Probiotics for Pathogen Control in Poultry and Livestock

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Introduction

Enteric viruses and bacteria are a major cause of death, disease and poor performance in food producing animals. Furthermore, the major food-borne human pathogens commonly associated with meat products are essentially all enteric organisms. Control of enteric pathogens in food-producing animals is essential both for improvement of animal health and productivity as well as to increase the safety of products for human consumption. Vaccination and/or antibiotics have been used effectively for control of enteric pathogens in food animals. However, concerns about antibiotic residues in meat products and increasing development of antibiotic resistance in pathogenic bacteria have stimulated interest in alternatives to mass therapy with antibiotics for pathogen control in food animals (Wilson & Salyers, 2002).

A number of options have been explored as alternatives to antibiotics including dietary manipulations, chlorates and other small molecules, organic acids, bacteriophages, antimicrobial peptides and the subject of this discussion, probiotics. A commonly used definition of probiotics is provided by Fuller who states probiotics are "live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance" (Fuller, 1989). Reported beneficial effects include increased feed intake, feed efficiency, average daily gain, improved carcass characteristics, and decreased morbidity (Patterson & Burkholder, 2003; Reid *et al.*, 2003). Note that the above definition of probiotics makes no claim about pathogen reduction, a distinction that may have significant regulatory implications (Reid *et al.*, 2003). Furthermore, confusion is introduced by various synonymous, partially synonymous and homophonic terms, such as direct fed microbials (DFM), competitive exclusion (CE) cultures, prebiotics and synbiotics. While the term DFM has been proposed by both the FDA and the American Association of Feed Control Officials (AAFCO) for feed products based on live microbial organisms and is certainly accurate, the more generic probiotic is in common usage in both the human and veterinary literature and will be used in this paper as defined above (AAFCO, 1999; FDA, 2003). CE cultures are derived from indigenous intestinal microflora of healthy animals, either as undefined mixtures or characterized as to the microbial organisms present (Nisbet, 1998). CE cultures represent an important subset of probiotics, distinct from single microorganism probiotics. Prebiotics are "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Gibson & Roberfroid, 1995; Rastall & Maitin, 2002). The term synbiotic is used to describe the combined application of pre- and probiotics to achieve a beneficial effect (Gibson & Roberfroid, 1995; Rastall & Maitin, 2002). The purpose of this paper is to examine the selection, possible modes of action and potential problems with probiotics for pathogen control, and to present some select examples of how probiotics have been applied towards control of specific pathogens in food animals.

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Characteristics of probiotics

What actually constitutes a probiotic? Empirically, based on the definition above, any live microbial preparation that provides any beneficial effect when fed is a probiotic. However, certain common characteristics have been described for probiotics and these are summarized in Table 1 (Dunne *et al.*, 1999). These properties of "ideal" probiotics relate to either practical issues (e.g. ability to be stored for prolonged periods) or to mechanism of action (e.g. production of antimicrobial substances). One characteristic commonly described as ideal for probiotics is that the microorganisms should be of host origin (Patterson & Burkholder, 2003; Simmering & Blaut, 2001). While use of only host origin microorganisms is the basis for use of CE cultures, and may lessen the possibility of adverse effects, increase colonization efficiency or make regulatory approval simpler, it is not inherently necessary for probiotic efficacy and thus has been intentionally omitted from Table 1. Furthermore, al-

though bacteria and yeasts with probiotic properties are known to possess some or all of these properties, it is likely that this list does not include all important characteristics nor are all listed characteristics required for probiotic activity. While these characteristics provide some guidance, ultimately, efficacy of a probiotic preparation must be determined empirically.

Table 1. Characteristics of an ideal probiotic.

Non-pathogenic
Remain functional through processing and storage
Resist gastric acid and bile
Adhere to gut epithelium and/or mucus
Persist in the gastrointestinal tract, for at least short periods
Produce antimicrobial substances
Modulate immune responses
Alter microbial activities

Selection of probiotics

With all the bacteria and yeasts in the world, how does one select the microbial content of candidate probiotic preparations? For CE cultures, by definition, the starting material is uncharacterized intestinal microflora from healthy animals. This material is then maintained and grown in various culture systems until a stable population is achieved. The microbial content of the CE culture may be characterized by isolation of individual bacterial species or by molecular methods such as 16S ribosomal RNA gene sequencing (reviewed in Collins & Gibson, 1999), though partially or uncharacterized CE cultures have certainly been used successfully. A second approach is to screen bacterial or fungal genera that have been previously shown to have probiotic activity. A list of commonly utilized strains is presented in Table 2. Representative strains are evaluated for specific desired activities in the host of interest to identify candidate probiotics. While possible in theory, few examples of truly random searches for probiotic candidates are present in the literature. A subject beyond the scope of this paper, but one that will be of increasing interest is the development of engineered probiotic strains. A few examples of these exist already, such as an *E. coli* that expresses the Shiga toxin receptor to adsorb toxin from the gut of infected people or an attenuated *Salmonella* that competes for adhesion sites with pathogenic *Salmonellae* (Dueger *et al*., 2003; Paton *et al*., 2000). Regardless of efficacy, regulatory and public acceptance of engineered probiotics for use in food animals is likely to be problematic, in my opinion.

Table 2. Genera proposed for use as probiotics in food animals[1].

Bacteria	*Lactobacillus*
	Bifidobacterium
	Enterococcus
	Bacillus
	Escherichia
	Proteus
	Streptococcus
Fungi	*Saccharomyces*
	Aspergillus

[1]Modified from Reid and Friendship, 2002

There are functional, safety and practical considerations that must be addressed for a probiotic preparation to be deemed useful in a production setting (reviewed in Saarela 2000). Functional assays for probiotics range from empirical feeding trials in the target species to various *in vitro* screens for colonization or antimicrobial activity. Examples of *in vitro* screening tests include evaluating candidate probiotic strain inhibition of target pathogens by cross culturing on plates or in chemostat cultures, production of low molecular mass substances that are growth inhibitory to the target pathogen and selecting for adherence to intestinal epithelial cells. Practical aspects of probiotic development include acid tolerance to enhance survival on passage through the stomach, tolerance to bile for survival in the small intestine, and for ruminants, ability to survive passage through the complex milieu of the rumen. *In vitro* screening tests for acid and bile tolerance are relatively simple. Rumen survival requires either an effective artificial rumen or *in vivo* screening. These practical issues are not the most interesting scientific problems but are absolutely essential for development of effective probiotics. Our understanding of how probiotics exert their beneficial effects is incomplete (see below) and feeding trials are still the gold standard for evaluating probiotic effects.

Safety considerations are also clearly important in the selection of probiotics. Obviously, the probiotic strain or mixture should not be pathogenic itself. Nor should it contain any opportunistic organisms that, while not normally pathogenic, could cause disease in stressed, injured or otherwise compromised animals. Most of the common bacterial and yeast strains used as probiotics are non-pathogenic, though some, such as *Saccharomyces boulardii*, have caused infection in rare cases (Hennequin *et al*., 2000). A further concern is that the probiotic preparation should not contain mobile elements capable of transferring resistance to antimicrobials or virulence factors to other enteric bacteria, including potential pathogens (Ochman & Moran, 2001). Note that this is distinct from the probiotic necessarily being susceptible to common antimicrobials, since such resistance may be non-transferable. Feeding trials to evaluate the effect of probiotics on pathogens should also be designed to evaluate adverse effects on recipients as well as antibiotic resistance effects.

Mechanism of action of probiotics

Considerable knowledge has been developed on the interaction of commensal, symbiotic and pathogenic microorganisms in the gastrointestinal tract (Hooper & Gordon, 2001; Levin & Antia, 2001; Russell & Rychlik, 2001). There are several postulated mechanisms by which probiotics exert their effect on susceptibility to pathogens. However, for most probiotics known to be effective, the actual mechanism of action is unknown. It is likely for most probiotics that combinations of factors are responsible for activity. This hypothesis is supported by the greater efficacy re-

ported for probiotic preparations consisting of mixtures of bacteria, and CE cultures, which are likely to affect a variety of processes in pathogen survival, colonization and pathogenesis (Reid & Friendship, 2002). There is considerable research interest in this area and both the proof for these mechanisms and identification of new mechanisms for probiotic activity are likely to be forthcoming. The following is a short discussion of some of the commonly proposed mechanisms for probiotic activity.

Competition for receptors.

Probiotic bacteria have been shown to compete with pathogens for receptors on the surface of intestinal epithelial cells. They are also likely to compete in some cases for other binding sites, on mucus, extracellular matrix, food particles or other sites in the gastrointestinal tract (Lee *et al.*, 2000; Ouwehand *et al.*, 1999; Styriak *et al.*, 2003; Tuomola *et al.*, 1999). The effect of competition for receptors is one of the justifications for use of *in vitro* binding to epithelial cells cultures as a screen for probiotic candidates, the other being colonization ability. Competition for receptors may be part of the basis for early expression of probiotic effect (within 4 hours of administration) observed in some experiments. A related mechanism is degradation of host receptors by probiotic microbes. This has been demonstrated for *Saccharomyces* and *Clostridium difficile* toxins in humans (Castagliuolo *et al.*, 1999).

Competition for nutrients.

The stable intestinal microflora is a complex system in which essentially all available nutrients are utilized (Holzapfel *et al.*, 1998). Effective competition for available nutrients is hypothesized to be a mechanism of probiotic activity. However, direct proof of this mechanism of action *in vivo* is lacking (Rolfe, 2000).

Production of antimicrobial substances.

Probiotic microorganisms have been shown to produce a variety of potentially antimicrobial substances. These include low molecular weight (LMW) substances such as organic acids, alcohols, hydrogen peroxide, ammonia, methylhydantoin, lipoteichoic acid, siderophores and diacetyl (Helander *et al.*, 1997). Other as yet uncharacterized LMW antimicrobial compounds may also be produced (Bernet-Camard *et al.*, 1997; Lievin *et al.*, 2000). Production of bacteriocins is thought to contribute to antimicrobial activity of some probiotics (Zamfir *et al.*, 1999).

Immune enhancement.

Probiotics can have stimulatory effects on both antigen specific and non-specific immune functions (Isolauri *et al.*, 2001; Lopez-Varela *et al.*, 2002). Experiments in mice and humans indicate an increase in macrophage numbers and phagocytic activity with probiotic feeding. Probiotic effects on production of cytokines, such as interferon-gamma and tumor necrosis factor, which are involved in modulation of both specific and non-specific immune responses, have also been described. There is evidence for probiotic-enhanced specific local and systemic IgA responses. This may occur through increased number and activity of antigen presenting cells such as macrophages, altered cytokine expression or direct processing of antigens by the probiotic organisms. Interestingly, both strain and dose of *Lactobacilli* influenced the type and extent of dendritic cell activation in mice (Christensen *et al.*, 2002). Similarly, probiotic *Lactobacilli* can shift the helper T cell response to a more aggressive antibacterial Th1-type response (Cross, 2002). Evidence for specific attribution of immune effects to probiotic activity against pathogens is somewhat sparse, though at least one study correlated probiotic-induced increased levels of specific IgA with resistance to *Salmonella typhimurium* in mice (Perdigon *et al.*, 1991).

Modulation of epithelial cell gene expression and function.

As alluded to above, probiotics can affect host cell function, either through direct actions on the host cells themselves or indirectly through moderation of the effects of pathogens, toxins or other stimuli (Hooper & Gordon, 2001; Schiffrin & Blum, 2002). For example, a study of *Lactobacillus rhamnosus* found a protective effect against epithelial cell apoptosis induced by inflammatory cytokines mediated by induced expression of the *Akt* gene. This effect should increase epithelial cell survival in an inflamed intestinal wall, thus maintaining the epithelial barrier and reducing disease and secondary infections (Yan & Polk, 2002). Other recent studies indicate that constituents of *Lactobacillus* cell wall modulate the response of intestinal epithelial cells to lipopolysaccharide from either *E. coli* or *Salmonella*, moderating potentially injurious inflammation (Vidal *et al.*, 2002). Further examples of host cell modulation by probiotics exist and, as additional molecular studies are performed, a more comprehensive understanding of the complex cross talk between probiotics, gut microflora and host cells will emerge.

Effectiveness of probiotics for pathogen control

The question that often gets asked about probiotics is do they work? When food animal producers ask this question they generally mean would this treatment be ***effective*** on my animals to prevent infection and/or disease? Effectiveness is different from ***efficacy***, roughly defined as a statistically significant effect in an experimental or controlled setting. Effectiveness implies that one can define adequately an effective outcome. Is a 10% reduction in prevalence of *Salmonella* adequate, is it cost-effective or will it demonstrably improve human or animal health? These complex questions affect all interventions in preharvest food safety. A framework for evaluating the effectiveness of probiotic interventions is provided though evidence based medicine (EBM), an approach that relies on evaluating the available data to make decisions about treatment (McQueen, 2001). However, not all data is created equal, thus various criteria for the strength of evidence have been proposed (Table 3). Study design is a key criterion for evaluating strength of evidence (Agency for Healthcare Research and Quality, 2002). Study populations, sample size (power), appropriate control groups, assigning treatment groups, blinding of observers, and appropriate statistical evaluation are design

criteria that affect how an intervention is evaluated. Appropriate diagnostic criteria and methodology are also important. Publication of studies in high quality, peer-reviewed journals is essential, both for the rigor provoked by the review process and to ensure availability so that researchers and potential users of the probiotic can critically review these results.

Table 3. Type and strength of evidence for effectiveness of interventions.

Level	Definition
I	Strong evidence from at least one published systematic review of multiple well-designed randomized controlled trials
II	Strong evidence from at least one published properly designed randomized controlled trial of appropriate size and in appropriate clinical setting
III	Evidence from published well designed trials without randomization, single group pre-post, cohort, time series or matched case-control studies
IV	Evidence from well-designed non-experimental studies from more than one center or research group
V	Opinions of respected authorities based on clinical or experimental evidence, descriptive studies or reports of expert consensus committees.

I indicates strongest evidence, V indicates weakest.
Modified from: Bandolier volume 6, July 1994,
http://www.jr2.ox.ac.uk/band6/b6.html

A simple search of the literature demonstrates that most probiotic studies in food animals are relatively small scale experimental challenge designs. These types of experiments are informative and, in fact, are essential to screen probiotics for any effect on pathogens. However, these challenge studies often do not account for factors likely to be important in a production setting. For example, pathogens of a given type may vary widely genetically and phenotypically in the field, whereas usually only one strain of pathogen is used in an experimental challenge study. Other factors include feed differences, presence of unrelated pathogens or microflora, stress, genetic differences between host populations and prevalence of pathogen in the population or environment. Fully randomized controlled trials (RCT) cut across populations to account for these factors but are generally large, expensive undertakings. For these reasons, RCT are uncommon for probiotics, indeed for most interventions applied to food animals. Still, study designs approaching RCT are required to evaluate the effect of probiotics in non-selected animal populations, under typical production conditions and with exposure to the pathogen of interest at varying doses and times.

It is beyond the scope of this discussion to review all the probiotic and CE literature for food animals. To illustrate some of the issues involved in evaluating probiotic interventions, two important food safety-related pathogen problems were selected as examples, *Salmonella enterica* serotype Enteritidis (SE) in chickens and *E. coli* O157:H7 (O157) in cattle. For details on these food-borne pathogens in food animals please refer to recent reviews (Guard-Petter, 2001; Renter & Sargeant, 2002). Studies published in the last five years that evaluated prevalence of O157 or SE in feces or gut contents of cattle or chickens, respectively, in response to probiotic administration alone, were included in the analysis. The maximum effect (% reduction in prevalence) was calculated and the statistical significance of the difference between probiotic and control groups evaluated by Chi-square test. The number of individuals in the probiotic group (n) and the number of individuals that represent the difference between control and probiotic group (n x effect) are also presented.

Summaries of O157 studies are presented in Table 4 (Brashears *et al.*, 2003; Elam *et al.*, 2003; Tkalcic *et al.*, 2003; Zhao *et al.*, 2003). It is clear that even for closely related probiotics in solidly designed and executed studies, such as those of Elam (2003) and Brashears (2003), there can be considerable variability in probiotic effectiveness. This is also true for experimental challenge models of Tkalcic (2003) and Zhao (2003), though the differences here can likely be attributed to differing age of cattle (neonatal versus weaned) and small sample sizes. There is a general trend towards effectiveness for these probiotics against O157 regardless of statistical significance but much larger studies are required before such probiotics could be recommended for general use.

Table 4. Effect of probiotics on fecal prevalence of *E. coli* O157:H7 in cattle.

		Prevalence (%)						
Study type	Probiotic genera	Control	Probiotic	Max. effect	p-value	n	n x effect	Reference
III	*Lactobacillus, Proprionibacterium*	28	14	50.9	>0.07*	60	8	Elam, 2003
III	*Lactobacillus*	65	45	30.8	<0.006	60	12	Brashears, 2003
V	*Escherichia*	67	0	100	<0.05	6	6	Tkalcic, 2003
V	*Escherichia*	57	29	49.1	>0.05*	7	2	Zhao, 2003

Table 5. Effect of probiotics on SE prevalence in chickens.

		Prevalence (%)						
Study type	Probiotic genera	Control	Probiotic	Max. effect (%)	p-value	n	n x effect	Reference
III	*Avigard*	87	0	100	<0.05	*	*	Davies, 2003
V	*Lactobacillus*	100	100	0	>0.05	5	0	LaRagione, 2004
V	*Undefined CE*	100	0	100	<0.05	50	50	Andreatti-Filho, 2003
V	*Avigard*	75	10	87	<0.05	10	7	Seo, 2000
V	*Broilact*	40	10	75	>0.05	10	3	Fukata, 1999

* not calculable from the data available

Recent studies of probiotic effects on SE in poultry are summarized in Table 5 (Andreatti Filho *et al.*, 2003; Davies & Breslin, 2003; Fukata *et al.*, 1999; La Ragione *et al.*, 2004; Seo *et al.*, 2000). A similar trend towards effectiveness is seen for the mixed CE probiotics, where the Lactobacillus strain had no effect on SE. It is worth noting that this Lactobacillus strain did show efficacy against Clostridium perfringens in this study. The magnitude of the CE effect against SE suggests that these products may be effective but again the small sample sizes and restricted nature of the study groups limit the conclusions that can be drawn from this data.

These summaries are not intended to represent a systematic review of the literature on either the probiotics tested or the effect of probiotics on either SE or O157. A number of potentially relevant studies were excluded due to incompatible outcome definitions, significant treatment differences or other considerations. It is also possible that more extensive studies have been performed but have not been published due to intellectual property or other concerns. However, the included studies are representative of the types of studies used to support use of probiotics for pathogen control present in the peer-reviewed literature and serve to illustrate the complexity of answering the question, do probiotics work for pathogen reduction?

Conclusions

The concept of probiotics for pathogen reduction in food animals is well established. Probiotics offer an attractive alternative to antibiotics and vaccines for decreasing the prevalence of food-borne pathogens in livestock and poultry. However, the available literature on probiotics for pathogen control is inadequate to critically evaluate their effectiveness. For probiotic candidates that demonstrate a high degree of efficacy in experimental models, randomized controlled trials should be performed to provide clear guidance for producers as to the effectiveness of these products. The feasibility of this degree of rigor in clinical evaluation is indicated by at least one FDA-approved RCT that was performed for a probiotic directed against *Salmonella typhimurium* in chicks (Corrier *et al.*, 1995). The need for alternatives to current pathogen control methods in food animals requires investment in adequate evaluation of promising probiotic technologies.

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