Pre-Slaughter Control of Food-Borne Bacterial Pathogens in Poultry without Antibiotics

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Summary

There is growing concern with regard to the continued emergence of antibiotic resistant bacteria, which pose a significant risk to both human and animal health. Antibiotics have been used as therapeutic agents in agriculture since the 1940 s and in subtherapeutic doses as food additives to improve feed conversion of livestock and domestic poultry since the 1950's. However, the use of antibiotics in the poultry industry, both at therapeutic and sub-therapeutic levels, is thought by some to add to the problem of the emergence of antibiotic resistant bacteria (Anonymous, 1999). The emergence of antibiotic resistant bacteria has been the basis for considerations to eliminate or severely restrict the use of antibiotics in the poultry industry. Such restriction would make the poultry industry vulnerable to an increase in bacterial disease of food safety importance. These diseases would significantly increase the risk of pathogen contamination of poultry products, increase the cost of poultry production, and decrease poultry welfare. Therefore, there is a real need to find effective and practical alternatives to antibiotics for poultry production.

Endemic *Salmonella* and *Campylobacter* continue to be predominant food-borne pathogens worldwide, and poultry and poultry products are a prevailing vehicle for disease (Bean and Griffin, 1990; Persson and Jendteg, 1992). This manuscript explores two alternative strategies for controlling food borne pathogens. The first strategy, competitive exclusion (CE), relies on administration of carefully selected beneficial bacteria to day-of-hatch chicks or poults to accelerate intestinal maturity and reduce the prevalence of *Salmonella* and *Campylobacter* infection. Without such treatment, neonatal chicks and poults are susceptible to infection by very low numbers of pathogens. The concept of accelerating development of normal enteric microflora, reducing the susceptibility of young poultry to infection, is illustrated through the use of competitive exclusion products. These products are mixtures of bacteria with the ability to reduce or exclude pathogenic colonization in chicks or poults. While competitive exclusion has been shown to have tremendous potential, existing cultures lack consistent efficacy, may be expensive, difficult to administer, or could potentially harbor unknown pathogens. Importantly, several effective competitive exclusion cultures, developed principally to target *Salmonella*, have also been demonstrated to be efficacious against a wide range of enteric bacterial pathogens. This latter observation underscores the fact that the mechanisms of competitive exclusion are not specific to any one group of pathogens and provides an example of how this concept could be used to combat disease-causing and performance-reducing enteric infections of poultry.

The second strategy currently under exploration in our laboratories is the use of bacteriophages (viruses that infect and kill only bacteria, with no potential to harm animals or plants) that are targeted specifically against *Salmonella* or *Campylobacter*. Specific bacteriophage therapy has been reported in numerous research studies to be potentially efficacious as an alternative to the use of antimicrobial drugs for the control of enteric disease in animals and man. Despite the encouraging published research, there is still a surprising dearth of information relating to appropriate methods for selection of bacteriophages for treatment of specific diseases or to reduce or eliminate food borne pathogens in poultry. The potential value of custom bacteriophage selection for therapeutic treatment of antibiotic resistant should be considered because of the individual value of poultry flocks, especially in cases of chronic or potentially catastrophic disease problems. With careful biological characterization of bacteriophages and evaluation of characteristics predictive of therapeutic efficacy, it is quite possible that bacteriophage therapy will become an accepted alternative for some disease problems of commercial poultry. Presently, we have isolated bacteriophage for *Salmonella* or *Campylobacter* from a variety of environmental sources and have extensive preliminary data on their efficacy *in vitro*. As bacteriophages are highly specific for individual bacterial isolates, it will be necessary to develop and perfect methodologies for rapid selection, amplification and

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use of phages that are effective against specific *Salmonella* or *Campylobacter* isolates. Because of the increasing concerns about antibiotic resistance, we believe these are important alternatives with considerable potential to combating food borne pathogens.

Issues Driving Development of Antimicrobial Alternatives in the U.S.A.

Food borne illness is a significant worldwide public health problem. The United States Council for Agricultural Science and Technology, in a 1994 report entitled Food Borne Pathogens: Risks and Consequences estimated that as many as 9,000 deaths and 6.5-33 million illnesses in the United States each year are caused by ingestion of contaminated foods. Recent disclosures of bacterial contamination of poultry food products has begun to erode the public confidence to the point that chicken is no longer considered an economical wholesome food source by some consumers. In 1996, the Foodborne Diseases Active Surveillance Network (FoodNet) collected data on 9 foodborne diseases in several sites within the United States (USDA-FSIS, 1997). Since the start of this program, *Campylobacter* and *Salmonella* have been the leading causes of laboratory-confirmed foodborne illness. In 1997, *Campylobacter* (3,966 cases) and *Salmonella* (2,204 cases) accounted for over 76% of the confirmed foodborne-related diseases in the United States (USDA-FSIS, 1998). The poultry industry has demonstrated that chicken can be economically produced, making it possible to continue meeting the needs of a growing global population. However, a major concern for poultry producers is the continued cost-effective production of poultry in the absence of antibiotics, which may be mandated in the near future. Even in countries where antibiotic use in poultry may not be eliminated by regulatory mandate, emerging antimicrobial resistance issues and a lack of new antimicrobial drugs has reduced the effectiveness of existing drugs.

In countries where regulatory actions mandate reduced pathogen loads, additional costs are incurred by the processor through government mandates to control the spread of poultry-associated food borne pathogens through programs such as HAACP. From a more global perspective, one study calculated an annual *Salmonella*-related loss of approximately 1.4 billion dollars in lost human productivity, medical expenses and increased animal production costs in the United States alone (Madie 1992). For these reasons, identification of alternative pathogen control strategies that can offer the producer an economic alternative and yet also be readily accepted by consumers has become an important priority. Control of bacterial pathogens in the commercial poultry setting has a potential two-fold benefit; reduction of the impact of low level disease on performance, and reduced potential of poultry products to cause food-borne illness in humans.

Competitive Exclusion

Neonatal chicks are susceptible to infection by very low numbers of *Salmonella* and *Campylobacter*, with increasing resistance as the birds, and presumably their normal enteric microflora mature (Byrd *et al.*, 1998, Mead, 1998, Young *et al.*, 1999). The concept of accelerating development of normal enteric microflora, thereby increasing the resistance of young poultry to infection, was first described by Nurmi and Rantala (1973) following a significant *Salmonella infantis* outbreak in Finland.

There are several proposed mechanisms of protection provided by effective competitive exclusion cultures (Nurmi *et al.*, 1992; Corrier and Nisbet, 1999), including competition for binding sites, competition for nutrients, and production of antibacterial compounds. Since that time, numerous mixed and undefined cecal cultures have been demonstrated to provide marked protection against *Salmonella* infection (Pivnick and Nurmi, 1982; Mead and Impey, 1986; Bailey, 1987; Stavric and D aoust, 1993).

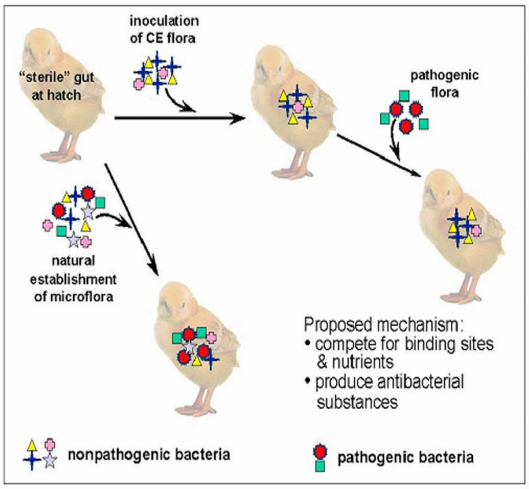


Figure 1.

Cecal cultures can also protect against *Campylobacter* although the effect is inconsistent (Newell and Wagenaar, 2000). These undefined cultures have generally relied on periodic amplification in specific pathogen free chickens and production of batch cultures. Because of public pressure for reducing food borne illness in many European countries, coupled with increasing European restrictions on use of antimicrobial drugs in poultry, these undefined cultures have met with considerable acceptance in commercial poultry operations in several countries (Mead, 1998). Furthermore, commercial producers of undefined cultures have found surprising market share in several developing countries presumably due to improved performance associated with treatment. However, concerns regarding the safety of application of undefined cultures with regard to the possibility of introducing a known or emerging pathogen into poultry flocks has prevented general acceptance and Food and Drug Administration approval of undefined competitive exclusion cultures in the United States (Byrd, 1999).

More recently, an effective defined (PREEMPTTM MS Bioscience, Madison, Wis.) and an undefined (Mucosal Starter Culture, Continental Grain, Chicago, Ill) culture were developed by USDA-ARS scientists (Corrier, *et al.*, 1995; Blankenship, *et al.*, 1993). Following demonstration of prophylactic (but not therapeutic) efficacy, PREEMPTTM was licensed by the United States Food and Drug Administration (FDA). Furthermore, Mucosal Starter Culture may eventually meet with FDA approval as the culture originates from the same seed culture in each batch culture, without additional amplification in chickens, thus greatly reducing concerns with regard to unintentional pathogen introduction. Concerns relating to cost, storage requirements, and ease of administration have limited commercial acceptance of PREEMPTTM to date within the United States, although other cultures, not currently approved for use in the United States, have gained some acceptance in many countries world-wide.

Unpublished data from our laboratories indicate that occasional adult chickens can be identified which have enteric microflora with the ability to displace enteric *Salmonella* in infected chicks. Therefore, it may be possible to identify cultures with the ability to actually treat infected poultry flocks through *in vitro* screening, an attribute that would greatly increase the usefulness and appeal of competitive exclusion. The only effective defined culture currently available in the United States was produced from a randomly selected chicken without regard to any special attributes of that animals protective microflora. Thus, there is considerable appeal to using cultures originally selected by screening microflora from a relatively large number of chickens for *in vitro* efficacy against *Salmonella* and *Campylobacter*. By pre-selecting cultures with marked prophylactic and/or therapeutic characteristics *in vitro*, the resulting product may be enhanced with regard to efficacy in chicks.

Significant progress has been made in the identification and testing of cecal cultures against both *Salmonella enteritidis* and *Campylobacter jejuni*. The *in vitro* procedure developed by our laboratory have allowed sampling ceca from numerous poultry to identify cultures with efficacy against *Salmonella enteritidis*. Using an indicator for H2S production (suggestive of *Salmonella* growth) we have demonstrated that we can rapidly screen mixed cecal microflora for the presence of organisms capable of eliminating *Salmonella in vitro*.

To date, we have identified over 125 isolates capable of competing against *Salmonella enteritidis, in vitro*. These cultures have been identified to species level, grown as related groups and tested on chicks for prophylactic efficacy. These preliminary results related to protection against *Salmonella enteritidis* infection have provided an important bench mark and encouragement to begin development of a separate screening procedure for the identification of cultures capable of reducing *Campylobacter, in vitro*. Use of this *in vitro* assay has, to date, successfully identified more than 30 cultures effective against *C. jejuni in vitro*. Preliminary results indicate that these cultures are capable of excluding both *Salmonella enteritidis* (Table 1 and 2) and *Campylobacter* from the gut of neonatal chicks.

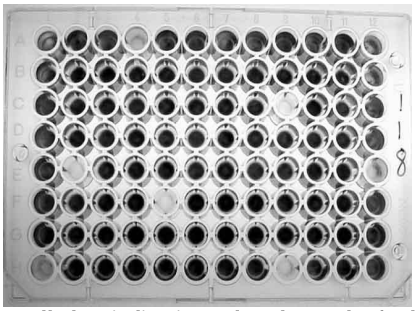
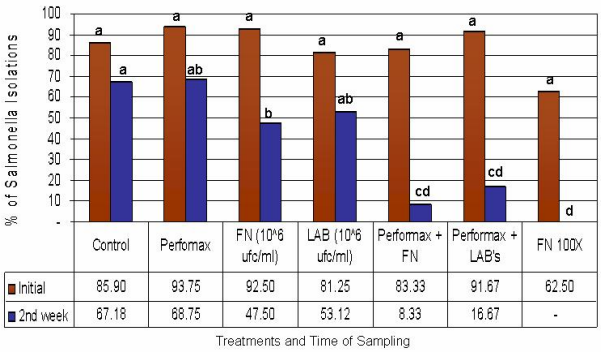


Figure 2. 96 well plate indicating reduced growth of Salmonella enteritidis (SE) in the presence of competitive exclusion were placed in each well, and Salmonella enteritidis was also placed in all wells. Dark wells indicate the presence of SE through production of H2S, while SE growth has been inhibited in clear wells. Bacteria isolated from the clear wells were further isolated and evaluated for efficacy.

Very recently, our laboratory has evaluated a defined culture consisting of nine bacterial organisms in a large commercial turkey field trial in the United States involving approximately 38 million turkeys. In these studies, performance was enhanced ($0.096 per bird due to feed efficiency improvement), mortality was reduced, total medication cost was reduced, and *Salmonella* detection on carcasses at the processing plant was reduced by almost 50%. Also, as part of this study, *Salmonella*-infected flocks were identified at least 2 weeks prior to slaughter and treated with the bacterial culture. Treatment resulted in eliminating incidence of recovery of *Salmonella* in over 80% of the drag swabs (environmental samples) as compared to controls (Figure 3). These results provide some indication that this culture and others may be effective for actually treating enteric infections of poultry.

Figure 3. Effect of Beneficial Bacteria Alone or in Combination with Perfor-Max on Salmonella Isolation in Commercial Turkey Houses



Bacteriophage Therapy

Bacteriophages are considered more plentiful in the biosphere than any other group of organisms (Kokjohn, *et al.*, 2000). Discovered more than 80 years ago by H. D. Herelle and F. Twort (Kutter, 1997), bacteriophage therapy has re-

ceived little attention in the Western world, perhaps because of continuous antimicrobial development and inconsistent results (Soothill, 1992).

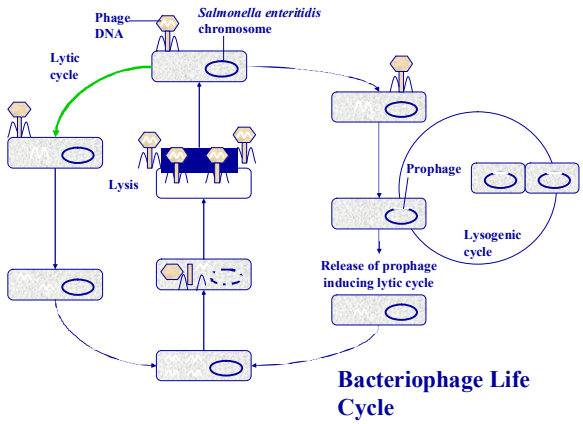


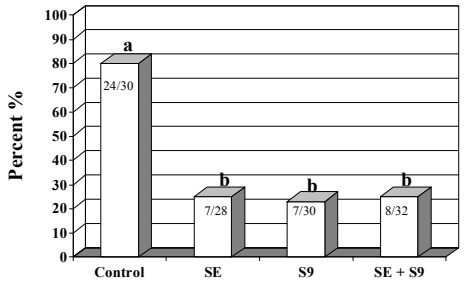
Figure 4.

Despite the fact that the Western world has largely ignored bacteriophages, human bacteriophage therapy in Eastern Block countries has been reportedly practiced for more than 50 years with considerable success (Kutter, 1997). The potential benefits of bacteriophage use in the poultry industry were first explored in 1925 when phages specific for *Salmonella pullorum* were successfully isolated from the feces of hens who had recovered from an infection. It was demonstrated that two of the bacteriophages could lyse 31 different strains of *Salmonella pullorum*, and could be maintained by passing the phage into fresh media daily. However, in treating chicks with bacteriophage only limited success was achieved. While not completely clearing the infection, phage treated groups experienced a postponed death and lessened bacillary diarrhea (Pyle, 1925).

It was not until the 1980's that interest in bacteriophage applications for animal infections was renewed. Smith and Huggins (1982) successfully treated *Escherichia coli* infections in mice, calves, piglets, and lambs with bacteriophage. Importantly, they reported that in their mouse model phage effectiveness *in vitro* was correlated with efficacy in vivo. In these studies, a single bacteriophage was as efficacious as multiple doses of streptomycin, and more effective than multiple doses of tetracycline, ampicillin, chloramphenicol, or trimethoprim (Smith and Huggins, 1982). Additionally, they found that when challenging calves with *E. coli*, treated calves had higher levels of phage resistant mutant bacteria, yet these bacteria were less virulent, so that they did not colonize the small intestine and induce diarrhea (Smith and Huggins, 1983).

Preliminary work in our laboratories has demonstrated that relatively large numbers of bacteriophages, capable of effectively lysing specific *Salmonella enteritidis* or *Campylobacter* can be quickly isolated from municipal sewage using conventional soft overlay techniques. Bacteriophages were purified from environmental samples likely to contain target organisms, with or without prior amplification by host suspension addition, by filtration through a 0.2 micron filter to remove bacterial colonies. Serial (limit) dilution and combination with the specific target organism (e.g. *Salmonella enteritidis*) in soft liquid agar (0.65%) allowed for the identification of clonal lytic bacteriophage plaques. In a preliminary study two concentrations of a single wild type phage (PHL 4) were evaluated for the ability to reduce or eliminate *Salmonella enteritidis* from broiler carcass rinse water. Commercially processed 8 week broiler carcasses collected on day of processing were individually rinsed with 100 ml of sterile distilled water. Rinse water was pooled aliquoted and inoculated with *Salmonella enteritidis* and phage. Bacteriophage significantly reduced *Salmonella* levels at the higher concentration tested (Table 3).

More recently we have demonstrated that host specificity of bacteriophage is somewhat related to bacteriophage concentration and numbers, and that higher concentrations of bacteriophage can kill a broader range of related hosts. Very recently, using a cocktail of 72 bacteriophages originally isolated against *Salmonella enteritidis*, we effectively eliminated *Salmonella* contamination on processed poultry carcasses in a commercial processing plant (Figure 5).



[a-b] Different superscripts indicate significant ($p<0.05$) differences in incidence.

Figure 5: Positive recovery of *Salmonella* from positive flocks rinsed with water and bacteriophage specific for each *Salmonella* isolate.

While bacteriophages have been successfully used for the treatment (and cure) of a variety of bacterial diseases of mammals, bacteriophage therapy is largely unevaluated in poultry and nothing is known regarding the appropriate characteristics predictive of an effective therapeutic bacteriophage treatment. Ongoing experiments have demonstrated successful treatment of respiratory colibacillosis with a bacteriophage administered by aerosol. Preliminary experiments with enteric salmonellosis indicate that not all bacteriophages are effective within the environment of the gastrointestinal tract of birds. Very recent data from our laboratories would suggest the great majority (but not all) wild-type bacteriophages are highly sensitive to rapid and marked declines in environmental pH, such as may be experienced by ingested bacteriophages as they pass the poultry proventriculus on their way to the site of enteric colonization. This provides a potential, if not probable, explanation for this partial success in treating high-virulence *Salmonella*-infected poultry. This observation, and determina-

tion of *in vitro* characteristics predictive of in vivo efficacy may be the key to providing consistently effective bacteriophage treatments for bacterial enteric diseases. These experiments are ongoing.

Conclusions

Competitive exclusion cultures can greatly increase resistance to a number of enteric bacterial pathogens of poultry. While therapeutic treatment with antibiotics can reduce or eliminate CE efficacy, re-treatment is possible given the availability of inexpensive products. The possiblity of developing therapeutically efficacious CE cultures is exciting and work in this area is ongoing. Bacteriophage therapy offers a totally different approach from the selective toxicity of antimicrobial chemicals. Bacteriophages have the potentially to be amplified to very high numbers (10 billion PFU/ml in only 1.5 hours) for application to poultry flocks. The limiting issues for bacteriophage therapy in poultry is the development of appropriate strategies to rapidly isolate and amplify appropriate bacteriophage. At present, isolation and amplification can be performed very quickly, but some of the bacteriophage isolated using the existing schemes are inappropriate for oral administration. Ongoing work is focused on determination of characteristics which will allow for more discrete selection of appropriate bacteriophage for administration to poultry. Certainly the need exists for antibiotic alternatives in poultry. The near future will reveal whether competitive exclusion and bacteriophage therapy will be viable alternatives to existing therapies.

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