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Alternative Strategies for *Salmonella* Control in Poultry

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1. Introduction

Salmonella enterica serovars continue to be among the most important foodborne pathogens worldwide due to the considerable human rates of illness reported and the wide range of hosts that are colonized by members of this genus, which serve as vectors and reservoirs for spreading these agents to animal and human populations. Furthermore, public concern for the appearance of resistant strains to many antibiotics, particularly among zoonotic pathogens such as common *Salmonella* isolates, is also challenging the poultry industry to find alternative means of control (Boyle, Bishop, Grassl, & Finlay, 2007). For example, in January 2006 Europe implemented a complete ban on growth promoting antibiotics in animal feed (Anadon, Martinez-Larranaga, & Aranzazu Martinez, 2006). Thus, while attempting to control human foodborne pathogens poultry producers are simultaneously challenged to improve production in the face of increasing feed costs while using fewer antibiotics due to increased restriction of antimicrobial usage. These regulations were implemented because of export market restrictions and consumer or customer preferences in local markets. For these reasons continued research on sustainable alternatives to antibiotic growth promoters for animal production such as probiotics or direct fed microbials (DFM) consisting of live or dead organisms and spores (Patterson & Burkholder, 2003), non-traditional chemicals (Ko, Mendoncam, Ismail, & Ahn, 2009), bacteriophages (Andreatti Filho et al., 2007; Bielke, Higgins, Donoghue, Donoghue, & Hargis, 2007; J. P. Higgins et al., 2005; J. P. Higgins, Andreatti Filho et al., 2008), organic acids and other plant extracts and essential oils (Aengwanich & Suttajit, 2010; Allen-Hall, Arnason, Cano, & Lafrenie, 2010; Bagchi et al., 2000; Kubena, Byrd, Young, & Corrier, 2001; Over, Hettiarachchy, Johnson, & Davis, 2009; Van Immerseel et al., 2006), and vaccines (Kremer et al., 2011; O'Meara et al., 2010; Wolfenden et al., 2010; Van Immerseel et al., 2005; Dueger et al., 2001, 2003) are increasingly more important. These potential solutions have emerged in the last decade as tools that could be potentially useful in the near future for pathogen control and poultry performance improvement.

Probiosis, although not a new concept, has only recently begun to receive an increasing level of scientific interest. In agriculture, probiotics and DFMs used in animal feed are becoming accepted as potential alternatives to antibiotics for use as growth promoters, and in select cases, for control of specific enteric pathogens (Anadón, Rosa Martínez-Larrañaga, & Aranzazu Martínez, 2006; Boyle et al., 2007; Cartman, La Ragione, & Woodward, 2008; Vila et al., 2009; L. D. Williams, Burdock, Jimenez, & Castillo, 2009). For these reasons the

development of new and more effective probiotic products that can be licensed for animal use continues to receive considerable interest (Hong, Duc le, & Cutting, 2005; Hong, Huang, Khaneja, Hiep, Urdaci, & Cutting, 2008a; Jadamus, Vahjen, & Simon, 2001; Osipova, Makhailova, Sorokulova, Vasil'eva, & Gaidarov, 2003; P. Williams, 2007b; Wolken, Tramper, & van der Werf, 2003).

Currently, there is no universal class of probiotic bacterium. However, the most common types that have been indisputably effective involve LAB. These bacteria are found normally in the gastrointestinal tract (GIT) of vertebrates and invertebrates, and the use of some LAB cultures are able to restore the natural microflora within the gut (Shahani & Ayebo, 1980). Lactic acid bacteria include the genera *Lactobacillus*, *Pediococcus*, and others that have long been associated with health benefits and which have been used for fermentation of certain foods. While speciation of members of these genera is difficult and inconsistent, these organisms are considered uniformly safe and are not associated with disease in healthy animals or humans (Tellez et al., 2006).

A second classification of probiotic cultures are those microorganisms that are not normally found in the GIT (such as allochthonous flora). For example, *Saccharomyces boulardii*, a strain of yeast found on some tropical fruits, has been shown to be effective in preventing the recurrence of *Clostridium difficile* infections (Czerucka, Piche, & Rampal, 2007) and some colibacillosis in humans (Czerucka & Rampal, 2002). Other allochthonous probiotic microbes are the spore-forming bacteria, normally members of the genus *Bacillus*.

2. Lactic acid bacteria-based probiotic for *Salmonella* control and performance in poultry

The selection of individual enteric bacteria capable of inhibiting *Salmonella* growth *in vitro* and the ability of selected oxygen-tolerant bacteria to also protect neonatal poult and broilers from *Salmonella* infection following challenge has been a goal of multiple research laboratories (Menconi et al., 2011; Vicente et al., 2008; Bielke et al., 2003; Hollister et al., 1999; Corrier et al., 1998; Hume et al., 1998). Tellez and co-workers (2006) evaluated a simple method to select for individual enteric bacteria capable of inhibiting *Salmonella* growth *in vitro* and the ability of selected oxygen tolerant bacteria, in combination, to protect neonatal poult from *Salmonella* infection following challenge. Concurrently, they also worked toward the isolation, selection, further evaluation and combination of LAB to control additional foodborne pathogens. Extensive laboratory and field research conducted with this defined LAB culture has demonstrated accelerated development of normal microflora in chickens and turkeys, providing increased resistance to *Salmonella spp.* infections (Farnell et al., 2006; J. P. Higgins et al., 2007; J. P. Higgins et al., 2008; J. P. Higgins et al., 2010; S. E. Higgins et al., 2008; Vicente et al., 2008). Published experimental and commercial studies have shown that these selected probiotic organisms are able to reduce idiopathic diarrhea in commercial turkey brooding houses (S. E. Higgins et al., 2005). Large scale commercial trials indicated that appropriate administration of this probiotic mixture to turkeys and chickens increased performance and reduced costs of production (Torres-Rodriguez et al., 2007a; Torres-Rodriguez et al., 2007b; Vicente et al., 2007a; Vicente et al., 2007b; Vicente et al., 2007c).

These data have clearly demonstrated that selection of therapeutically efficacious probiotic cultures with marked performance benefits in poultry is possible, and that defined cultures can sometimes provide an attractive alternative to conventional antimicrobial therapy (see <http://www.pacificvetgroup.com/> for more information).

3. Mechanism of action of probiotics against *Salmonella*

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. Amongst the many benefits associated with the consumption of probiotics, modulation of the immune system has received considerable attention (Borchers, Keen, & Gershwin, 2002; Borchers, Selmi, Meyers, Keen, & Gershwin, 2009).

Previously, it was thought that administration of bacteria such as probiotics to neonates directly reduced infection by pathogens due to competition amongst the bacteria for attachment sites and nutrients and, that beneficial bacteria would out-compete pathogens within the GIT. This competition, coined as “competitive exclusion” was first described in 1973 by Nurmi and Rantala (Nurmi & Rantala, 1973). Their data indicated that early administration of beneficial bacteria to chicks prevented infection by pathogens. Since Nurmi and Rantala proposed competitive exclusion could be used as a method to prevent *Salmonella* infection, numerous researchers have reported the ability of live bacterial cultures to also reduce colonization of opportunistic microorganisms in the gastrointestinal tract (Callaway et al., 2008; Wagner et al., 2003; Hollister et al., 1999; Corrier et al., 1998; Hume et al., 1998; Nisbet et al., 1998) and probiotic organisms (J. P. Higgins et al., 2010; S. E. Higgins et al., 2008; Vicente et al., 2008; J. P. Higgins et al., 2007; Bielke et al., 2003; Patterson & Burkholder, 2003). Yet, understanding of how probiotics mediate these health benefits, specifically reduction of *Salmonella* infection, is very limited.

Balanced gastrointestinal microflora and immune-stimulation are major functional effects attributed to the consumption of probiotics (Amit-Romach, Uni, & Reifen, 2010; Boirivant & Strober, 2007; Boirivant, Amendola, & Butera, 2008; Flint, O'Toole, & Walker, 2010; Flore, Francois, & Felicite, 2010; Ibrahim et al., 2010; Klein, Sanders, Duong, & Young, 2010; Nayak, 2010). Many probiotic effects are mediated through immune regulation, particularly through balance control of pro-inflammatory and anti-inflammatory cytokines (Di Giacinto, Marinaro, Sanchez, Strober, & Boirivant, 2005; Foligne et al., 2010; Hacini-Rachinel et al., 2009; Jobin, 2010; Li, Xia, & Li, 2009). However, several animal and human studies have provided unequivocal evidence that specific strains of probiotics are able to stimulate multiple aspects of innate immunity (Amit-Romach et al., 2010; Boirivant & Strober, 2007; Boirivant et al., 2008; Farnell et al., 2006; Romanin et al., 2010; Weiss et al., 2010) as well as to increase humoral immunity (Fang, Elina, Heikki, & Seppo, 2000; Galdeano, de Leblanc Ade, Carmuega, Weill, & Perdigon, 2009; Leblanc, Fliss, & Matar, 2004; Nermes, Kantele, Atosuo, Salminen, & Isolauri, 2011).

Using a *Salmonella* challenge model, an effective LAB probiotic, administered 2 hours after *Salmonella* challenge, had no effect during the first 12 hours on increasing cecal colonization by this pathogen, although marked and rapid decreases were observed between 12 and 24 hours post-challenge (J. P. Higgins et al., 2007; J. P. Higgins et al., 2010). Later, using the same model and microarray analysis of gut mRNA expression, gene expression differences in birds treated with a *Lactobacillus*-based probiotic were compared to saline treated birds. At 12h post-probiotic treatment, 170 genes were significantly different ($P < 0.05$), but by 24h post treatment, the number of differentially regulated genes were 201. Pathway analysis revealed that at both time points, genes associated with the NF κ B complex were significantly regulated, as well as genes involved in apoptosis. Probiotic-induced differential regulation of the genes *GAS2* and *CYR61* may result in increased apoptosis in the ceca of chicks. Because *Salmonella* is an intracellular pathogen, it was suggested that increased apoptosis may be a mechanism by which B11 reduces *Salmonella* infection (S. E. Higgins et al., 2011).

4. Comparisons between genotypic 16S rRNA, MIDI, and biolog identifications of FloraMax™ lactic acid bacteria

A well-characterized LAB-based probiotic has been investigated in numerous studies (Tellez et al., 2006; Torres-Rodriguez et al., 2007a; Torres-Rodriguez et al., 2007b; Vicente et al., 2007a; Vicente et al., 2007b; Vicente et al., 2007c) and has now been commercialized (Pacific Vet Group USA Inc., Fayetteville AR 72703). Struggles with speciation of the LAB isolates during development of this product illustrate the well recognized problem for speciation of LAB. The identification techniques of choice for many facultative anaerobes are biochemical analyses, but the standard identification system for lactic acid bacteria is cellular fatty acid profiling. Nevertheless, these phenotypic methods can yield variable results. Genotypic methods that rely on comparisons of 16S rRNA sequences from unknown bacteria are proving to be valuable for use in a wide range of genera and are not sensitive to variable culture conditions. Genotypic 16S rRNA identification of organisms from probiotic cultures may be more consistent than the current standard microbial techniques applied separately to different microbial groups. However, this approach comes with its own limitations and issues. As identification is based on specific sequence homology as compared with a known database of microflora previously identified through conventional methodologies, the speciation is dependent upon the closest match with what was previously identified, correctly or incorrectly, in the database. As databases constantly expand and change, the same sequence submission over time may match other names with greater homology. Thus, at this moment, it is nearly impossible to really know the speciation of LAB except under specific examples with very highly characterized isolates. In fact, 16S rRNA sequencing of isolates from internationally-known name brands of commercially-produced yogurt with live cultures has consistently resulted in database matches with LAB species that are labeled as other species on the yogurt labels (unpublished). Thus, while 16s RNA sequencing can positively identify one LAB isolate as unique among several, true accuracy of homology comparisons is a somewhat subjective exercise.

Even though there are many new experimental molecular identification techniques, such as microarray hybridization, sequence analysis of 16S rRNA is the predominant molecular technology presently available for microbial identification of these commensal microorganisms (Wagner et al., 2003), even with the known problem of database accuracy and consistency over time. The detailed information needed to identify each species represented in a commercial probiotic product can only be fully obtained from the 16S rRNA at the level of the nucleotide sequence. As an example, an identification scheme was designed using the MIDI System ID from two different private laboratories (Micro Test Lab Inc., Agawam, MA 01001, USA; and Microbial ID Inc., Newark, DE 19713, USA) the Biolog ID System (Biolog, Inc., Hayward, CA 94545, USA) and compared those results with the 16S rRNA Sequence Analyses (Microbial ID Inc., Newark, DE 19713, USA) for identification of the individual component bacteria present in the commercial probiotic FloraMax™ (Table 1). The results of that study showed that the complex populations of bacteria present in FloraMax™ are not easy to accurately identify, especially with phenotypic techniques. Conventional technologies can detect human pathogens, because they are well-established in comparative databases, but emerging and opportunistic pathogens are not. Despite the fact that uncertainty exists between different methods of identification of non-pathogenic probiotic bacteria, identification of known pathogens is much more consistent. Therefore, the use of fully defined cultures for competitive exclusion or probiotic use are still inherently safer than undefined cultures or those where organisms are identified after the culture has been produced.

LAB ID	16S RNA Sequencing (FIRST 500 bp) Microbial ID Inc.	Midi system ID Micro Test Lab Inc.	Midi system ID Microbial ID Inc.	Biolog ID Dept. of Poultry Sc. U. of Arkansas
18	<i>Pediococcus parvulus</i>	<i>Enterococcus cecorum</i>	<i>Lactobacillus gasseri</i>	Unable to identify
24	<i>Weissella confusa</i>	<i>Lactobacillus casei</i>	<i>Lactobacillus casei</i>	<i>Clostridium clostridiiforme</i>
27	<i>Weissella confusa</i>	<i>Lactobacillus casei</i>	<i>Lactobacillus casei</i>	<i>Weissella confusa</i>
29	<i>Pediococcus parvulus</i>	<i>Lactobacillus delbreuckii-bulgaricus</i>	<i>Lactobacillus delbreuckii-bulgaricus</i>	<i>Lactobacillus hamsteri</i>
36	<i>Lactobacillus salivarius</i>	<i>Lactobacillus cellobiosus</i>	<i>Lactobacillus casei</i>	<i>Weissella confusa</i>
37B	<i>Weissella confusa</i>	<i>Pediococcus acidilactici</i>	<i>Pediococcus ruminis</i>	Unable to identify
40	<i>Weissella confusa</i>	<i>Lactobacillus casei</i>	<i>Lactobacillus cellobiosus</i>	<i>Weissella paramesenteroides</i>
44	<i>Weissella paramesenteroides</i>	<i>Lactobacillus fermentum</i>	<i>Lactobacillus fermentum</i>	Unable to identify
46	<i>Lactobacillus salivarius</i>	<i>Lactobacillus helveticus</i>	<i>Lactobacillus sanfranciscensis</i>	<i>Lactobacillus salivarius</i>
48	<i>Lactobacillus salivarius</i>	<i>Lactobacillus helveticus</i>	<i>Lactobacillus gasseri</i>	<i>Lactobacillus salivarius</i>
52	<i>Pediococcus parvulus</i>	Unable to identify	<i>Lactobacillus cellobiosus</i>	Unable to identify

Table 1. Comparisons between MicroSeq , MIDI, and Biolog identifications of FloraMax™ lactic acid bacteria¹

5. *Bacillus* spore-based probiotic for *Salmonella* control and performance enhancement in poultry

In spite of the success showed by the development of the LAB probiotic for use in commercial poultry as described above, there is still an urgent need for commercial probiotics that are shelf-stable, cost-effective and feed-stable (tolerance to heat pelletization process) to increase compliance and widespread utilization. Among the large number of probiotic products in use today some are bacterial spore formers, mostly of the genus

¹Adapted from Tellez et al., 2006

Bacillus. Used primarily in their spore form, some (though not all) have been shown to prevent selected gastrointestinal disorders and the diversity of species used and their applications are astonishing. While not all *Bacillus* spores are highly heat tolerant, some specific isolates are the toughest life form known on earth (Vreeland, Rosenzweig, & Powers, 2000) and can be used under extreme heat conditions. Several studies have shown that either live vegetative cells or endospores of some isolates can prevent colon carcinogenesis (Parket et al., 2007) or discharge antimicrobial substances against Gram-positive bacteria, such as *Staphylococcus aureus*, *Enterococcus faecium*, and *Clostridium difficile* (O'Mahony et al., 2001). These results provided evidence of colonization and antimicrobial activity of probiotic bacteria, thus, products containing *Bacillus* spores are used commercially as probiotics, and they offer potential advantages over the more common LAB products since they can be used as direct feed microbials (Anadón et al., 2006; Barbosa et al., 2005; Duc le et al., 2004; Hong et al., 2005; Hong et al., 2008a; Hong et al., 2008b; McNulty et al., 2007; Osipova et al., 2003; P. Williams, 2007a; Wolken et al., 2003). There is scientific evidence suggesting that some but not all isolates of ingested *B. subtilis* spores can, in fact, germinate in the small intestine (Casula & Cutting, 2002; Casula & Cutting, 2002; Duc le & Cutting, 2003; Hoa et al., 2001). Together, these studies not only show that spores are not transient passengers in the gut, but they have an intimate interaction with the host cells or microflora that can enhance their potential probiotic effect. Several commercial spore-forming *Bacillus* cultures have been shown to reduce food borne pathogens (Aureli et al., 2010). However, cost issues associated with achieving necessary concentrations of spores in feed have greatly limited commercial acceptance in the animal industry (Hong et al., 2005). While the majority of clear-cut research with regard to beneficial probiotic cultures has focused on LAB, as discussed above, a major question in several laboratories is whether or not selected spore-former bacteria (genus *Bacillus* or related) can be as effective as the best known LAB cultures. Recently, one *Bacillus subtilis* spore isolate was as effective as a well-established LAB-based probiotic for *Salmonella* reduction in poultry (Wolfenden R.E. et al., 2010; Shivaramaiah et al., 2011), and was equal to bacitracin for prevention of experimental necrotic enteritis, and was able to markedly reduce necrotic enteritis issues in large scale feed trials (unpublished from the author's laboratory).

Other isolates or combinations of isolates with increased potency and efficacy may be identified with continued research. Some of these environmental *Bacillus* isolates have been evaluated *in vitro* for antimicrobial activity against selected bacterial pathogens, heat stability, and the ability to grow to high numbers. Unpublished experimental evaluations have confirmed improved body weight gain as well as *Salmonella sp.* or *Clostridium perfringens* reduction in commercial turkey and broiler operations when compared with medicated (nitarson) or control nonmedicated diets respectively. Indeed, preliminary data suggests that these isolates could be an effective alternative to antibiotic growth promoters for commercial poultry.

Importantly, improved efficiency of amplification and sporulation is absolutely essential to gain widespread industry acceptance of a feed-based probiotic for ante mortem foodborne pathogen intervention, as well as cost effectiveness. Recently, both vegetative growth and sporulation rates have been optimized, which may lead to new efficiencies for commercial amplification and manufacture of a cost-effective product at very high spore counts (Wolfenden R.E. et al., 2010). In order to select even more effective isolates, current research is focused on the mechanistic action of new *Bacillus* candidates. Preliminary studies indicate

a potential mechanistic action of these new *Bacillus* candidates at least partially involve rapid activation of innate host immune mechanisms (system or responses) in chickens and turkeys (unpublished data). This data provides an exciting possibility for identification of vastly superior and more potent probiotics in the near future.

6. Prospects of bacteriophage therapy to control gastrointestinal disease

6.1 Overview

During the last approximately 60 years, there have been sporadic published reports of efficacy in treating Enterobacteriaceae infections systemically and within the gastrointestinal tract. While a number of reports have rather consistently indicated that systemic or tissue-associated infections were treatable by parenteral administration of appropriate bacteriophage cocktails, reports of successful treatment of enteric Enterobacteriaceae are much more sporadic, and are interspersed with a number of reports of failed attempts for enteric treatment. The following sections will discuss selected successes and failures and describe the possible differences in these studies and the potential for development of more effective strategies.

6.2 Successes

The bacteriocidal effects of bacteriophages have long been studied for their usefulness in treating gastrointestinal infections. Early studies originating from the former Soviet Union, Eastern Europe, and Eastern Asia suggested bacteriophages could prevent and treat *Vibrio cholera* infections (Dubos et al, 1943; Dutta, 1963; Sayamov, 1963; and Marčuk et al, 1971). In the 1980s Slopek and co-workers (1983a-b, 1984, 1985a-c, 1987) published numerous papers showing the promising results of treating septic patients with bacteriophages. While the validity of these studies has been questioned, in part due to relaxed scientific rigor in these regions during the time when these studies were completed (Merril et al, 2003; Alisky et al, 1998) and are not often cited by bacteriophage researchers in recent years, they have served as an inspiration for continued research into the possibility that bacteriophages can cure gastrointestinal diseases in humans and animals.

Smith and Huggins (1982) compared the efficacy of phages with that of antibiotics in treating both generalized and cerebral infections in mice. They isolated anti-K1 bacteriophages that were able to lyse K1-positive *E. coli*. These bacteriophages were able to cure infection caused by K1-positive, even when used at a low titer. The bacteriophages were more effective than several antibiotics for curing mice. Smith and Huggins (1983) also successfully used bacteriophage therapy to treat calves, pigs, and lambs that had been infected with *E. coli*. Perhaps key to their success, they selected a bacteriophage that would lyse *E. coli* and also selected a second bacteriophage that would lyse the target *E. coli* that had become resistant to the first bacteriophage. In 1987, Smith and Huggins used bacteriophages to treat calves with *E. coli*-caused diarrhea. They selected their bacteriophages by administering *E. coli* to a calf followed by a bacteriophage cocktail. Bacteriophages able to survive the gastrointestinal tract were collected in the feces 24 hours post-administration. These bacteriophages were used to treat subsequent calves. Calves given bacteriophages within 24 hours of the onset of diarrhea recovered within 20 hours. Also, sick calves placed on litter that had been sprayed with bacteriophages recovered from diarrhea. Smith and Huggins noted that during the period of disease, bacteriophages continued to persist in the feces, but after recovery, bacteriophage numbers dropped dramatically.

Biswas et al. (2002) successfully cured *Enterococcus faecium*-infected mice with bacteriophage therapy. Mice were treated with bacteriophages just 45 minutes after infection with bacteria. Treatment at a multiplicity of infection (MOI) level of 0.3 to 3.0 was able to cure all of the infected mice. However, lower MOIs of 0.03 to 0.003 resulted in just 60% and 40% survival of mice, respectively. They also noted that bacteriophage treatment could be delayed for up to five hours after infection. However, if treatment was delayed for 18 or 24 hours, only 50% recovery was seen.

Berchieri et al. (1991) treated broiler chickens infected with *Salmonella typhimurium* (ST) with bacteriophages and found that the levels of ST could be reduced by several logs, and mortality associated with ST was reduced significantly. However, ST was not eliminated and it returned to its original levels within six hours of treatment. Also, the bacteriophages did not persist in the gastrointestinal tract for as long as the *Salmonella* was present. In fact, bacteriophages persisted only as long as they were added to the feed. In order to be effective, bacteriophages had to be administered in large numbers, and soon after infection with ST.

In 1998 Barrow et al. prevented morbidity and mortality in chickens using bacteriophages lytic for *E. coli*. When chickens were challenged intramuscularly with *E. coli* and simultaneously treated with $10^6 - 10^8$ pfu of bacteriophages the mortality was reduced by 100%. This study also demonstrated that bacteriophages can cross the blood brain barrier, and furthermore that they can amplify in both the brain and the blood. Similarly, a number of other researchers have shown that bacteriophages can be useful for treating non-enteric *E. coli* infections. Extensive research about the effects of bacteriophages on colibacillosis in broiler chickens has shown that bacteriophages can treat respiratory infections (Huff et al, 2002a-b; Huff et al, 2003a-b). Treatment was most successful when bacteriophages were directly applied to the infected area or injected into the bloodstream. This observation is consistent with previous research discussed above.

However, such successes do not necessarily translate into effective enteric treatments. Host-associated pressure against pathogen infections may predispose systemic bacteriophage therapy toward success. In these cases, where bacteriophage(s) are used to treat systemic or tissue-associated infections, an acute efficacy of merely reducing the infection load by 90% or more, could greatly reduce mortality and reduce the duration and magnitude of disease. In the intestinal lumen, host pressures against the infection may not be as severe and many Enterobacteriaceae are capable of free living status within the gut without eliciting robust acquired immune responses from the infected animal. In these cases, a temporary reduction in enteric colonization may not be as likely to be curative, as discussed below.

6.3 Failures

As the history of published successful bacteriophage treatments of enteric disease is reviewed, it is readily evident that such reports, while often dramatic in effect, are relatively sporadic during the last approximately 60 years. Given that experimental failures frequently are not published, as the cause of failure can often not be ascertained, the authors suspect that history is replete with unpublished examples of failures to treat enteric Enterobacteriaceae infections.

Our laboratory, and others, have demonstrated that resistance to bacteriophages selected against *Salmonella* isolates quickly occurs, often in a single passage (Bastias et al, 2010). When bacteriophage cocktails of 71 different bacteriophages selected for treatment of experimental *Salmonella enteritidis* infections in chickens, a brief reduction in enteric

colonization was noted during the first 24 hours, but rebound levels were similar to controls within 48 hours, even with repeated or continuous dosage of the bacteriophage cocktail (Higgins et al, 2007). Because of the demonstrated temporary reduction in enteric colonization in these studies, effective bacteriophages were demonstrably able to pass to the lower gastrointestinal tract. As continued treatments failed to maintain this reduction, development of resistance by the enteric *Salmonella enteritidis* is the most likely explanation. In order to potentially deliver higher levels of bacteriophage, several attempts to protect the bacteriophage cocktail through the upper gastrointestinal tract were made in our laboratory. Pre-treatment of infected poultry with antacid preparations designed to reduce the acidity of the proventriculus (true stomach) were successful in increasing the number of administered bacteriophage that successfully passed into the intestinal tract, but this treatment did not improve the outcome of bacteriophage treatment of *Salmonella enteritidis* infection (Higgins et al, 2007).

An alternative approach is to select for alternative non-pathogenic bacteriophage hosts which could potentially “carry” bacteriophage through the gastrointestinal tract and, with continuous dietary administration of the non-infected alternative host bacterium, provide a means of amplification within the gut of the host (Bielke et al., 2007a). Bielke and co-workers demonstrated that non-pathogenic alternative hosts can be selected for some bacteriophages that were originally isolated using a *Salmonella enteritidis* target (2007b). This approach, which has potential utility for amplification of large numbers of phage without the necessity to thoroughly separate bacteriophage from a pathogenic target host, was also used to create a potential “Trojan Horse” model for protecting the bacteriophages through the upper gastrointestinal tract, thus potentially providing a vehicle for enteric amplification of those surviving bacteriophages. In these studies, neither the Trojan Horse approach, nor the continuous feeding of the alternative host bacteria as a source of enteric amplification, were effective in producing even more than a transient reduction in enteric *Salmonella* infections. Through these failures, many investigators have concluded that the escape of even a minority of target bacteria within the enteric ecosystem allows for almost immediate selection of resistant target bacteria and rebound to pre-treatment levels of infection may even exceed the levels of non-treated controls in some cases.

6.4 Potential strategies to overcome failures

Bacteriophage resistance is an important component of therapy to overcome before bacteriophages can really be a viable antimicrobial for infection. The generation time for bacteria is typically short enough that mutants with bacteriophage resistance can emerge within hours (Higgins et al, 2007; Lowbury and Hood, 1953). One possibly strategy to overcome this problem is administration of multiple bacteriophage isolates for treatment, but resistance is difficult, if not impossible, to predict and combining the correct cocktail of bacteriophages to overcome resistance would be a blind guess in most cases.

The most success is likely to come from treating points in the system that are continually bombarded with bacteria that have not been previously subjected to the bacteriophages being used for treatment. Also important for this system is keeping exposure of the bacteria to bacteriophages to a minimal amount of time. If the bacteriophages interact with the bacteria for long periods of time, the bacteria will become resistant. Food and meat processing facilities are an excellent example. As live animals enter a processing facility, the bacteria have not likely been exposed to the bacteriophages used to treat the infection. This greatly increases the chances of success.

Higgins and co-workers (2005) successfully treated turkey carcasses at a processing facility with bacteriophages specific to the *Salmonella* to which they were infected. This process was effective when either an autogenous bacteriophage treatment targeted to the specific *Salmonella* strain infecting the turkeys was used, or a cocktail of nine wide host-range *Salmonella*-targeting bacteriophage were used. Similarly, a bacteriophage treatment for cattle carcass contamination has been effective at reducing the *E. coli* 0157:H7 load at processing has been developed and commercially licensed in the United States. These successes avoid development of bacteriophage resistance by applying treatment at a single point during production, in an environment where proliferation of the target organism is extremely limited. In this way, since the target organism is never intentionally exposed twice to the same treatment, resistance is unlikely to ever increase beyond the naturally-occurring resistance to the bacteriophage (or cocktail) used.

One of the most well documented successes of published treatment of enteric Enterobacteriaceae infections with bacteriophages was the study of Smith and Huggins (1983) as described above. It is notable that in this successful study, the bacteriophage cocktail used was a combination of two bacteriophages, but the second was isolated using the target organism which was resistant to the first bacteriophage. This approach of selecting for bacteriophage isolates using target bacteria that are resistant to sequential bacteriophage treatments was not used in the work of Higgins et al (2007), or in several other published studies. Higgins and co-workers (2007) used a collection bacteriophages, independently isolated from different sources and with several different plaque morphologies, suggesting that a number of different bacteriophages were employed – and failed to persistently reduce enteric colonization.

It is possible that one of the most notable exceptions to the many failures to treat enteric Enterobacteriaceae infections during recent years, that of Smith and Huggins (1983), provides a singular clue as to the potential for enhancing the likelihood of enteric Enterobacteriaceae efficacy. It is possible that selection of multiple bacteriophages for the same target cell phenotype results in selection of bacteriophages that are effective through identical mechanisms of adhesion, penetration, replication, and release. When new bacteriophages are isolated for efficacy against sequentially resistant isolates of the target bacteria, and these are combined for administration as a cocktail, the ability of the target cell to shift phenotype may be severely limited, resulting in a much larger proportion of target cell reduction, thereby increasing the probability of elimination or cure.

Clearly, widespread bacteriophage treatments with Enterobacteriaceae have not been adopted for any animal species during the last 60 years and successful research in this area has been modest and sporadic. Nevertheless, the occasional reports by reputable scientists in solid journals must indicate that there is potential for improved therapeutic efficacy of bacteriophages for this purpose. With the diminution of new antimicrobial pharmaceuticals and the widespread resistance among many pathogenic enteric Enterobacteriaceae, a breakthrough in this area is sorely needed.

7. Vaccination for control of *Salmonella* in poultry

Killed whole-cell bacterins and live attenuated vaccines are the most common types of vaccines currently used in the poultry industry. Vaccination programs depend on the recognition of specific antigens, called epitopes, by the immune system of the host to prevent or reduce the spread of pathogenic viruses and bacteria. Because there are a large

number of *Salmonella* serovars, each with individual epitopes that do not elicit cross-protection against other serovars, there has been little traditional emphasis on development of generic *Salmonella* vaccines. Primarily, killed vaccines, which generally must be administered parenterally (through injection), have been applied to protect against systemic infections, and although they have been shown to reduce colonization and shedding, the protection provided by these vaccines has limited ability to stop intestinal colonization. They predominantly stimulate both humoral (circulating IgM and IgG) and cell-mediated responses, but are quite ineffective at generating mucosal immunity as secretory IgA antibody stimulation is very low through this type of vaccination. This is important because, whereas both systemic (humoral and cell-mediated) and mucosal immunity can reduce the chances of disease and mortality, only the mucosal portion of this adaptive immune response is capable of protecting animals from infection. The key to inducing both an adaptive systemic and mucosal response has traditionally been through the use of the mucosa as a “portal of entry” for live but weakened (attenuated) vaccines. However, the use of such vaccines for protection against *Salmonella* infection have been tremendously limited due to the very large number of different antigens presented by the more than 200 serotypes that can infect domestic animals and man, with more than 38 of these commonly infecting poultry within the United States, as discussed below (Hargis et al., 2010).

One approach to solving the problem of serotype variation among the common paratyphoid strains of *Salmonella*, which are often not a disease-causing problem for poultry but rather create a source of foodborne illness for consumers, is the identification of “universal epitopes” that are shared among all *Salmonella* isolates. This concept has been established for a number of pathogens and is based on the identification of a minor surface structure (antigen or epitope) which does not cause robust immune reaction during infection, but which can be targeted for protection if the antigen is presented in a way that tricks the animal into responding robustly. Some of these are relatively minor antigens which are highly conserved among related organisms - usually because they involve biological function. Since small peptide sequences that are biologically functional cannot vary in sequence, organisms that carry a mutation for such sequences are often either lethal or sufficiently detrimental to cause these to not be successful over time (Neiryneck et al., 1999).

A well-described example of this phenomenon is a small 23 amino acid peptide on the surface of Type A Influenza viruses named M2e. This peptide is part of an ion transport channel which is necessary for viral activation. Mutations in this sequence undoubtedly occur frequently, but since the 1918 Spanish Influenza outbreak, all Type A Influenza isolates share a highly conserved core sequence for this peptide (Layton et al., 2009). Although natural influenza infection does not result in a robust immune response to this peptide sequence, tricking the animal into producing a robust response has resulted in protective immunity in several animal species (Neiryneck et al., 1999; Mozdzanowska et al., 2003; Fiers et al., 2004; Zou et al., 2004). In recent years, the rapid increase in molecular biological techniques has led to the development of more sophisticated vaccines, of which live recombinant bacterial vectored vaccines are one of the most promising (Ashby et al., 2005; Zhang et al., 2006; Duc et al., 2007; Kajikawa et al., 2007; Uyen et al., 2007; Yang et al., 2007; Huang et al., 2008; Liu et al., 2008; Ceragioli et al., 2009; Deguchi et al., 2009).

This type of vaccine uses a genetically modified bacterium to express a heterologous antigen. Oral live attenuated *Salmonella* vaccine vectors expressing recombinant foreign antigens have previously been shown to stimulate systemic, mucosal, humoral, and cell-mediated immune responses against *Salmonella* (Mollenkopf et al., 2001; Koton and

Hohmann, 2004; Ashby et al., 2005). *Salmonella* vectors have the potential advantage of being extremely inexpensive to manufacture and, because they do not have to be injected and can be administered by spray or drinking water, they are much more acceptable for widespread administration to commercial poultry.

Currently, some laboratories are exploiting this concept by identifying candidate antigens/epitopes that are evolutionarily conserved between the many different serotypes of *Salmonella* and which do not elicit a robust response when animals are infected with wild type *Salmonella* (or vaccinated with conventional vaccines), but which may protect against infection when delivered in an appropriate way using a recombinant vaccine platform (Wolfenden, RE et al., 2010; Kremer et al., 2011). Recently, bacterial carriers of antigens (vectors), including *Salmonella* Enteritidis and *Bacillus subtilis*, have been manipulated to express protein antigens to protect against bacterial, viral, and protozoal pathogens (Layton et al., 2009; O'Meara et al., 2010; Kremer et al., 2011; Layton et al., 2011). These vaccines have an advantage over many other types of vaccines in that they are able to be delivered directly to a mucosal surface via nasal, ocular, or oral administration. Because most pathogens invade the host through a mucosal surface, an enhanced mucosal immune response is the only portion of acquired immunity that can markedly reduce the probability of an animal or flock to become infected, as discussed above. While prevention of morbidity and mortality alone are useful traits of conventional vaccines for most poultry disease-causing agents, in the case of the common *Salmonella* serotypes which cause foodborne illness, these isolates generally cause little or no disease in the animals. Thus, recombinant vaccines that are able to provide wide-range protection against common *Salmonella* serotypes of poultry, by mucosal presentation, may be a critical component for controlling this problem in the next few years.

Along with presentation of conserved antigens through mucosally-administered recombinant vaccines, there is a need to trick the immune system of the animal to respond robustly to these recombinant bacteria that are not capable of infecting or causing disease. Co-expression of molecules that may enhance the immune response or may be recognized by receptors located on the mucosal surface of the gastrointestinal tract is a promising area of work. Several such molecules may enhance the response to these recombinant vaccines (Layton et al., 2009; O'Meara et al., 2010; Wolfenden et al., 2010).

Presently, there are no broad-spectrum recombinant vaccines approved for use in agricultural animals to protect against the wide range of serotypes which plague poultry producers worldwide. Specific serotype vaccines, such as *S. Enteritidis* or *S. Gallinarum*, have gained considerable acceptance in countries with endemic problems with these more devastating serovars, particularly in breeders and table egg production chickens (see Shivaprasad, 1997, for a review). These vaccines generally do not provide robust protection against infection with even the identical serotype, and even less protection against heterologous serotypes (Hargis et al., 2010). However, there is a general consensus that some protection is provided and for valuable birds, these vaccines may offer a much-needed modicum of protection, though often through reduced persistence and shedding of the organism, thus limiting spread. For example, studies have shown that oil emulsion *Salmonella* Enteritidis bacterins administered to breeders caused a three log₁₀ cfu/g cecal content reduction in recovery from progeny chicks (Inoue et al., 2008), and a two log₁₀ cfu/g cecal content reduction in breeders after molting (Nakamura et al., 2004). Thus, these vaccines have value at the present time, especially for breeders and at-risk laying hens.

Live-type vaccines with gene deletions assuring avirulence while allowing immunogenicity have been reported (Curtiss and Kelly, 1987; Dueger et al., 2003), and other specific deletion

mutants have been proposed (Zhang-Barber et al., 1999; Sydenham et al., 2000). Day-of-hatch chicks vaccinated with this type of attenuated *Salmonella* vaccine have been shown to have serological protection to homologous and heterologous *Salmonella* serotypes, possibly through a mechanism similar to competitive exclusion (Hassan and Curtiss, 1994; Hassan and Curtiss, 1997; Dueger et al., 2003; Holt et al., 2003; Bohez et al., 2008). Furthermore, maternal antibodies can be demonstrated in eggs and chicks from breeders vaccinated with this vaccine. These antibodies were reported to reduce *Salmonellae* colonization and to provide protection to laying hens up to 11 months post-inoculation (Hassan and Curtiss, 1997). However, susceptibility to antimicrobial agents commonly used in poultry production can reduce or eliminate the efficacy of live vaccines, and these vaccines are subject to the serotype limitations as discussed above.

Autogenous vaccines provide for yet another mechanism for vaccinating poultry. In many (but not all) countries, there are regulatory provisions under certain circumstances for production of specific killed vaccines using the specific isolate plaguing a given poultry flock or complex. These “autogenous” *Salmonella* isolates are typically grown, killed and mixed with an adjuvant (a chemical that potentiates the immune response) for parenteral administration. Some veterinarians associated with valuable breeder flocks believe that these vaccines are highly preferred for vaccination against endemic and common serotypes for which no commercial vaccine exists.

Taken together, there are tremendous future opportunities for manipulating the acquired immune response, particularly the mucosal secretory IgA response, for reducing *Salmonella* infections in poultry. However, current vaccine availability is limited and progress is greatly needed on two fronts: 1) improving mucosal immune responses for *Salmonella* vaccines; and 2) targeting shared protective epitopes for broad-spectrum serotype coverage for the paratyphoid *Salmonellae* that currently plague poultry producers world-wide. Currently-available commercial vaccines are enjoying significant popularity due to the intense regulatory pressures facing meat and egg producing poultry, although applications are generally limited to breeder or layer flocks except under intense regulatory pressure.

8. Conclusions

The interest in digestive physiology and the role of microorganisms has generated data whereby human and animal well-being can be enhanced and the risk of disease reduced. New molecular techniques that allow an accurate assessment of the flora composition, resulting in improved strategies for elucidating mechanisms. Given the recent international legislation and domestic consumer pressures to withdraw growth-promoting antibiotics and limit antibiotics available for treatment of bacterial infections, probiotics can offer alternative options. New advances in the application of probiotics, are directed to produce significant changes in gut physiology and provide even higher levels of health as well as increase performance parameters in poultry.

Metchnikoff founded the research field of probiotics, aimed at modulating the intestinal microflora (Dobrogosz, Peacock, & Hassan, 2010; Schmalstieg & Goldman, 2010; Weissmann, 2010). However, other parts of the body containing endogenous microflora or problems relating to the immune system may also be candidates for probiotic therapy. Research has shown that probiotics have potential for human health issues such as: vaginal candidiasis (Ehrstrom et al., 2010; Ya, Reifer, & Miller, 2010); dental caries (Chen & Wang, 2010; Stamatova & Meurman, 2009); allergies (Gourbeyre, Denery, & Bodinier, 2011; Schiavi, Barletta, Butteroni,

Corinti, Boirivant, & Di Felice, 2010b); autoimmune diseases (Lavasani et al., 2010; Tlaskalova-Hogenova et al., 2011); urogenital infections (Pascual, Ruiz, Giordano, & Barberis, 2010; Ruiz et al., 2009); atopic diseases (Hoang, Shaw, Pham, & Levine, 2010; Nermes et al., 2011); rheumatoid arthritis (Lee et al., 2010; Mandel, Eichas, & Holmes, 2010); and respiratory infections (Harikrishnan, Balasundaram, & Heo, 2010; Silvestri et al., 2010). Current research is still heavily biased toward gastrointestinal applications for probiotics, such as: chronic constipation (Bu, Chang, Ni, Chen, & Cheng, 2007; Coccorullo et al., 2010); chronic diarrhea (Preidis et al., 2011; Swidsinski, Loening-Baucke, Verstraelen, Osowska, & Doerffel, 2008); inflammatory bowel disease (Ng, Chan, & Sung, 2011; Vanderpool, Yan, & Polk, 2008); irritable bowel syndrome (Camilleri & Tack, 2010; Enck, Klosterhalfen, & Martens, 2011); and food allergy (Gourbeyre et al., 2011; Schiavi, Barletta, Butteroni, Corinti, Boirivant, & Di Felice, 2010a), but the possibilities for impacting many areas of health are numerous. Much research has been completed in efforts to understand and apply the natural benefits of non-pathogenic bacteria, but there is much still to do.

New approaches to vaccination-based prophylaxis for *Salmonella* infection in poultry offer tremendous hope that highly effective vaccines may be on the horizon for commercial poultry. However, currently available and autogenous vaccines for *Salmonella* offer a modicum of protection that is generally only useful for breeders and laying hens at this time.

Although there are occasional successes with treatment of enteric *Salmonella* infections in live birds with bacteriophage cocktails, as described above, resistance to bacteriophage lysis generally develops very quickly, leading most scientist to conclude that these offer little promise for treating *Salmonella* infections in live poultry. However, when broadly-effective bacteriophage cocktails have been applied to poultry carcasses at processing, these cocktails have been highly efficacious and potentially cost-effective for inducing marked reductions in *Salmonella* contamination (Higgins et al., 2005). This latter approach has the probability of avoiding the resistance issues associated with treatment of live animals in that *Salmonella* contaminants would only be exposed to the bacteriophage cocktail at a single point in the vertical production scheme, thereby avoiding re-introduction of resistant *Salmonella* isolates into the integrated poultry production operation.

The scientific progress outlined in this chapter show highly encouraging progress toward intervention methods for *Salmonella* infections of poultry, and opportunities that are just becoming available to potentially impact poultry as a source of *Salmonella*-related food borne illness. *Salmonella* infections of poultry continue to be hugely problematic in both developed and developing countries. To date, no single “silver bullet” has been identified which can be applied commercially to eliminate this risk for this important and healthy human food source. Nevertheless, several tools, as described above, have been shown to be highly effective in reducing *Salmonella* levels in poultry production operations worldwide, particularly when used in combination. New probiotic/DFM products, with isolate selection based on better understanding of the mechanisms of efficacy, along with eventual regulatory approval and commercialization of exciting new vaccine technologies may make a tremendous impact in the very near future.

9. References

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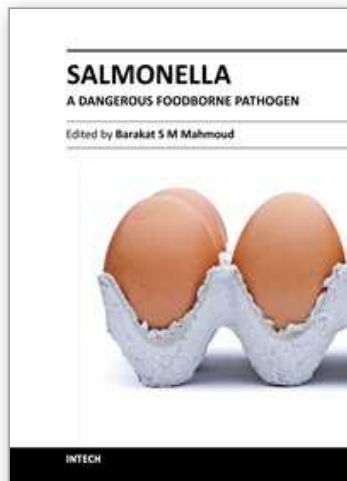
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Salmonella - A Dangerous Foodborne Pathogen

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More than 2,500 serotypes of Salmonella exist. However, only some of these serotypes have been frequently associated with food-borne illnesses. Salmonella is the second most dominant bacterial cause of food-borne gastroenteritis worldwide. Often, most people who suffer from Salmonella infections have temporary gastroenteritis, which usually does not require treatment. However, when infection becomes invasive, antimicrobial treatment is mandatory. Symptoms generally occur 8 to 72 hours after ingestion of the pathogen and can last 3 to 5 days. Children, the elderly, and immunocompromised individuals are the most susceptible to salmonellosis infections. The annual economic cost due to food-borne Salmonella infections in the United States alone is estimated at \$2.4 billion, with an estimated 1.4 million cases of salmonellosis and more than 500 deaths annually. This book contains nineteen chapters which cover a range of different topics, such as the role of foods in Salmonella infections, food-borne outbreaks caused by Salmonella, biofilm formation, antimicrobial drug resistance of Salmonella isolates, methods for controlling Salmonella in food, and Salmonella isolation and identification methods.

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