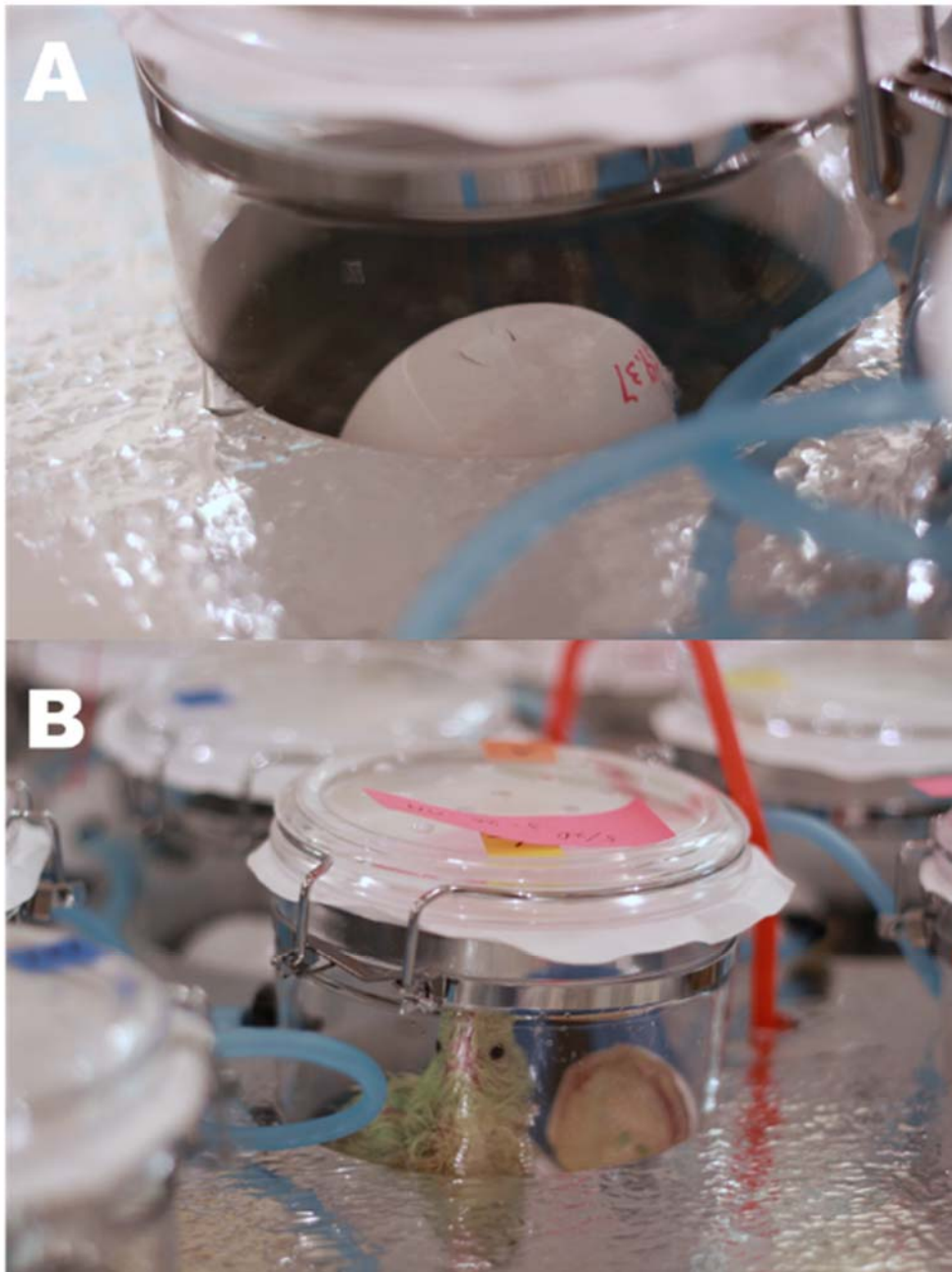
Figure 8.16 Clear floating jar brought flexibilities on observations and sampling. Start of pipping could be observed and recorded bird by bird (Panel A) and sampling could be performed at exact hatching time (Panel B).
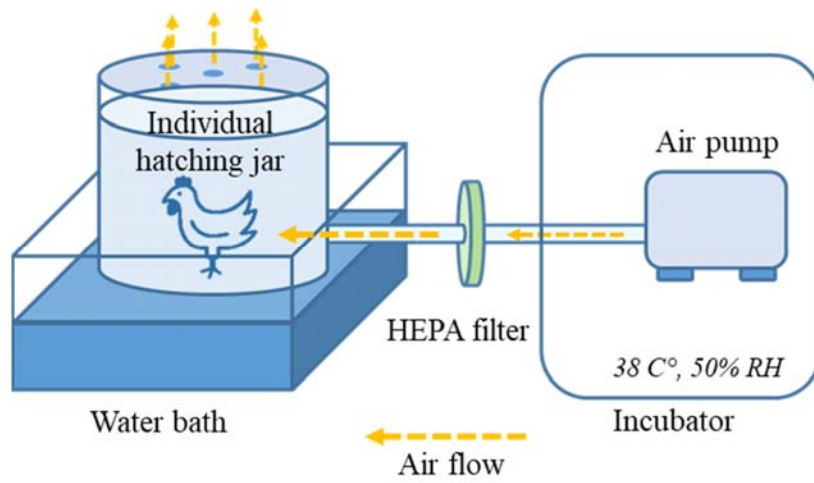
Figure 8.17 Simple diagram of the sterile individual hatching jars used in Chapter 3 and Chapter 4.