INTERACTION OF *BACILLUS SPP*. AND *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM IN IMMUNE/INFLAMMATORY SIGNALING FROM SWINE INTESTINAL EPITHELIAL CELLS

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ABSTRACT

Previous research evaluated a laboratory strain of *Bacillus licheniformis* (BL) in a model swine epithelium and found it exerted anti-inflammatory effects on *Salmonella enterica* serovar Typhimurium (S)-induced secretion of interleukin-8 (IL-8). The current investigation evaluated the anti-inflammatory actions of *Bacillus* bacteria available commercially as feed additives for the swine industry. Three isolates were obtained from the product, two *Bacillus subtilis* (BS1 and BS3) and one *Bacillus licheniformis* (BL2). Swine jejunal epithelial IPEC-J2 cells were seeded into wells on permeable membrane supports and allowed to form confluent monolayers. Treatments included apical pretreatment with BL, BS1, BL2, or BS3 for 17 h without S, and the same *Bacillus* treatments but with 10^8 CFU S added in the final 1 h of *Bacillus* incubation. Two additional treatments included negative control wells receiving no bacteria (C) and positive control wells receiving only S. Following bacterial incubation, wells were washed and fresh media containing gentamicin was added. Cells were incubated for an additional 5 h, after which apical and basolateral media were recovered for quantitation of IL-8 and bacitracin. In addition, inserts with epithelial cells that had received S were lysed and lysates cultured to determine treatment effects on S invasion. Exposure to S alone provoked an increase in IL-8 secretion from IPEC-J2 cells compared to C wells ($P < 0.001$ for both the apical and basolateral directions). Pre-treatment with each *Bacillus* isolate followed by challenge with S reduced S-induced IL-8 secretion in both apical and basolateral compartments compared to the wells receiving only S ($P < 0.001$; except for BS3 apical, $P < 0.01$). Secretion of bacitracin could only be detected in BL2 and BL2+S. Fewer S colonies could be cultured from lysates of BL2+S than S, BS1+S, and BS3+S treatments ($P < 0.001$). Results suggest that *Bacillus subtilis* and *Bacillus licheniformis* have the ability to intervene in secretion of the neutrophil chemoattractant IL-8 from swine intestinal epithelial cells. This effect on chemokine secretion by gastrointestinal epithelial

cells in vitro could not be explained solely by production of bacitracin or reduced invasion of epithelial cells by S.

Keywords: *Bacillus*, *Salmonella*, Swine

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Chapter 1:

Review of the Literature

INTRODUCTION

The use of in-feed antibiotics in animal production has been a common practice, over the last several decades, in both the United States and worldwide. In 1969, The Swann committee report initiated the first inquiry on the impact of the use of non-therapeutic antibiotics on human health and the environment (Swann, 1969). Sweden, in 1986, was the first country to ban all growth promoting antibiotics and was followed shortly by all the members of the European Union. Currently, the United States allows the use of in-feed antibiotics but the debate around their possible ban is intense. The public and several major fast food chains, such as McDonalds and Chipotle, are pushing the livestock industry to discontinue this practice and use "antibiotic free" production as a marketing tool. For example, a billboard posted along Interstate 70 in Kansas advertising Chipotle Mexican Grill displayed the following message (seen in April, 2007): "Did you want antibiotics with your lunch? We didn't think so. All meats served in Kansas City are naturally raised".

Although the use of in-feed antibiotics remains the dominant form of production for nursery pigs in the United States, there is a growing market for pork produced without in-feed antibiotics. Thus, the industry is seeking alternatives to the use of in-feed antibiotics, which provide the same health benefits and ensure the same performance during production but are safer for the environment. One of the possible alternative feed additives is direct-fed microbials, also known as probiotics.

In the current chapter, an overview is presented of the debate over antibiotics vs. probiotics, including the main legislation and relevant regulations. Then, information is provided about probiotics and more specifically about probiotics containing *Bacillus* spore forming bacteria. The focus is mainly on their benefits within the gastrointestinal tract and their effects on livestock performance. In Chapter 2, new data are presented on the interaction

of *Bacillus spp*. and *Salmonella enterica* serovar Typhimurium in immune/inflammatory signaling from a model swine gastrointestinal epithelium in vitro.

PROBIOTICS VS. ANTIBIOTICS

Probiotics are defined as ‘live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001). This concept has a long history of health claims. For example, in a Persian version of the Old Testament (Genesis 18:8), it states "Abraham owed his longevity to the consumption of sour milk." However, the interest in probiotics for use as direct- fed microbials for livestock is only a few decades old.

Antibiotics. There is a considerable body of literature documenting the use of antibiotics as feed additives in livestock. The term antibiotic refers to natural or synthetic compounds that in, low concentrations, inhibit the growth of or kill microorganisms. They are used to treat bacterial infection in humans and animals. Over the years, the increase in prophylactic use has led to concerns for the potential development of bacterial antibiotic resistance.

The efficacy of antibiotics is associated with the dosage and duration of the treatment. The violation of this simple association may, over time, result in the problematic development of antibiotic resistance and increased risk of bacterial outbreak. For this reason, it is indeed prudent for the livestock industry to be proactive in considering the potential ramifications of the improper use of antibiotics.

In the United States, the use of antibiotics in human medicine is not the most important when compared to use in livestock and poultry production. Meat production is estimated to account for up to 70% of the total United States antibiotic consumption and is by far the greatest single consumer of antibiotics (Mellon et al., 2001). Moreover, this consumption is mainly for non-therapeutic use. A few decades ago, the main concern of the livestock industry was to provide customers more products at a lower price. Statistics,

compiled in the 1990s (Miller et al., 2003), show that, for an average swine facility, in-feed antibiotics boost the daily growth and reduce death rates during production. Similar studies have been conducted in other animal species and resulted in the same conclusions (Cook, 2004; Graham et al., 2007; Samanidou et al., 2008). Later, large-scale production and the consumer demand for product consistency was an additional stimulus for the use of in-feed antibiotics to provide standard-sized market animals. Collectively, the confinement meat production industry had found a way to ensure the growth and reasonably consistent end weight of animals and decrease the risk of diseases even in high confinement husbandry.

Despite the advantages of the use of in-feed antibiotics in meat production, contemporary market demands, including the emerging natural and organic markets, are bringing greater and greater pressures to eliminate the practice all together. Studies have reported the occurrence of bacterial antibiotic resistance due to the use of the same antibiotics in both humans and animals (Wegener, 2003). Furthermore, the presence of animal antibiotic residues have been found in vegetables (Kumar et al., 2005a), water (Yang et al., 2003; Kumar et al., 2005b) and air (Chapin et al., 2005). Such results call the use of antibiotics into question and encourage the meat industry to find alternative solutions. Currently the trend is to promote a drastic decrease of the use of antibiotics, both in humans and in livestock production.

Relevant legislation and regulations on antibiotics. In 1986, Sweden banned all growth-promoting antibiotics on the basis of "precautionary principle". Then, from 1997 to 1999, the European Union banned the use of avoparcin, bacitracin, spiramycin, tylosin and virginiamycin, on the same basis (Casewell et al., 2003). Since January 2006, all growth promoting antibiotics are forbidden in European meat production. Only the use of antibiotics as a curative is authorized in livestock but in a strictly controlled fashion (Samanidou et al., 2008). Following the example of the European Union, the World Health Organization has

called for a ban on antibiotics which are used in both animal and human therapy. In the United States, the Centers for Disease Control recognized antimicrobial use in food animals as the main cause of antibiotic resistance among food-borne pathogens (Mellon, 1998). However, the use of growth promoting, in-feed antibiotics is still not banned in the United States and controversy continues to surround this issue (Samanidou et al., 2008).

Antibiotics for growth promotion. The lack of "scientific" proof concerning the benefits for human health and our environment after a ban of growth promoting antibiotics is not the only point of the debate. The use of in-feed antibiotics, as mentioned previously, promoted positive effects on growth and health status in the herd. A ban of this practice will have an economic impact on the cost of production. The opponents to a change in the practice argue that if there is no proven benefit for human health, why should we increase the cost of production and the cost of the final product. Economists from Europe and the United States have attempted to establish an economic model to asses this cost, but the multitude of factors entering in the calculation made the task difficult. Moreover, the differences of practices, techniques and markets between countries did not allow the publication of a worldwide model that was reasonably valid (Hayes et al., 1999; Graham et al., 2007).

After the European withdrawal of many antibiotics in 1990's and the announcement of a complete ban for growth promotion in 2006, the French swine technical institute led a study on such implementation effects on pig production cost. This study was held in a grower-finisher pig unit (only weaned piglets and fattening) and considered the following factors: growth rate, feed efficiency and occurrence of sanitary problems. Three different situations were analyzed: the total suppression of growth promoting antibiotics, the use of antibiotic still allowed in 2001 (na-salinomycin, flavophospholipol, avilamycin) and the use of alternatives products (e.g. enzymes, acidifiers, probiotics). The cost of production per pig, compared to the initial situation (which allowed in-feed antibiotics), were shown to be increased by 12.40

F (approximately 2.02 USD; 6.55957 F = 1.0667 USD) with a complete ban of in-feed antibiotic and within a range of 1.50 to 17.20 F in the two other situations (Gourmelen et al., 2001). Such results cannot be applied directly to United States production. However, the use of the European model, even if not completely accurate, can still be helpful in assessing the economic change induced by a potential ban of in-feed antibiotics in the United States.

In 1999, using the Swedish pork industry historical data, a study on the United States swine industry concluded that the ban of in-feed antibiotics will, in the most likely case, increase the cost per head from $5.24 to $6.05. However the diminution of supply due to the decrease of productivity would increase the retail price of pork ($0.05 per pound) and so the net profit for the producer should decline only by $0.79 per head (Hayes et al., 1999). It is important to note that this economic impact was calculated for an average farm and that it might differ greatly regarding the individual situation of each producer (e.g. density of population, quality of facilities quality, quality of sanitation, etc.).

Even if the United States legislation still allows the use of growth promoting antibiotics, the debate is not over. Without a doubt, a ban of in-feed antimicrobials will provoke a change for the United States industry and be a potential challenge. For this reason, it is important to continue to search for efficient alternatives to in-feed antibiotics to be prepared for this transition.

Probiotics. The growing concerns regarding the use of in-feed antibiotics together with the precedent set by the European Union, and the positions articulated by the World Health Organization and the Centers for Disease Control, collectively provide some impetus to the United States to look for alternatives to this practice. To this end, probiotics are among the alternatives that have received substantial attention. The concept of using natural bacteria, with antimicrobial capacity, to substitute for antibiotics, fulfills the expectations of the public and safety organizations. Ironically, part of the effectiveness of some probiotics in affecting

the microbial population in the gut may be due, in part, to their "natural" ability to produce antibiotic compounds.

Principle of probiotic use. Since birth, the gastrointestinal system is colonized by a microbial flora that is recognized to contribute in a positive way to the health and digestive/absorptive functions of the digestive tract. Highly processed and sterilized foods induce a deficiency in essential microorganisms (e.g. *L. plantarum*, *L. rhamnosus*, *L. casei*, and *L. acidophilus*) in modern human diets, and our organisms are more susceptible to unbalance (Bengmark, 2000). When a change in the microflora occurs, the ingestion of beneficial bacteria, via probiotics, helps individuals recover their normal balance more quickly. However, the physiological effects of probiotic bacteria are not entirely understood. Bacteria are thought to exhibit powerful antipathogenic and anti-inflammatory capabilities (Isolauri et al., 2002).

In humans and animals, probiotics are used to prevent and treat a wide variety of conditions like gastrointestinal disorders and antibiotic-associated diarrhea (Hibberd et al., 2008). They are available mainly as viable preparations in foods (yogurt, fermented dairy drink, etc.) or as dietary supplements (usually in the form of capsules as complementary or alternative medicines) and "claim" to improve the health status of the consumer (Salminen et al., 1998). The FDA's regulation for dietary supplements is different than for medication. Dietary supplements can be sold with no or limited research documenting their efficacy. Products marketed as probiotics can originally contain the bacteria or the bacteria can be added during the preparation. Probiotic products constitute a new and expanding market. Because of this heightened interest, a large number of bacteria are now considered as probiotics or potential probiotics (Holzapfel et al., 1998).

Strains used in probiotic preparations. An important point to emphasize is that a product containing bacteria is not automatically a probiotic. The bacteria have to be viable at the time of use and in sufficient quantity to confer a physiologic health benefit (Reid et al., 2005).

Thus, not every bacterium can be used in probiotic preparations. A bacterial strain intended for probiotic use should have the ability to survive through the gastrointestinal tract; to colonize it; to be nonpathogenic; to be able to interact with the intestinal epithelium; and to have a proven health benefit. Moreover the strain should maintain its effectiveness and potency until the end of the product shelf life (Goldin, 1998).

Table 1. Microorganisms considered as probiotics[1]

***Lactobacillus* species**	***Bifidobacterium* species**
L. acidophilus	*B. adolescentis*
L. amylovorus	*B. animalis*
L. casei	*B. bifidum*
L. crispatus	*B. breve*
L. delbrueckii subsp. *Bulgaricus*	*B. infantis*
L. gallinarum	*B. lactis*
L. gasseri	*B. longum*
L. johnsonii	
L. paracasei	
L. plantarum	
L. reuteri	
L. rhamnosus	

[1]Modified from Holzapfel et al., 2001.

In 1908, lactic acid producing bacteria were the first bacteria officially recognized for their health benefits in fermented milk products (Metchnikoff, 1908). These bacteria ferment the lactose into lactic acid (Axelsson, 1998) which inhibits the growth of many undesirable microorganisms. The main lactic acid producing bacteria used in probiotics belong to the *Lactobacillus* or *Bifidobacterium* species (Table 1) which commonly inhabit the healthy gut

and vagina. *Lactobacilli* predominate in the small intestine and *Bifidobacterium* predominate in the large intestine.

Species other than lactic acid bacteria are also used in probiotic production. For example, non pathogenic yeast, such as *Saccharomyces boulardii*, which proliferate along the entire gastrointestinal tract, or non lactic acid bacteria, such as *Bacillus spp.*, which are often referred as soil-based probiotics. With the increasing interest in probiotics, new probiotic strains continue to emerge. However, the efficacy of each new strain is often not fully characterized.

Legislation and regulations for the use of probiotics. Currently, neither the United States nor the European Union has a legal definition of the term "probiotic", so the marketing of products considered as probiotics is largely unregulated (Sanders, 2008). The World Health Organization, as well as the FDA, have developed guidelines for the evaluation of probiotics in food. They mainly recommend that the producer perform efficacy testing of the product in vitro and in animal models, labeling strain identification and safety evaluation (Sorokulova, 2008). Even so, the industry is only responsible for ensuring safety of its customers and consistency of the product with the scientific definition of probiotics (Sanders, 2008). The specific probiotic activities of each species within each genus of bacteria make the establishment of a general rule for probiotic products very complicated. Moreover, there are no standardized protocols to assess microbial safety and efficacy within the product that makes the potential legislation more difficult to enforce.

Probiotic products. Despite the ambiguous demonstration of efficacy, several probiotics are already in the market for both human and animal uses. The primary interest in probiotics for human consumption is the market targeted towards promoting enhanced gastrointestinal health. The United States sales of probiotics were estimated in 2005 at $764 million with an increase up to $1.1 billion expected by 2010 (Hibberd et al., 2008).

Probiotic products in humans have been investigated for the prevention and treatment of acute infantile diarrhea, nosocomial infantile diarrhea, Crohn's disease, and atopic eczema (Ezendam et al., 2006). Results from these studies have shown significant improvement of these various conditions in patients receiving probiotics (Isolauri et al., 2002). Table 2 summarizes the main probiotic products currently commercialized in the US for human use.

Although probiotic use in livestock and companion animals, like humans, could be presumed to improve gastrointestinal health, ultimately, at least for growing livestock, the hope is that probiotics might result in stimulation of growth performance. Probiotics too are seen by some to fit nicely into natural production practices for livestock and as a substitute for in-feed antibiotics. However, most studies to date fail to document comparable growth stimulation from probiotic treatment compared to in-feed antibiotics.

Presently, the main target market for probiotics in livestock diets are in swine and poultry, although some studies have been published for ruminants (Wallace et al., 1992) and aquaculture (Wang et al., 2008). Probiotics intended for use in animal production have slightly different requirements compared to probiotics that are intended for human use. The main difference is in the quantity needed and the process of production.

Table 2. Major probiotics found in the United States[1]

Strain	Commercial products	Source	Indication
Saccharomyces cerevisiae (boulardii)	Florastor (powder)	Biocodex (Creswell, OR)	Antibiotic associated diarrhea (*C. difficile*)
B. infantis 35264	Align® (capsules)	Procter & Gamble (Mason, OH)	Irritable bowel syndrome symptoms
B. lactis Bb-12	Good Start Natural Cultures® (infant formula)	Nestle (Glendale, CA) Chr. Hansen (Milwaukee, WI)	Immune support
L. casei Shirota *B. breve* strain Yakult	Yakult® (daily dose drink)	Yakult (Tokyo, Japan)	Immune support, Gut transit time and bowel function
L. casei DN-114 001 ("L. casei Immunitas™")	DanActive® (fermented milk)	Danone (Paris, France)	Keeping healthy and infant diarrhea, antibiotics associated diarrhea (*C. difficile*)
B. animalis DN173 010 ("Bifidis regularis™")	Activia® (yogurt)	Dannon (Tarrytown, NY)	Gut transit time and bowel function
L. reuteri RC-14™ L. rhamnosus GR-1™	Fem-Dophilus® (capsules)	Chr. Hansen (Milwaukee, WI) Urex Biotech (Ontario, Canada) Jarrow Formulas (Los Angeles, CA)	Vaginal applications
L. johnsonii Lj-1 (same as NCC533; formerly *L. acidophilus* La-1)	LC1®	Nestlé (Lausanne, Switzerland)	
L. rhamnosus GG	Culturelle® (capsules)	Valio Dairy (Helsinki, Finland)	Immune support, infant diarrhea, antibiotics associated diarrhea (*C.difficile*)
L. rhamnosus GG	Dannon Danimals® (drinkable yogurt)	Valio Dairy (Helsinki, Finland)	Immune support
B. lactis Bb-12	LiveActive (cheese)	Kraft (Canada, United States)	Immune support

[1] Modified from http://www.usprobiotics.org/products.asp.

THE *BACILLUS* GENUS USE IN PROBIOTIC PRODUCTION

Despite the common use of indigenous bacteria from the gastrointestinal tract as initial sources of probiotic isolates, some non-indigenous bacteria can present interesting characteristics and probiotic potential. *Bacillus* organisms are an example of environmental bacteria that have been exploited for their potential in probiotic applications.

Bacillus genus. The genus *Bacillus* consists of a large diversity (more than 100 species) of Gram-positive aerobic bacteria capable of producing endospores that are resistant to extreme environmental conditions.

Ubiquitous in nature (soil, water and air), *Bacillus* species are mostly harmless, with the notable exceptions of *Bacillus anthracis* and *Bacillus cereus*. These organisms are well known for their extreme pathogenic potential. Because *Bacilli* are found in high numbers in the environment, the daily intake of these bacteria, via our gastrointestinal and respiratory tract, is important. Yet, they are not considered as part of the indigenous flora (Sorokulova, 2008). Recent studies suggest that they might be adapted to the intestinal ecosystems (Jensen et al., 2003). Moreover, their capacity to sporulate enhance their capacity to gain access to the lower gastrointestinal tract because of their ability as spores to escape destruction in the gastric environment.

The genus *Bacillus* is extensively used in the fermentation industry to produce enzymes (proteases, α-amylases, glucose isomerase, and pullulanase) and nucleic acid bases (inosine, a flavor enhancing nucleotide). These bacteria are also used to produce polypeptide antibiotics against other bacteria and fungi (SCAN, 2000). Among the *Bacillus* genus, three *Bacillus* species: *Bacillus cereus, Bacillus licheniformis* and *Bacillus subtilis,* have been and are currently investigated intensively.

Bacillus cereus group. The *Bacillus cereus* group is composed of *Bacillus anthracis*; *Bacillus cereus*; *Bacillus mycoides*; *Bacillus thuringiensis*; *Bacillus weihenstephanensis*; and *Bacillus pseudomycoides* (Fritze, 2004). *Bacillus cereus* strain has been shown to grow in anaerobic conditions and to be internalized by epithelial cells (Schierack et al., 2007). *Bacillus cereus, Bacillus mycoides* and *Bacillus thuringiensis* comprise bacterial species attributed to gastrointestinal infections. Consequently the *Bacillus cereus* group is known for outbreak and the Scientific Committee on Animal Nutrition (SCAN) strongly discourages its use in animal feed (SCAN, 2000).

Bacillus subtilis group. *Bacillus subtilis* and *Bacillus licheniformis* belong to the *Bacillus subtilis* group that shares 72% genotypic homology to the *Bacillus cereus* group. However, the distinction between these two groups is dramatic. Moreover, the *Bacillus subtilis* group is classified as "Generally Regarded As Safe" (GRAS) by the Food and Drug Administration (Zheng et al., 1999). The only cases of outbreak due to bacteria from the *Bacillus subtilis* group were reported in immunosuppressed individuals or following trauma (US Environmental Protection Agency, 1997a, b).

Bacillus licheniformis and *Bacillus subtilis* are two soil microorganisms. Both species are known as aerobic, anaerobic facultative and are able to grow over a wide range of temperatures and both are spore formers (U.S. Environmental Protection Agency, 1997a, b). These characteristics allow them to temporarily proliferate and inhabit the gastrointestinal tract of humans or animals even in absence of oxygen (Leser et al., 2008). Like almost all species of the *Bacillus* genus, they produce hemolysin, with lytic activity for epithelial cells, and several protease and amylase enzymes that participate in nutrient conversion. They are also able to synthesize lecithinase that can disrupt the cell membrane of mammalian cells (U.S. Environmental Protection Agency, 1997a, b).

Bacillus and sporulation. When conditions are not favorable for the growth of vegetative cells, sporulation begins. The lysis of each vegetative cell gives rise to a metabolically dormant spore (1 - 1.2 µm). The spores consist of multiple layers surrounding the nucleotide which make them extremely resistant to heat, radiation, desiccation, extremes in pH and toxic chemicals. Germination of the spores and "new" growth of vegetative cells, can be triggered by stomach acid (Leser et al., 2008). The ability to sporulate is a key advantage for *Bacillus* for use in the livestock and companion animal industry. Sporulation ensures that bacteria stay viable through the production processes, such as pelletization at high temperature and pressure, and have a long shelf-life; moreover, it allows a reduction of the cost of probiotic production (Nicholson et al., 2000).

Bacillus and antimicrobial agents. Sporulation is not the only characteristic of the *Bacillus* genus that makes it attractive to the direct-fed microbial industry. *Bacillus* bacteria also produce secondary metabolites. These metabolites, unlike primary metabolites, are not directly involved in physiologic development of the microorganism. Secondary metabolites take multiple forms such as pigments, toxins, enzymes, pheromones and, antibiotics. Their production occurs when the growth rate and the nutrient availability are decreasing (Demain, 1998), or in other terms, during the stationary growth phase (or idiophase) of the bacteria. Among the secondary metabolites, the production of antimicrobial agents appears to be an important criterion in the evaluation of the potential use of *Bacillus* strains as probiotics.

Antimicrobials synthesized by Bacillus. The genus *Bacillus* produces various classes of antibiotics (Table 3), such as cyclic or linear oligopeptides, basic peptides and aminoglycoside antibiotics, with the predominant class being the peptide antibiotics (Torsten, 2005).

Table 3. Some antibiotics elaborated by *Bacillus* genus[1]

***Bacillus* Species**	**Antibiotic produced**	***Bacillus* Species**	**Antibiotic produced**
Bacillus brevis	Gramicidin S, Linear gramicidin	*Bacillus circulans*	Butirosin
	Tyrocidine		Circulin
	Brevin		Polypeptin
	Edeine		EM-49
	Eseine		Xylostatin
	Bresseine	*Bacillus laterosporus*	Laterosporamine
	Brevistin		Laterosporin
Bacillus subtilis	Mycobacillin	*Bacillus cereus*	Biocerin
	Subtilin		Cerexin
	Bacilysin		Thiocillin
	Bacillomycin	*Bacillus polymyxa*	Polymixin
	Fungistatin		Colistin
	Bulbiformin		Gatavalin
	Bacillin		Jolipeptin
	Subsporin	*Bacillus licheniformis*	Bacitracin
	Bacillocin		Licheniformin
	Mycosubtilin		Proticin
	Fungocin	*Bacillus thiaminolyticus*	Octopytin
			(Thianosine)
	Iturin		Baciphelacin
	Neocidin	*Bacillus pumilis*	Micrococcin P
	Eumycin		Pumilin
Bacillus mesentericus	Esperin		Tetain

[1] Modified from Katz et al, 1977.

Low molecular weight and hydrophobic or cyclic structures, with unusual constituents like D-amino acids, are common characteristics of peptide antibiotics normally synthesized by *Bacillus*. Moreover, they are generally resistant to hydrolysis by peptidases and proteases of animal and plant origins (Katz et al., 1977) and are synthesized by ribosomal or nonribosomal mechanisms (Mannanov et al., 2001).

The different strains of the *Bacillus subtilis* synthesized more than twelve antibiotics (Torsten, 2005). *Bacillus subtilis* (and less often *Bacillus licheniformis*) is used as a source of surfactin. This lipopeptide antibiotic has exceptional surfactant activity and emulsification properties. Surfactin also demonstrated other properties among which were antitumoral, antiviral, and antibacterial activities (Schallmey et al., 2004).

Bacillus licheniformis does not produce as many antibiotics as *Bacillus subtilis*. However, it inhibits the growth of various fungi and is used as a biocontrol agent of several fungal pathogens (Lebbadi et al., 1994). *Bacillus licheniformis* is known especially for its ability to synthesize bacitracin.

The Bacillus antibiotic bacitracin. Bacitracin was discovered in 1945 (Johnson et al., 1945); it is a non ribosomal peptide antibiotic produced by *Bacillus licheniformis spp.* and some strains of *Bacillus subtilis* (Murphy et al., 2007).

In nature, bacitracin is found in different "forms". The commercial bacitracin is a mixture of nine bacitracins, where bacitracin A is predominant. Bacitracin is neutral, soluble in water and non-toxic for eukaryotic cells (Johnson et al., 1945). Bacitracin is considered as a metalloantibiotic because it needs a metallic ion (zinc, copper, nickel or manganese ions) to exert its biologic activity (Ming et al., 2002).

The multi-enzyme complex, bacitracin synthetase ABC, is responsible for the formation of bacitracin. Three peptide synthetases: BacA, BacB and BacC compose the multi-enzyme complex. Bacitracin synthetase ABC catalyses the incorporation of amino

acids (L or D), the formation of the thialozine ring between isoleucine and cysteine, the chain elongation and finally the liberation of the peptide chain (Murphy et al., 2007). Many studies reported that the production of bacitracin was only observed when culture conditions supported sporulation (Bernlohr et al., 1959). Bacitracin production seems to share common factors with the sporulation process. However, production of antibiotic is not a requirement for sporulation to occur (Torsten, 2005).

Production of bacitracin, as other antibiotics, takes place during the early stage of sporulation when the microorganism has passed the rapid growth phase (Katz et al., 1977). The deficiency of nutritional components slows down and/or arrests the growth and initiates the biosynthesis of bacitracin. This delay in production is vital for the bacteria as microorganisms appear to be sensitive to bacitracin during their growth and acquire resistance during the idiophase (Martin et al., 1980).

Bacitracin interferes with bacterial cell wall synthesis (Storm, 1974). The bacterial cell wall consists of interlocking chains of identical peptidoglycan monomers. Peptidoglycans are built up from a backbone of repeating units of N-acetylglucosamine and N-acetylmuramic acid, connected by a glycosidic bond between carbon 1 and 4. N-acetylmuramic acid carries a amino acid side chain, varying among bacteria, that forms covalent bonds with the adjacent peptidoglycan chains (Horton et al., 2006).

Peptidoglycan prevents osmotic lysis. Without a strong cell wall, the bacterium would burst from the osmotic pressure of the water flowing into the cell. Interference with this process results in a weak cell wall and lysis of the bacterium from osmotic pressure (Becker et al., 2006). Peptidoglycan monomers are synthesized in the cytosol of the bacterium. Synthesis begins with glucose that is readily converted into N-acetylglucosamine. Uracil diphosphate (UDP) is added to N-acetylglucosamine and will serve as a carrier of the growing peptidoglycan during its synthesis within the cytoplasm. Addition of phosphoenol

pyruvate (PEP) converts UDP- N-acetylglucosamine into UDP- N-acetylmuramic. Then, amino acids: L-alanine, D-glutamic acid, diaminopimelic acid and finally two D-alanines are successively added and form the UDP- N-acetylmuramic-peptide, which is transported to the membrane (Linnett et al., 1973). UDP- N-acetylmuramic -pentapeptide is attached to N-acetylglucosamine and transported across the cytoplasm by the phosphorylated bactoprenol (P-bactoprenol; membrane carrier molecule). After passing the cytoplasmic membrane, the P-bactoprenol is released and the peptidoglycan monomer formed can be added to the growing peptidoglycan chain. The P-bactoprenol returns to the cytoplasmic membrane after being dephosphorylated and is ready to carry a new monomer. Bacitracin inhibits this dephosphorylation. The P-bactoprenol is not able to return the membrane and the transport is interrupted. Consequently, in absence of this building block, peptidoglycan synthesis is stopped (Katayama et al., 2003) and the cell will die. As noted above, bacitracin inhibits the synthesis of the bacterial cell wall and induces the death of the cell by osmotic lysis. Compared to Gram-negative bacteria, Gram-positive are more sensitive to bacitracin.

Bacillus licheniformis, as a source of bacitracin and as Gram-positive bacterium, has to itself be resistant to its own antibiotic. Ironically, *Bacillus licheniformis* cells, when they are in vegetative growth, are sensitive to bacitracin. Such an effect is no longer observed during antibiotic synthesis (idiophase). This resistance is due to the active ABC-type efflux system (Harel et al., 1999). Previous studies have demonstrated that the activity of the ABC transporter is correlated to the level of resistance of the bacteria (Podlesek et al., 1995).

Bacitracin is used in veterinary medicine, in combination with others antibiotics as a wound powder and for intramammary treatment of mastitis in lactating cows and in dry cow therapy. Bacitracin was used worldwide as a feed additive for poultry, pigs, calves, and lambs. However, in 1998, the European Union withdrew the authorization for its use as a growth promoter (The Council of the European Union, 1998). In the United States, bacitracin

is still used as a growth promoter in swine and poultry production (Graham et al., 2007; Samanidou et al., 2008).

Bacillus and immunomodulation. Even though *Bacillus* bacteria are generally regarded as originating in the soil, they do temporally inhabit the gastrointestinal tract and can be considered as part of the commensal flora. In spite of this, the oral absorption of *Bacillus* spores, and *Bacillus* bacteria, induce an immune response by the organism. For this reason, they are said to be immunogenic (Huang, 2008). Animals lacking microflora demonstrate nutritional issues and weak immune systems (Macpherson et al., 2004). Thus, the immune response developed by the organism in the presence of *Bacillus* spores or bacteria, plays an important role in the development of the gut-associated lymphoid tissue.

Bacillus bacteria are transported from the lumen of the intestine into the Peyer's patches by the M cells. As soon as they reach the intracellular compartment of the Peyer's patches, they interact with antigen presenting cells (APC; macrophages and dendritic cells), B cells and T cells (Huang, 2008). As a result of this interaction, B cells synthesize immunoglobulins (Ig) specific to the spore: IgG or IgA. They stimulate the recruitment of cytotoxic T lymphocytes (CTL) and the destruction of intracellular microorganisms (Hong et al., 2005). Moreover, *Bacillus* provoke the synthesis of IL-6, IL-1, IFN-γ and TNF-α in gut-associated lymphoid tissue and in peripheral blood that stimulate macrophages and natural killer cells (Hong et al., 2005). The recruitment and activation of immune and inflammatory cells might increase the resistance, of the body, to infection (Duc et al., 2004), strengthen the immune system and perhaps "prime" it for an acquired response (Huang, 2008). In addition, vegetative cells of *Bacillus subtilis* (and perhaps *Bacillus licheniformis*) carry peptidoglycans and lipoteichoic acids in their membrane lipoproteins. These compounds are recognized by Toll-like receptors (TLRs), respectively TLR2 and TLR4. The binding of these membrane

compounds to the receptor trigger the upregulation of gene transcription, by NF-κB, for TLR2 and TLR4 (Huang, 2008).

PROBIOTIC ATTRIBUTES OF *BACILLUS*

The features of the *Bacillus* genus, described in the previous sections, allow these bacteria to stay viable in the gut, to resist bile and acid, to synthesize secondary metabolites and to have a high biological activity. All these characteristics are important requirements for a probiotic strain (Sorokulova, 2008) but *Bacillus* bacteria have to be demonstrated, in vitro and in vivo, to be safe and effective before being use as a probiotic for humans or animals. For that reason, studies were conducted to determine the benefit of the use of *Bacillus* bacteria as a probiotic component in various species. The following provide an overview of the results and of the *Bacillus* probiotics developed to date.

Initial studies were conducted to demonstrate the positive effects of *Bacillus* bacteria on health status and performance. Once completed, additional studies were conducted to determine the mechanisms responsible for these effects.

Monogastrics. In grower finisher pigs (Alexopoulos et al., 2004b) supplementation of the ration with spores of *Bacillus licheniformis* and *Bacillus subtilis* improved significantly feed conversion ratio, average daily gain (ADG) and carcass quality. The optimization of the gut microflora balance by the uptake of *Bacillus* bacteria may be an explanation of these results, although direct measurements were not made to determine the underlying mechanisms.

In sows, like in grower and finisher pigs, the feed consumption in sows during lactation was significantly increased by the consumption of *Bacillus* probiotic (Alexopoulos et al., 2004a),. This increase may be related to the enhancement of the sows' appetite. The weight loss and the body condition were improved in the sows treated with *Bacillus* bacteria. The greater appetite and the higher concentration of serum cholesterol and total lipids, after

mid-lactation, also may have contributed to positive effects. As shown in previous studies (Kyriakis et al., 1992; Alexopoulos et al., 1998), greater concentrations of serum cholesterol and total lipids allow better performance of the animal. There is some suggestion too that sows' milk also may be improved by the *Bacillus* supplement. The concentration of milk fat and protein were increased suggesting that the nutritive value of the milk might have been improved by *Bacillus* supplementation. This enrichment may be the result of the relationship between mammary gland activity and absorbed nutrients. *Bacillus* bacteria also demonstrated an effect on sow fertility. The number of sows returning to estrus was increased in the treated group. A hypothesis for this phenomenon is the relation between improved body condition at weaning and the weaning to estrous interval, conception rate and survival of embryos. The last beneficial effect, observed in sows was the change in composition of the bacterial load in feces. The presence of fewer pathogenic microorganisms and a higher numbers of bacilli spores may create "safer" waste and therefore a lower threat to the environment.

In piglets, probiotics appeared to have direct and indirect effects (Alexopoulos et al., 2004a). Direct effects result from the feeding of piglets with probiotics containing *Bacillus* spores. For example, *Bacillus cereus* var. *toyoi* has been tested as a probiotic supplement and has shown multiple benefits on piglet growth and health status. Observed effects included a decrease in mortality, an increase of weight gain and improved feed conversion ratio. *Bacillus cereus* also contributes to the reduction in the incidence of liquid feces and post-weaning diarrhea (Alexopoulos et al., 2001; Baum et al., 2002; Schierack et al., 2007). The major causes of diarrhea in piglets are most often *E. coli* and *S. typhimurium*. Even if *Bacillus* strains are more effective against Gram-positive bacteria, they have some effectiveness against Gram-negative bacteria and may inhibit bacterial responsible for the induction of diarrhea. Another hypothesis is the establishment of favorable conditions for the growth of *Lactobacilli* by the germination of the *Bacillus* spores. The development of *Lactobacilli* has

been shown to fight against harmful bacteria. The last explanation is the production of second metabolites by the *Bacillus* bacteria, such as antimicrobials or bacteriocins, that can inhibit pathogens (Guo et al., 2006). The indirect effects come from the probiotic diet of the sows. The absorption of *Bacillus* spores by the mother induced health benefit for the litter such as reduction of diarrhea and mortality. The possible explanation is that piglets benefit from improved quality milk with higher fat content. This high nutritional milk has a positive effect on the growth of the litter (Haydon et al., 1988; Kyriakis et al., 1992). Moreover, swine rearing environments with good sanitation and management appeared to influence the colonization of the gut of the new born when sows were fed *Bacillus* probiotic and appeared to confer them with a more favorable starting microflora (Alexopoulos et al., 2004a).

In broiler production, the acquisition of a balanced microflora, as rapidly as possible, is imperative due to the short lifetime of the animals (around 42 d). *Bacillus subtilis* has an antagonist activity against a broad range of pathogens, such as *Campylobacter*, *E. coli* O78:K80, *Salmonella enterica* serotype *Enteritidis*, *L. monocytogenes* and *Clostridium perfringens* (Barbosa et al., 2005). The antagonist activity may be due to competitive exclusion and production of second metabolites (antibiotics, bacteriocins) by *Bacillus subtilis*. This mechanism was observed on *Helicobacter pylori* activity which is inhibited by *Bacillus subtilis* bacteriocin (Barbosa et al., 2005).

As in pigs, *Bacillus subtilis* spores (Maruta et al., 1996) significantly improved body weight and feed conversion ratio (in the period from 21 to 42 d), although treatment did not affect the mortality rate (Hooge et al., 2004). Different trials (in Brazil and in the United States) compared the performance of broilers receiving *Bacillus subtilis* spores (*Bacillus subtilis* DSM17299, GalliPro® from Chr Hansen at 8 x 10^5 CFU/g feed) and broilers receiving antibiotic growth promoter (Virginiamycin, Bacitracin-MD, or Avilamycin). All the results were consistent that there was no significant difference between the two groups.

Bacillus subtilis spores can substitute for antibiotic growth promoters and suport the growth and health status benefits, at least under conditions of this experiment in broilers (Hooge et al., 2004).

Ruminants. The effect of probiotics has been largely studied in monogastric livestock, although a few investigations have been completed in ruminants. The effects of *Bacillus* probiotic supplementation, in general, have been positive.

In calves (from birth to 5 wk of age), like in monogastric species, the reduction of diarrhea and growth performance were improved by the use of probiotic supplement (Donovan et al., 2002). Moreover, these benefits were not significantly different from calves receiving antibiotics. *Bacillus* bacteria also have shown some effect on immunity in calves, notably a reduction in the incidence of scours (Novak et al., 2007). Similar effects, as in calves, were observed in lambs, but in contrast to piglets, there was no benefit on the survival of the lambs. The daily milk yield of ewes receiving *Bacillus* supplement was improved and the milk quality was enhanced compared to the control group. Probiotic supplementation, as in sows, appears to improve the fat and protein content of the milk. This is an important feature, because ewe's milk intended for cheese production must have a high concentration of fat and protein.

Commercialized Bacillus probiotics. The mechanism of action of *Bacillus* probiotics is not fully understood. Still, products have been declared safe for the use in humans and animals. The ability of *Bacillus* bacteria to sporulate provides these organisms a clear advantage compared to other bacteria used in probiotic formulation. Many probiotics made up of *Bacillus* species are already in the worldwide market as is evident in Table 4.

Table 4. Some commercialized *Bacillus* products[1]

Specie	Name	*Bacillus* strain	Country	Indication
Human	Cerobiogen	*Bacillus cereus*	Sanofi-Winthrop SpA, Milan, Italy	Intestine infections, diarrhea, acute and chronic enteritis in children and adults
	Enterogermina	*Bacillus clausii*		
	Subtyl	*Bacillus subtilis*	Biophar Co. Ltd., Na Thang, Vietnam	
	Domuvar	*Bacillus clausii*	BioProgress SpA, Anagni, Italy	
	Lactipan *plus*	*Bacillus subtilis*	Instituto Biochimico Italiano SpA, Milan, Italy	
	Biosubtyl	*Bacillus pumilis*	Biophar Co. Ltd., Na Thang, Vietnam	
	Bactisubtil	*Bacillus cereus*	Marion-Merrell-Dow Laboratories, France	
	Medilac	*Bacillus subtilis R0179* *(and enterococcus faecium)*	Hanmi Pharmaceutical Co., Ltd., Korea and China	
	Nature's First Food	42 strains including *Bacillus laterosporus, polymyxa, subtilis* and *pumilis*	Nature's First Law, San Diego, CA	
Human and animal	Biosporin, Subalin, Gynesporin	*Bacillus subtilis* and recombinant *Bacillus* strains	D. K. Zabolotny Institute of Microbiology and Virology, Ukraine	
Swine	BioPlus 2B	*Bacillus licheniformis* *Bacillus subtilis*	Chr. Hansen	Improved feed conversion and animal performance Increased weight gain Reduced mortality rate
Broiler and swine	Calsporin	*Bacillus subtilis* C-3102	Calpis Japan	
Broiler	Gallipro	*Bacillus subtilis*	Chr. Hansen	

[1]Modified from http://www.agronavigator.cz/UserFiles/File/Agronavigator/Kvasnickova/Probiotics_Prebiotics_3.pdf.

INTESTINAL EPITHELIUM AND BACTERIAL INTERACTION

Probiotics, as noted previously, have the capacity to improve the intestinal functions and therefore the overall health of the animals. An effective and healthy gastrointestinal tract is benefitial to all animal production situations, to guarantee the best absorption of nutrients and optimum efficiency of feed use.

The swine gastrointestinal tract. Pigs have a single stomach compartment, as humans and chickens, characteristic of the monogastric. The gastrointestinal tract of the pig is composed of a stomach, a small intestine divided in three parts (duodenum, jejunum, and ileum), a large intestine, and a rectum.

The digestive process consists of the breaking down of the feed into smaller components that can be absorbed, used and excreted by the body. A first digestion of the feed occurs in the stomach, where acids and enzymes break down proteins, however the major site of nutrient absorption and digestion is the small intestine. The three segments of the small intestine receive secretions from the intestinal wall, the pancreas and the liver. The wall of the small intestine is organized in four layers: the mucosa, the submucosa, the muscularis and the serosa. The most inner layer is the mucosa and is divided in three sublayers: the muscularis mucosa, the lamina propia and the epithelium (Cunningham et al., 2005).

The intestinal epithelium is the primary physical barrier between the lumen of the intestine and the underlying mucosa. It regulates the passage of components into the intracellular compartment and protects the organism against bacterial invasion. The epithelium is composed of a continuous sheet of single-layered epithelial cells considered as mediators of the early innate immune response. The luminal surface is largely increased by the presence of finger-like projections creating villi, at the apical cells surfaces, and crypts of

Lieberkühn. This wide surface area of exchange enhances the nutrients' absorption (Cunningham et al., 2005).

Aside from its digestive functions, the small intestine is involved in the immune system and lymphoid tissues. The Peyer's patches are lymphoid follicles located in the mucosa and extending into the submucosa of the small intestine. They are part of the so-called gut associated lymphoid tissue and contain antigen presenting cells (APCs), B cells and T cells. A distinct follicular epithelium is found overlying the luminal surface of the Peyer's patches. This epithelium contains M cells that are able to sample antigen directly from the lumen and direct it to invaginations, at the basolateral surface, containing APCs (Srinivasan et al., 2006).

Within the APCs, dendritic cells seem to play a key role in the control of bacterial invasion. These cells are found in the Peyer's patches and in the lamina propria (Biedzka-Sarek et al., 2006). They are derived from the bone marrow and interact with the pathogenic bacteria present in the lumen (direct sampling) or that have breached the first line of defense: the epithelial cells (Fagarasan et al., 2003). Dendritic cells engulf the pathogen and present the antigen at their surface, allowing the activation of T cells (Stagg et al., 2004).

The intestinal microflora. Shortly after birth, the gastrointestinal tract is colonized by an extremely diverse commensal bacterial population. This flora allows the digestion of compounds, such as cellulose, that require specific sets of enzymes. The bacteria benefit from the stable synergistic habitat and the energy provided by ingested food (Macpherson et al., 2004). The balance between harmless and pathogenic bacteria, within this flora, is a feature of a normally functioning gastrointestinal tract. In addition to the beneficial effect on access to nutrients, the bacteria seem to have an action on intestinal physiology, morphology, mucus secretion, metabolism and immune functions (Shirkey et al., 2006). These statements are consistent with the observation that germ-free animals have an undeveloped mucosal immune

system with hypoplastic Peyer's patches and a reduced number of IgA-producing plasma cells in the lamina propia (Macpherson et al., 2004). When the organism experiences a stressor, the balance is altered and intestinal disorders occur that impact nutrient conversion, average daily gain and survival rate (Isolauri et al., 2002). The exact mechanism by which commensal bacteria exert their positive effect is still unknown. One hypothesis is that commensal bacteria compete with pathogenic bacteria for adhesion to common receptors in the intestinal epithelium; this phenomena is termed competitive exclusion (Schierack et al., 2006).

Salmonella invasion, dissemination and immune activation. Unfortunately, commensal bacteria are not the only micro-organisms that access the gastrointestinal tract. Pathogenic bacteria are responsible for the induction of disease. Among the intracellular pathogenic bacteria, Gram-negative *Salmonella enterica* serovar Typhimurium is the main pathogen responsible for infectious gastroenteritis in humans and enterocolitis in pigs (McCormick et al., 1993). Weaned pigs receiving a single oral inoculation of *Salmonella enterica* serovar Typhimurium had fever, inappetence and slowed growth (Balaji et al., 2000).

The activation of the innate immune system occurs through the recognition of antigen by pattern recognition receptors. These receptors are expressed by cells involved in the first line of defense, including dendritic cells and mucosal epithelial cells. Toll-like receptors are one type of pattern recognition receptor implicated in the recognition of bacterial lipoproteins and lipopolysaccharide (LPS). *Salmonella* bacteria have LPS within their outer membrane and flagellin is a major structural component of bacterial flagella. These bacterial elements, representing highly conserved molecular components, can be detected by TLR4 (CD284) and TLR5, respectively (Srinivasan et al., 2006). Once the flagellin has been recognized by TLR5, it upregulates the transcription of the chemoattractant IL-8 by epithelial cells (McCormick et al., 1993), and the secretion of the chemokine CCL20 that recruits immature

DCs (Schierack et al., 2006). However, TLR4 and TLR5 are strategically localized within the epithelium to limit the activation by the commensal bacteria. For example, TLR4 are only found in basal cells of the intestinal crypt, an area that is inaccessible to bacteria under normal circumstances (Srinivasan et al., 2006). In addition, the current dogmatic view is that TLR5 is only expressed on the basolateral surface of intestinal epithelial cells (Vijay-Kumar et al., 2008).

Salmonella have the ability to invade the epithelium by various mechanisms. These organisms deceptively gain phagocytic entry into enterocytes by reorganization of the host cytoskeleton (Schierack et al., 2006). The modification in the cell structure induces the formation of large macropinosomes that allow the entrance of the pathogen into the intracellular area (Ly et al., 2007). The *Salmonella* internalized by this process remain enclosed in a vacuole, a so-called *Salmonella*-containing vacuole (SCV). The bacteria can take control of this vacuole and protect themselves from destruction (Ly et al., 2007). *Salmonella* can also be detected directly from the intestinal lumen by DCs or use M cells as a gate (Biedzka-Sarek et al., 2006). In this case, M cells internalize the bacteria and transport them to the Peyer's patches. At this location, the bacteria interact with a rich population of macrophages and DCs. The *Salmonella* antigen are internalized in the DCs, processed and presented to naïve $CD4^+$ T cells (cell-mediated immunity) and B cells (humoral immunity). In this context, naïve $CD4^+$ T cells are activated and migrate to the site of inflammation. Additionally, B cells undergo maturation, and differentiate into IgA^+ B cells and proliferate (Fagarasan et al., 2003). Afterward, IgA^+ B cells migrate to the mesenteric lymph nodes (MLNs), proliferate further and finally differentiate into IgA-producing plasma cells that are able to secrete IgA into the intestinal lumen (Fagarasan et al., 2003).

Over the years, *Salmonella* have developed very efficient mechanisms to escape the immune defenses and can induce the apoptosis of M cells, macrophages and DCs. When

apoptosis occurs, it either results in the release of pro-inflammatory cytokines: IL-1β and IL-18, and in the recruitment of new DCs; or completely silences the immune response (Biedzka-Sarek et al., 2006). Besides, M cell destruction triggers a discontinuity in the epithelium and allows the entrance of additional bacteria (Srinivasan et al., 2006)

The knowledge of the interaction between bacteria (commensal or pathogen), the enterocytes and the immune system have tremendously increased in the last decade. However many mechanisms are still uncertain and need further investigations to be understood. In this perspective it is important to develop *in vitro* models that facilitate the study of mucosal immunity and the bacterial interface.

Modeling bacterial interaction with epithelial cells using IPEC-J2. IPEC-J2 cells are non tumorigenic epithelial cells from the pig jejunum. They have been used as an in vitro model epithelium for the study of functional characteristics of the swine intestinal epithelium (Schierack et al., 2006).

In vitro, IPEC-J2 form a monolayer of cuboidal cells organized by polarization and covered by microvilli on the apical surface. Tight junctions are found at the apicolateral membrane and define the permeability function of the membrane. They are also involved in characteristic immune signaling responses (Skjolaas et al., 2007). IPEC-J2 cells have demonstrated a capacity to release, in a dose-dependent manner, IL-8 in the presence of *Salmonella* enteric serovar Typhimurium. This release occurs in a polarized fashion toward the basolateral direction (McCormick et al., 1993; Schierack et al., 2006; Skjolaas et al., 2007).

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Chapter 2:

Interaction of *Bacillus spp.* and *Salmonella enterica* Serovar Typhimurium in Immune/Inflammatory Signaling from Swine Intestinal Epithelial Cells

ABSTRACT: Previous research evaluated a laboratory strain of *Bacillus licheniformis* (BL) in a model swine epithelium and found it exerted anti-inflammatory effects on *Salmonella enterica* serovar Typhimurium (S)-induced secretion of interleukin-8 (IL-8). The current investigation evaluated the anti-inflammatory actions of *Bacillus* bacteria available commercially as feed additives for the swine industry. Three isolates were obtained from the product, two *Bacillus subtilis* (BS1 and BS3) and one *Bacillus licheniformis* (BL2). Swine jejunal epithelial IPEC-J2 cells were seeded into wells on permeable membrane supports and allowed to form confluent monolayers. Treatments included apical pretreatment with BL, BS1, BL2, or BS3 for 17 h without S, and the same *Bacillus* treatments but with 10^8 CFU S added in the final 1 h of *Bacillus* incubation. Two additional treatments included negative control wells receiving no bacteria (C) and positive control wells receiving only S. Following bacterial incubation, wells were washed and fresh media containing gentamicin was added. Cells were incubated for an additional 5 h, after which apical and basolateral media were recovered for quantitation of IL-8 and bacitracin. In addition, inserts with epithelial cells that had received S were lysed and lysates cultured to determine treatment effects on S invasion. Exposure to S alone provoked an increase in IL-8 secretion from IPEC-J2 cells compared to C wells ($P < 0.001$ for both the apical and basolateral directions). Pre-treatment with each *Bacillus* isolate followed by challenge with S reduced S-induced IL-8 secretion in both apical and basolateral compartments compared to the wells receiving only S ($P < 0.001$; except for BS3 apical, $P < 0.01$). Secretion of bacitracin could only be detected in BL2 and BL2+S. Fewer S colonies could be cultured from lysates of BL2+S than S, BS1+S, and BS3+S treatments ($P < 0.001$). Results suggest that *Bacillus subtilis* and *Bacillus licheniformis* have the ability to intervene in secretion of the neutrophil chemoattractant IL-8 from swine intestinal epithelial cells. This effect on chemokine secretion by gastrointestinal epithelial

cells in vitro could not be explained solely by production of bacitracin or reduced invasion of epithelial cells by S.

Keywords: *Bacillus*, *Salmonella*, Swine

INTRODUCTION

The growth response of nursery pigs to in-feed antibiotics is well-documented (Dritz et al., 2002). To date, no single additive or class of additives has been identified to replace the growth response of nursery pigs to in-feed antibiotics. However, the search for non-antibiotic replacements continues, with the focus on direct-fed microbials representing a significant portion of that search. In general, evaluation of direct-fed microbials, at times referred to as probiotics, has been largely empirical. Little is actually known to suggest how direct-fed microbials may interact with enterocytes in the presence of pathogenic organisms that are presumably controlled (to some extent) by growth promoting levels of dietary antibiotics. One class of direct-fed microbials includes *Bacillus spp*. *Bacillus* bacteria are attractive because of their well-established ability to sporulate and their tendency to produce secondary metabolites (US Environmental Protection Agency, 1997a, b). To that end, our laboratory previously evaluated a laboratory strain of *Bacillus licheniformis* (BL) in a model swine epithelium and found it to intervene significantly in *Salmonella enterica* serovar Typhimurium (hereafter abbreviated *Salmonella typhimurium*) -induced secretion of interleukin-8 (IL-8) from gut epithelial cells (Skjolaas et al., 2007). Additional preliminary results suggested that the anti-inflammatory effects of BL were time-dependent (Godsey et al., 2007). The current investigation was undertaken to further evaluate the anti-inflammatory actions of *Bacillus spp*. in a model swine gut epithelium. We specifically sought to evaluate actions of *Bacillus* bacteria available commercially as direct-fed microbial feed additives for the swine industry.

MATERIALS AND METHODS

The aim of this study was to investigate the interaction of *Bacillus* bacteria and *Salmonella typhimurium* in immune/inflammatory signaling from swine intestinal epithelial

cells. Our previous investigation was limited to a laboratory strain of *Bacillus licheniformis* (Skjolaas et al., 2007). To gain access to *Bacillus* bacteria of relevance to the swine industry, we isolated strains from a commercial product (BioPlus® 2B, Chr. Hansen, Milwaukee, WI) for the current studies.

Bacterial isolation. A sample of the commercial feed additive (25 g) was solubilized in 225 mL of sterile water and mixed. Then, 1 mL of the solution was diluted in a 9 mL of trypticase soy broth (TSB; MP Biomedicals, LLC, Solon, OH). Following an overnight incubation at 37°C, a trypticase soy agar (TSA; MP Biomedicals, LLC, Solon, OH) plate was prepared using the broth and incubated overnight. Three different types of colonies were isolated. Colonies were forwarded to a commercial laboratory for identification (Silliker, Inc., St. Louis, MO). Specimens 1 and 3 were identified as *Bacillus subtilis* (hereafter, BS1 and BS3, respectively). Specimen 2 was identified as *Bacillus licheniformis* (hereafter, BL2).

The *Salmonella typhimurium*, and the *Bacillus licheniformis* (BL) isolates used for additional treatments were the same isolates utilized previously in our laboratory (Skjolaas et al., 2007). In brief, the *Salmonella typhimurium* was isolated form a clinical case of swine enteric disease and the BL isolate was a laboratory strain obtained commercially (American Type Culture Collection, Manassas, VA).

Growth curves. Growth curves were established for each bacterial isolate in TSB. For this purpose, the absorbance of the broth at 600 nm was measured followed by a standard bacterial plate count. After an overnight incubation at 37°C, colonies were counted and the bacterial population was estimated (Appendix A).

Bacterial sensitivity. The sensitivity of the bacteria, to common antibiotics, was assessed using a microplate assay. Bacteria were cultured on TSA and incubated overnight at 37° C. Then 3 to 5 colonies were picked and placed in distillated water to obtain a turbidity of 0.5 McFarland turbidity standard (= 10^8 CFU). One hundred microliters of the suspension was

added to 9 mL tube of Mueller-Hinton broth and 50 µL of the final solution were added in each well of the microplate containing antibiotics at various dilutions. The plates were incubated for 24 h at 37°C. The lowest antibiotic concentration that completely inhibited visible growth was considered to be the Minimum Inhibitory Concentration (MIC). Qualitative data concerning antibiotic sensitivity were used to ensure bacteria cultured out of epithelial cells were *Salmonella typhimurium* rather than one of the *Bacillus* ssp.

Culture of epithelial cells. The swine jejunal epithelial cell line, IPEC-J2, was used to assess the interaction of *Salmonella typhimurium* and the various *Bacillus* isolates (Rhoads et al., 1994). Culture conditions were identical to those described previously (Skjolaas et al., 2006, 2007), except that IPEC-J2 cells (passages 61 - 70) were cultured in 24 mm, six-well Costar Snapwells (Corning, NY) for exactly 7 d prior to execution of the experiment.

Exposure of IPEC-J2 cells to bacteria. Twenty-four hours before the beginning of the experiment, confluent IPEC-J2 cells were washed twice with PBS and fresh antibiotic-free media was added. *Bacillus* bacteria were grown on TSB to obtain the required concentration.

Design of the bacterial exposure of epithelial cells was patterned after our previously published study (Skjolaas et al., 2007). There were a total of 10 treatments and this required each replicate of the experiment to occupy two culture plates. Eight of the 10 treatments required pre-exposure of IPEC-J2 in the apical chamber to a 17 h incubation with *Bacillus* isolates (10^8 CFU/well). There were four *Bacillus* isolates, three of which were from the commercial product (BS1, BL2, and BS3) and one was the ATCC strain, BL, used previously (Skjolaas et al., 2007). After 16 h had elapsed, half of the wells containing *Bacillus* bacteria were treated apically with 10^8 CFU *Salmonella typhimurium* and the other half received a similar volume of sterile culture media. Thus, wells receiving *Salmonella typhimurium* were co-cultured with the respective *Bacillus* isolate in the final 1 h (a total of 17 h) of incubation. Recapping, the 8 treatment combinations were BL, BS1, BL2, and BS3 without and with

Salmonella typhimurium co-culture (BL+S, BS1+S, BL2+S, and BS3+S, respectively). The other two (of 10 total) treatments were negative control wells receiving no bacteria (C) and wells only receiving *Salmonella typhimurium* for 1 h (S).

Following the 1 h incubation after addition of *Salmonella typhimurium*, all wells were washed twice by over-flooding of PBS to remove the extracellular bacteria. New growth media containing 50 µg/mL gentamicin (Gibco, Grand Island, NY, USA) was added to both the apical and basolateral wells. Plates were returned to the incubator for an additional 5 h. Finally the media, from both apical and basolateral compartments, were collected and stored for later IL-8 determination as described previously (Skjolaas et al., 2006, 2007). An aliquot of the media was also used to determine concentration of bacitracin (detailed below).

Salmonella invasion into IPEC-J2 epithelial cells. Following removal of the media, inserts containing IPEC-J2 cells that had received treatment with *Salmonella typhimurium* were washed twice with PBS, placed in new plates and treated with 1 mL of 0.1% Triton X-100 . The Triton X-100 solution was pipeted up and down to thoroughly disrupt the epithelial cells. Dilutions of the cell lysate were then applied to TSA plates that contained 250 mg/mL sulfadimethoxine. All *Bacillus* isolates had previously been determined to be sensitive to this antibiotic, whereas our *Salmonella typhimurium* isolate was not. After an overnight incubation at 37°C, colonies were counted and the CFU of *Salmonella typhimurium*/mL of IPEC-J2 lysate was determined.

Bacitracin assay. Liquid chromatography coupled with electrospray ionization mass spectrometry (LCMS) was used to determine bacitracin production by the various *Bacillus* bacteria used in the experiment. Commercial bacitracin was purchased from Sigma-Aldrich (Vetranal, Analytical standard, Riedel-deHaen, Sigma-Aldrich). Bacitracin standard solutions were prepared by dilution of the commercial bacitracin in DMEM/F12 growth media (with gentamicin) at the concentration of 50, 100, 500, 1000, 5000 and 10000 ng/mL. Samples

(apical and basolateral media) were thawed and mixed thoroughly. Samples and standards were deproteinized by mixing 100 µL of media with 200 µL of methanol (100%). The mixtures were again mixed well and centrifugated for 5 min at maximum speed (13,000 rpm). Then, 200µl of the supernatant were transferred to injection vials for LCMS analysis.

The assay was optimized for bacitracin A only considering that bacitracin A is the predominant form of bacitracin produced (Konz et al., 1997). Chromatographic separation was performed on a Supelco Discovery C8 column (50 x 2.1 mm x 5uM; Sigma-Aldrich). The mobile phase was a mixture of acetonitrile (A) and 0.1% formic acid (B), and was delivered at a flow-rate of 0.4 mL/min under a gradient elution program (0 to 3 min: 5% A: 95% B; 3 to 5 min 30% A: 70% B; 5 to 6 min 5% A: 95% B; 6 min to the end 5% A: 95% B) at room temperature. A delay was observed between each injection to restore the initial conditions. The qualifying and quantifying ion mass to charge ration (m/z) used in the mass spectrometry interface were, respectively, 475.1 and 199.2. Settings, data acquisition, and processing were monitored by the software package: Analyst v.1.5 (Applied Biosystems). The time of retention of bacitracin was approximately 4 min. The results were expressed on count per second and converted to nanograms bacitracin /mL media, and then further to nanograms/well.

Statistical analyses. As noted previously, there were 10 well treatments and each well was considered an experimental unit. Each run of the experiment was conducted on 4 separate dates. Within each run, there were three replicate wells for each treatment. Thus, bars depicted in the figures generally represent the means of 12 observations. Technical difficulties prevented the inclusion of the *Salmonella typhimurium* invasion assay data from one run. Therefore, those means represent 9 observations.

Apical and basolateral concentrations of IL-8 were converted to nanograms/well to account for the difference in volume of the apical (1.5 mL) versus the basolateral chamber

(2.6 mL). Content of IL-8 in the apical and basolateral compartments were analyzed using the MIXED procedure (SAS Inst. Inc., Cary, NC). The model included fixed effects of treatment, secretion direction (apical or basolateral) and their interaction. Day was included in the model as a random effect. The MIXED procedure was also used to determine treatment effects for *Salmonella typhimurium* invasion into IPEC-J2 epithelial cells. In this case, treatment was the sole source of variation in the model. To ensure normality of the data, raw CFU values were square root transformed. Means (and SEM) were back transformed for presentation of the data. Bacitracin was only detected in media from wells containing the commercial BL2 isolate. The bacitracin data were analyzed using the MIXED procedure with treatment (with or without *Salmonella typhimurium*), secretion direction and the interaction in the model. All means are least-square means ± SEM. Comparisons of means was conducted only if a main effect or interaction was found to be significant ($P < 0.05$) in the model. Means were declared statistically different at $P < 0.05$.

RESULTS

Identification of bacterial specimens from commercial product. The three different colonies recovered from the sample were send to an accredited testing laboratory for microbiological and molecular (16S rRNA) analyses.

Table 5. Sample rRNA sequence comparison of specimens obtained from commercial product to library database.

Library database	Specimen 1		Specimen 2		Specimen 3	
	Match (%)	Mismatches	Match (%)	Mismatches	Match (%)	Mismatches
Bacillus subtilis subtilis ATCC=6051	99.93	2	98.1	12	99.99	2
Bacillus mojavensis	99.71	3	98.36	11	99.73	3
Bacillus subtilis spizizenii ATCC=6633	99.65	3	98.02	13	99.6	3
Bacillus subtilis spizizenii DSM=15029	99.50	4	98.18	12	99.59	4
Bacillus atrophaeus	99.23	5	97.63	15	99.39	5
Bacillus amyloliquefaciens	99.07	7	97.43	16	99.26	7
Bacillus vallismortis	99.05	8	97.52	16	99.19	8
Bacillus licheniformis	97.41	15	98.62	12	97.18	15
Bacillus sonorensis	96.86	17	98.50	12	96.59	17
Bacillus oleronius	94.65	28	93.35	34	94.16	28

rRNA derived for the bacterial samples were matched with the library sequence database to find the closest phylogenetic neighbors. None of the genetic profiles showed complete similarity with the library database. Specimens 1 (BS1) and 3 (BS3) had similarity scores with *Bacillus subtilis subtilis* (ATCC = 6051) of, respectively, 99.93% and 99.99% (Table 5); and the specimen was similar to *Bacillus licheniformis* at 98.1%. The precise alignments of each specimen, with its closest match, were then, analyzed and the results are summarized in Table 6.

Table 6. Location of mismatches of specimens obtained from commercial product compared to reference sequences[1]

Base number	0	1	28	31	47	49	52	138	159	175	239	241	257	422	439
Specimen 1													R	Y	
Bacillus subtilis subtilis ATCC=6051													A	T	
Specimen 2	R	W	A	T	G	T	Y	R	T	T		A			T
Bacillus licheniformis	G	T	C	C	T	G	C	G	G	C		G			Y
Specimen 3	W										R				
Bacillus subtilis subtilis ATCC=6051	T										A				

[1]The base number, in the top row, corresponds to the base position where a mismatch was observed. A = adenine; T = thymine; G = guanine; C = cytosine; R = A or G; W = A or T; Y = C or T.

The base differences that occur at the beginning (0 to 100) or at the end (400 to 500) of the sequence may be due to anomalies in the promoter attachment and have to be considered with

caution. The differences observed in the interior of the sequence are more likely to be accurate. Both BS1 and BS3 specimens exhibited 2 base differences compare to the *Bacillus subtilis subtilis* (ATCC = 6051). On the other hand, BL2 had a greater number of total mismatches (12) when compared to the *Bacillus licheniformis* from the library. However when looking at the alignment, many of these mismatches occur at the beginning and end of the sequence (base number: 0, 1, 28, 31, 47, 49, 52, and 439).

All phylogenetic analyses clearly assigned the bacteria to the *Bacillus* genus. Specimens BS1 and BS3 were more precisely identified as part of the *Bacillus subtilis* specie and BL2 was identified as a member of the *Bacillus licheniformis* specie.

Interleukin-8. In the absence of *Bacillus* co-culture, exposure to S alone provoked an increase in IL-8 secretion from IPEC-J2 cells (Figure 1) compared to control wells ($P < 0.001$ for both the apical and basolateral directions). Both the basal, unstimulated secretion of IL-8 in control wells and stimulated secretion in wells treated with S was greater in the basolateral than in the apical direction ($P < 0.05$ for control wells and $P < 0.001$ for wells treated with S). Treatment with both *Bacillus licheniformis* isolates (BL and BL2) decreased basal secretion of IL-8 when compared with control wells ($P < 0.05$). Pre-treatment with each *Bacillus* isolate followed by challenge with S reduced S-induced IL-8 secretion in both apical and basolateral compartment compared to the wells receiving only S ($P < 0.001$; except for BS3 apical, $P < 0.01$).

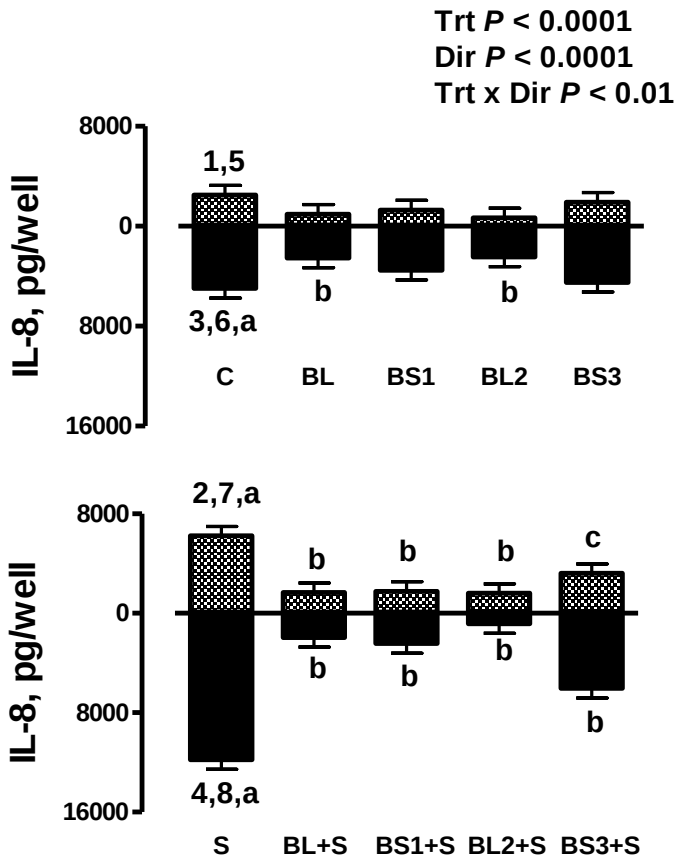

Figure 1. Polarized secretion of interleukin-8 (IL-8) from confluent porcine IPEC-J2 intestinal epithelial cells. Secretion into the apical chamber is represented by the shaded bars, while secretion into the basolateral chamber is represented by the solid bars. Bars represent the mean ± SEM of 12 replicate wells per treatment. Treatments included: control (C) with media alone, or 17 h apical incubation with 10^8 CFU/well *Bacillus licheniformis* ATCC strain (BL), *Bacillus subtilis* commercial isolate 1 (BS1), *Bacillus licheniformis* commercial isolate 2 (BL2), or *Bacillus subtilis* commercial isolate 3 (BS3) (top figure). Additional treatments (bottom figure) included all *Bacillus* treatments exposed to 1 h of co-culture with *Salmonella enterica* serovar Typhimurium (S) in the final hour of *Bacillus* incubation (BL+S; BS1+S; BL2+S; and BS3+S) or to *Salmonella enterica* serovar Typhimurium only for 1 hr (S). Media from the apical and basolateral compartments were removed and discarded. Cells were then washed, media containing gentamicin was added, and the cells were returned to the incubator. After 4 h, the experiment was terminated and media from the apical and basolateral compartments was removed for determination of IL-8. For analysis of the data, effects of treatment (Trt), secretion direction (Dir) and the interaction were included in the model. Numbers above and below bars represent comparisons among interaction means between and within C and S treatments: 1 vs 2, apical C vs apical S, $P < 0.001$; 3 vs 4, basolateral C vs basolateral S, $P < 0.001$; 5 vs 6, apical C vs basolateral C, $P < 0.05$; 7 vs 8 apical S vs basolateral S, $P < 0.001$. Letters above and below bars comparisons between C and other treatments (top figure) or between S and other treatments (bottom figure) within apical and basolateral means (a vs b, $P < 0.001$; a vs c, $P < 0.01$).

Effect of Bacillus bacteria on invasion of Salmonella typhimurium into IPEC-J2 cells. Plate counts of *Salmonella typhimurium* from lysates of cells pre-exposed to BL+S, BS1+S, and BS3+S were similar to plate counts from cells treated only with S (Figure 2). However

colonies of *Salmonella typhimurium* that could be isolated from epithelial cell lysates in the BL2+S treatment were reduced compared to S alone ($P < 0.001$).

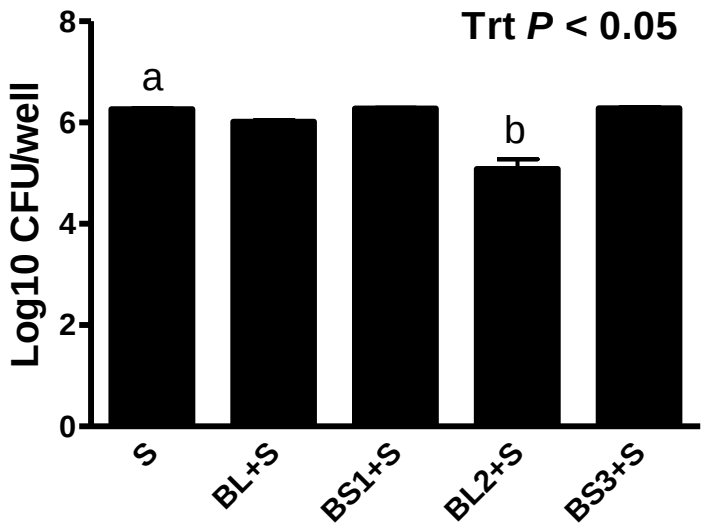

Figure 2. Invasion of *Salmonella enterica* serovar Typhimurium (S) into polarized confluent porcine IPEC-J2 intestinal epithelial cells. Bars represent the mean ± SEM of 9 replicate wells per treatment. Treatments included: apical 10^8 CFU S alone, or 17 h apical incubation with 10^8 CFU/well *Bacillus licheniformis* ATCC strain (BL), *Bacillus subtilis* commercial isolate 1 (BS1), *Bacillus licheniformis* commercial isolate 2 (BL2), or *Bacillus subtilis* commercial isolate 3 (BS3), then co-culture with S during the final hour of *Bacillus* incubation. Media from the apical and basolateral compartments were removed and discarded. Cells were then washed, media containing gentamicin was added, and the cells were returned to the incubator. After 5 h, the experiment was terminated. IPEC-J2 cells were lysed and the lysate cultured overnight on tryptic soy agar for the presence of S. Colonies of S were reduced in BL2+S compared to S ($P < 0.001$).

Production of bacitracin. Bacitracin could only be detected in media from IPEC-J2 cells exposed to BL2 and BL2+S (Figure 3). Secretion of bacitracin was similar for BL2 and BL2+S in both the apical and in the basolateral direction

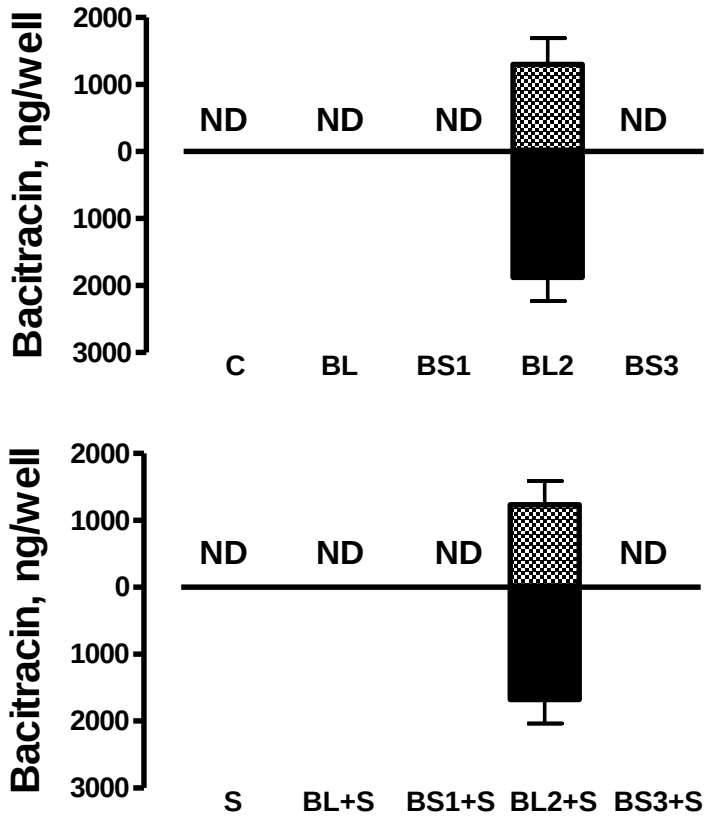

Figure 3. Polarized secretion of bacitracin from confluent porcine IPEC-J2 intestinal epithelial cells. Secretion into the apical chamber is represented by the shaded bar, while secretion into the basolateral chamber is represented by the solid bars. Bars represent the mean ± SEM of 12 replicate wells per treatment. Treatments included: control (C) with media alone, or 17 h apical incubation with 10^8 CFU/well *Bacillus licheniformis* ATCC strain (BL), *Bacillus subtilis* commercial isolate 1 (BS1), *Bacillus licheniformis* commercial isolate 2 (BL2), or *Bacillus subtilis* commercial isolate 3 (BS3) (top figure). Additional treatments (bottom figure) included all *Bacillus* treatments exposed to 1 h of co-culture with *Salmonella enterica* serovar Typhimurium (S) in the final hour of *Bacillus* incubation (BL+S; BS1+S; BL2+S; and BS3+S) or to *Salmonella enterica* serovar Typhimurium only for 1 hr (S). Media from the apical and basolateral compartments were removed and discarded. Cells were then washed, media containing gentamicin was added, and the cells were returned to the incubator. After 5 h, the experiment was terminated and media from the apical and basolateral compartments was removed for determination of concentration of bacitracin. Bacitracin was not detectable (ND) in media from treatments other than BL2 and BL2+S.

DISCUSSION

Previous studies demonstrated that a common swine pathogen of the gastrointestinal tract, *Salmonella typhimurium,* induced a proinflammatory response in the swine jejunal

epithelial cell line IPEC-J2 as evidenced by secretion of the neutrophil chemoattractant IL-8 (Skjolaas et al., 2006). Basolaterally polarized secretion of IL-8 has also been observed from epithelial cell lines from other species (McCormick et al., 1993; Vijay-Kumar et al., 2008). Of particular relevance to the current study, we previously reported that secretion of IL-8 from a model swine gastrointestinal epithelium was reduced substantially by pre-treatment with the ATCC 10716 strain of *Bacillus licheniformis* (Skjolaas et al., 2007). In the current study, we sought to investigate *Bacillus* strains of more direct relevance to the swine industry to evaluate if the anti-inflammatory action observed with the laboratory strain could be extended to *Bacillus* bacteria found in direct-fed microbial preparations. For this, we turned to the feed additive BioPlus® 2B. This is commercialized for use, not only in pigs, but also broilers and turkeys. It contains *Bacillus licheniformis* (DSM 5749) spores isolated from soil, and *Bacillus subtilis* (DSM 5750) spores isolated from soybean fermentation. BioPlus 2B contains at least 1.6 x 10^9spores/g of each *Bacillus* that are resistant to flavomycin and zinc-bacitracin. This product is reported to generally improve health, fertility and weight gain in swine production systems (Alexopoulos et al., 2004b; Jørgensen et al., 2006).

Our effort to obtain *Bacillus* bacteria from the product resulted in recovery of three isolates. The 16S rRNA genetic analysis revealed the presence of one *Bacillus licheniformis* (98.1%) and two *Bacillus subtilis* (99.93 and 99.99%). This is generally consistent with publically accessible information concerning the bacterial content of the product. However, the two *Bacillus subtilis* we recovered (BS1 and BS3) differed only by four bases, and two of those bases were found at the extremity of the sequence. The information provided by the manufacturer indicated that the product contained equal amounts of spores from *Bacillus licheniformis* and *Bacillus subtilis* spores. It could be that BS1 and BS3 are, in fact, the same *Bacillus subtilis* but given the slight difference underlined by the RNA analysis, we elected to evaluate the organisms separately.

In the current investigation, we again confirmed that the isolate of *Salmonella typhimurium* that we have used in many in vivo (Balaji et al., 2000; Burkey et al., 2004; Fraser et al., 2007) and in vitro studies (Skjolaas et al., 2006, 2007) stimulated polarized secretion of IL-8 from IPEC-J2 cells. This effect has been thoroughly documented in this swine derived cell line (Schierack et al., 2006) and cell lines from other species (Eckmann et al., 1993; McCormick et al., 1993). Of relevance to the major focus of the current study, we again observed that the ATCC BL isolate completely inhibited ST-induced secretion of IL-8 from IPEC-J2. Similarly, all isolates from the commercial feed additive behaved similarly to the ATCC BL isolate in blunting both apical and basolateral secretion of IL-8, although BS3 was somewhat less effective compared to the other strains. Of interest, both strains of *Bacillus licheniformis* (BL and BL2) even reduced basal IL-8 secretion from cells not stimulated with S. We had observed a similar effect previously, but only with *Lactobacillus reuteri* (Skjolaas et al., 2007).

One hypothesis to explain the effects of *Bacillus* bacteria to affect inflammatory signaling from enterocytes in vitro was that *Bacillus* prevented the ability of ST to attach and invade into the cell monolayer. To evaluate this possibility, we cultured lysates of IPEC-J2 after exposure to *Bacillus*. When compared to S, only BL2+S reduced colonies of *Salmonella typhimurium* that could be re-cultured out of IPEC-J2 lysates. Although graphically, the reduction does not appear to be substantial on a logarithmic scale, it suggests a marked decline in the number of *Salmonella typhimurium* breaching the epithelial barrier in the BL2+S treatment. Although this reduction may be related to other factors (discussed below), it does not explain the general ability of *Bacillus* to reduce secretion of IL-8 under these experimental circumstances because BL, BS1 and BS3 all reduced IL-8 without affecting invasion.

Among other secondary secretory components, *Bacillus* bacteria, including the ATCC BL isolate, is known to produce the polypeptide antibiotic bacitracin (Konz et al., 1997). We hypothesized that the ability of *Bacillus* bacteria, particularly *Bacillus licheniformis*, to affect *Salmonella typhimurium* to invade IPEC-J2 cells may simply be related to their ability to produce bacitracin. Indeed, BL2 produced bacitracin and did so in both the presence and absence of *Salmonella typhimurium*. Thus, the production of bacitracin may account for the reduced apparent invasion of *Salmonella typhimurium* in the BL2+S treatment. On the other hand, it is not clear that the levels of bacitracin in the media were sufficiently concentrated to exert a killing effect on *Salmonella*, although this must be considered. Of interest, we noted the gross appearance of biofilm associated with cultures of BL2 as we gained early experience growing the isolate. *Bacillus* bacteria are well-known producers of a variety of metabolites, including surfactin. *Bacillus subtilis*, for example, is known to produce a large array of secondary metabolites like mycosubtilin, iturin, and surfactin (Arima et al., 1968; Stein, 2005; Seydlova et al., 2008). Surfactin is a lipopeptide antibiotic and a powerful biosurfactant (Singh et al., 2004; Rodrigues et al., 2006; Nagorska et al., 2007). Surfactin, among its many properties, exhibits antimicrobial activities. Therefore, although it is possible, perhaps likely, that BL2 production of bacitracin contributed to reduced invasion of *Salmonella* into IPEC-J2, the ability to reduce IL-8 response to *Salmonella* more generally, we feel, must be related to other properties of *Bacillus* that will require additional investigation

Finally, we feel it is important to consider whether the effects of *Bacillus* on *Salmonella typhimurium*-induced IL-8 secretion we have observed here and previously (Skjolaas et al., 2007) have physiologic relevance. Or, could it be that these effects might simply be an artifactual consequence of the pre-treatment with *Bacillus* bacteria simply exhausting the nutrients in the media, leaving the cells less capable of secreting IL-8? On one hand, the

production of bacitracin is generally associated with the early stages of sporulation in *Bacillus licheniformis* (Bernlohr et al., 1959), and this, coupled with the obvious acidity (yellowing) that developed in the media by the time *Salmonella* were added might support such a conclusion. On the other hand, under identical experimental conditions, a lactic acid producing bacteria, *Lactobacillus reuteri*, produced substantial acidity in the media, but this condition alone failed to reduce the ability of *Salmonella typhimurium* to stimulate IL-8 secretion from IPEC-J2 (Skjolaas et al., 2007). Assuming our findings have relevance to the function of *Bacillus*-containing feed additives within the gastrointestinal tract, an important question that remains relates to the ability of these or any direct-fed microbial to colonize the gut in sufficient numbers to impact the interaction of the epithelium with enteropathogens to explain the reported benefits of probiotic bacteria.

Results of the current studies suggest that *Bacillus* bacteria, at least *Bacillus subtilis* and *Bacillus licheniformis*, have the ability to intervene in secretion of the neutrophil chemoattractant interleukin-8 from swine intestinal epithelial cells. This effect on chemokine secretion by gastrointestinal epithelial cells in vitro could not be explained by reduced invasion of epithelial cells by *Salmonella typhimurium*.

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APPENDIX A: BACTERIAL GROWTH CURVES

Characterization of the growth properties of the bacteria used for these studies was necessary in order to accurately estimate colony counts to be added to wells containing IPEC-J2 cells. These relationships had to be established for all of the *Bacillus* spp. and for the *Salmonella typhimurium* isolate because we had never grown these isolates in TSB media. Figures 4, 5, 6, 7 and 8 depict the regression of CFU on absorbance values read at 600 nm wavelength for *Salmonella typhimurium*, ATCC strain BL and the isolates from BioPlus® 2B, BS1, BL2, and BS3, respectively.

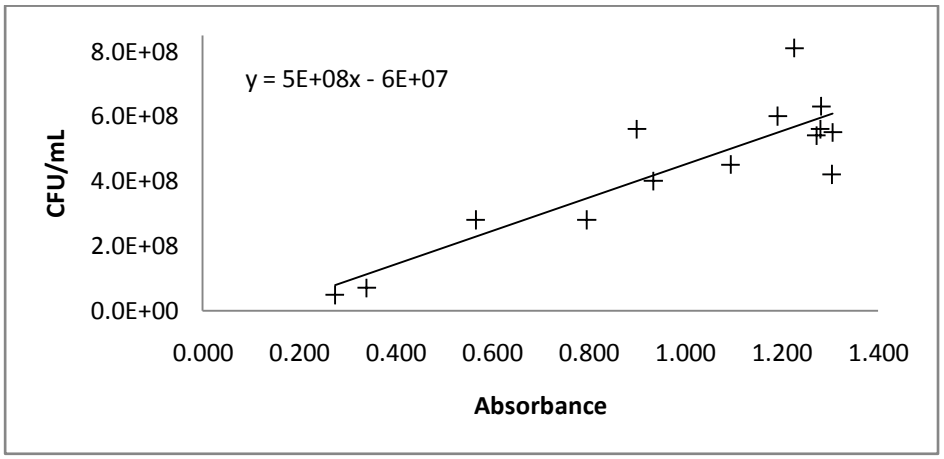

Figure 4. Relationship of colony forming units (CFU) of *Salmonella typhimurium* to OD 600. The fitted equation obtained from the data was: CFU/mL = (5.10^8 × OD600) – 6.10^7.

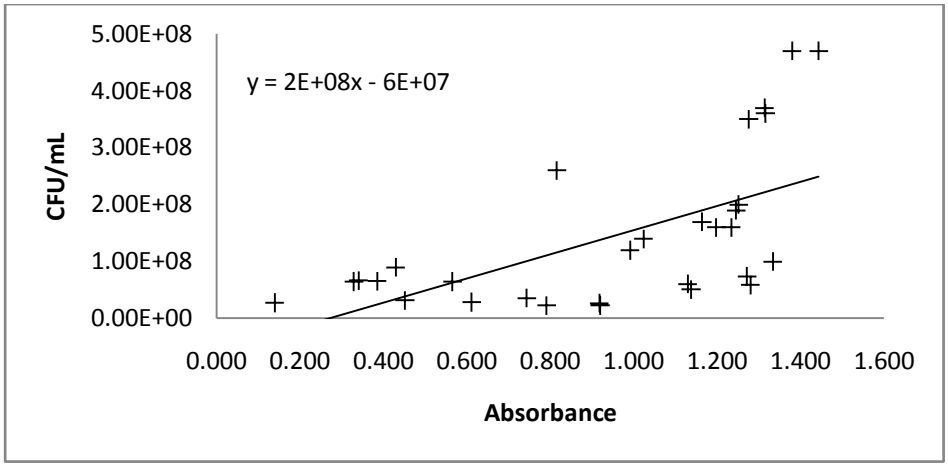

Figure 5. Relationship of colony forming units (CFU) of *Bacillus licheniformis* ATCC strain (BL) to OD 600. The fitted equation obtained from the data was: CFU/mL = (2.10^8 × OD600) – 6.10^7.

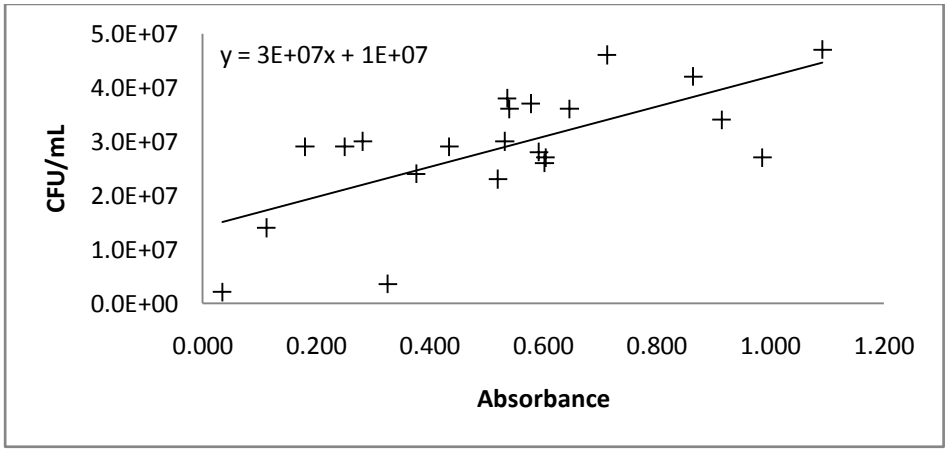

Figure 6. Relationship of colony forming units (CFU) of *Bacillus subtilis* isolate 1 (BS1) to OD 600. The fitted equation obtained from the data was: CFU/mL = (3×10^7 × OD600) + 1×10^7

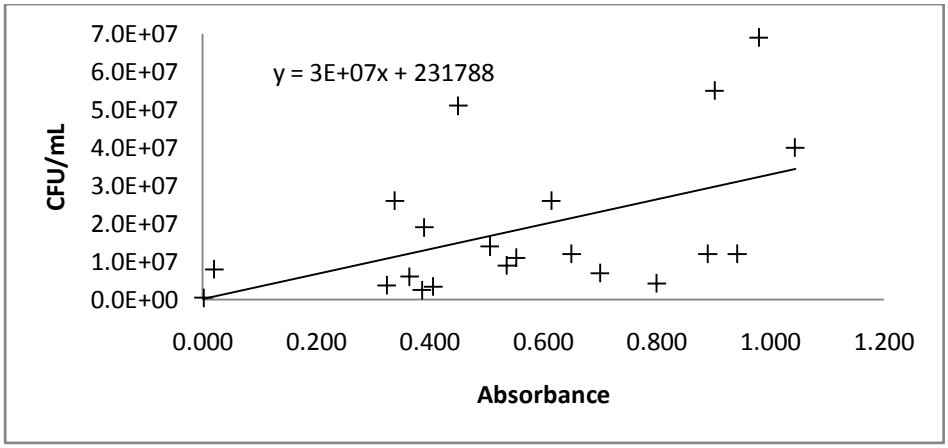

Figure 7. Relationship of colony forming units (CFU) of *Bacillus licheniformis* isolate 2 (BL2) to OD 600. The fitted equation obtained from the data was: CFU/mL = (3×10^7 × OD600) + 231788.

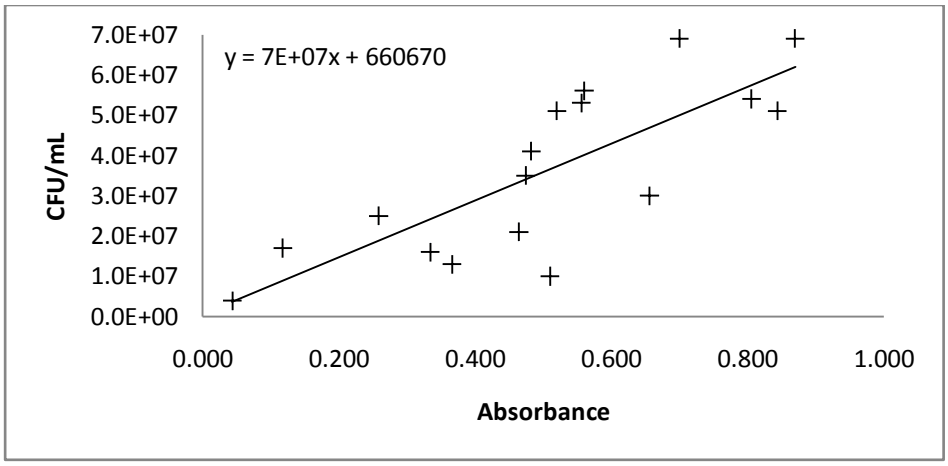

Figure 8. Relationship of colony forming units (CFU) of *Bacillus subtilis* isolate 3 (BS3) to OD 600. The fitted equation obtained from the data was CFU/mL = (7×10^7 x OD600) + 660670.