

# Mechanisms of action of nutritional tools to control intestinal zoonotic pathogens

F. VAN IMMERSEEL, V. EECKHAUT, E. TEIRLYNCK, F. PASMANS, F. HAESEBROUCK and R. DUCATELLE\*.

Faculty of Veterinary Medicine, Research Group on Veterinary Public Health and Zoonoses, Department of Pathology, Bacteriology and Avian Diseases, Gent University, Salisburylaan 133, B-9820 Merelbeke, Belgium.

\*Corresponding author: [Richard.ducatelle@UGent.be](mailto:Richard.ducatelle@UGent.be)

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Poultry can carry different zoonotic pathogens in their intestinal tract, including *Salmonella* and *Campylobacter*. Recent E.U. regulations constitute an incentive for the poultry producers to take measures aiming at reducing the degree of contamination in the live birds. Moreover, for *Salmonella* national control plans are implemented. Many different tools are available that can help reducing the level of contamination. Among these tools, numerous feed additives are on the market for which a claim is made with respect to reduction of carriage of *Salmonella* and other zoonotic agents. Most of these additives can be classified as prebiotics, probiotics, synbiotics, short chain fatty acids or mixtures of natural plant extracts. Most claims are based on trial and error experiments. In recent years however, both in vivo and in vitro experiments have provided a better understanding of the pathogenesis of the carrier state in chickens infected with different zoonotic agents. Simultaneously, insight has grown in specific and non-specific protection mechanisms in the chicken intestinal tract. By combining new information on the intestinal mucosal barrier with new data on pathogenesis of the infections, a rational explanation can be provided for the activity of certain feed additives. Some of the newly discovered mechanisms appear to be generic. Therefore in the near future it may be possible to exploit and to steer these intestinal mucosal protection mechanisms.

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**Keywords:** *Salmonella*; *Campylobacter*; prebiotics; probiotics; butyrate

## Introduction

Poultry meat is an important source of human pathogens entering the food chain. Most of these pathogens are bacteria, contaminating the chicken carcasses in the slaughterhouse (Corry and Hinton, 1997). Notorious amongst these zoonotic pathogens found on chicken carcasses are *Salmonella* and *Campylobacter*. The contamination rates of the fresh carcasses with some of these pathogens, including *Campylobacter*, but also *Listeria* spp., is high (Waldroup, 1996). In recent years *Helicobacter pullorum* is identified as yet another potential new zoonotic pathogen in raw poultry meat (Corry and Attabay, 2001). Finally also *Arcobacter butzleri* and related species can be isolated frequently from broiler carcasses (Houf et al., 2002).

Among the different pathogenic bacteria mentioned above, those belonging to the family Campylobacteraceae are the most important causes of human enteritis in Belgium in 2006, followed by *Salmonella enterica* (data from the Belgian Scientific Institute for Public Health in 2006). Two species, *Campylobacter jejuni* and *Campylobacter coli*, account for 89% of human campylobacteriosis (Vandenberg et al., 2004). Epidemiological studies suggest that poultry meat may be one of the most common sources of *Campylobacter* associated with sporadic gastroenteritis in humans (Lee and Newell, 2006). It is well documented that poultry meat also is a common source of *Salmonella*, causing gastroenteritis in humans. In contrast, the transmission of *Helicobacter pullorum* from poultry meat to humans still is controversial. Moreover, even if *Helicobacter pullorum* has been isolated as the

sole pathogen from a number of human patients with gastroenteritis, studies in our lab revealed no significant difference in the prevalence of *Helicobacter pullorum* among patients with gastrointestinal disease and clinically healthy persons (Ceelen et al., 2005). *Arcobacter* infections in humans, including *Arcobacter butzleri*, *Arcobacter cryaerophilus* and *Arcobacter skirrowii*, are mainly associated with enteritis and have been described in patients of different ages (Vandenberg et al., 2004). Although the source of infection is only occasionally identified, human infections with *Arcobacter* seem to occur through the consumption of contaminated water or food of animal origin (Lehner et al., 2005), with poultry meat being but one of many possible sources of infection.

Most of the above mentioned zoonotic or potentially zoonotic bacteria are known to colonize the chicken intestinal tract. Remarkable exceptions however are the *Arcobacter* spp., which commonly contaminate broiler carcasses, but for hitherto unknown reasons cannot be traced back in the live chickens (Van Driessche and Houf, 2007).

Several recently issued E.U. regulations, following on the original E.U. zoonoses directive 92/117, constitute a strong incentive for the poultry industry to take measures aiming at the control of zoonotic pathogens (reviewed by Ducatelle et al., 2006). For the time being the focus of the E.U. is on *Salmonella*. For the layers targets have been set for the step-wise reduction of the number of *Salmonella* Enteritidis and *Salmonella* Typhimurium positive epidemiological units. For the broilers, fresh meat that is contaminated with one of the 5 *Salmonella* serotypes considered of public health significance (Typhimurium, Enteritidis, Hadar, Infantis and Virchow), will no longer be allowed on the market from December 2010 onwards. On top of that, several E.U. regulations at this moment prohibit antimicrobial treatments of the end product. As a consequence, the poultry industry is urged to use control measures during the live phase of poultry production. Several tools are available which can help to reach the Community targets. In the layers, we showed that vaccination can drastically reduce the shedding of *Salmonella* Enteritidis in the eggs (Gantois et al., 2006b). In the broilers however, vaccination is not available yet, and may be very difficult to achieve (Bohez et al., in press; Adriaensen et al., in press). Taking into account that most of the important zoonotic pathogens colonize the intestinal tract in poultry, it seems logical that many efforts, to control zoonotic agents in live poultry, focus on nutritional strategies. These strategies not only include the use of feed additives, but also the selection of raw materials (feed composition) and even the feeding regime. There is a myriad of strategies available, some with well documented effects, some less well documented. Most have been selected by trial and error, and the observed effects are on an empirical basis. All this makes the choice of strategy extremely difficult. Recently for some of these control strategies the mechanism of action has been explained. The purpose of the present review is not to give an exhaustive list of available feed additives, but rather to give a brief overview of the known mechanisms of action. It is hoped that this will help the poultry producer to make a choice and combine different tools that have synergistic effects and avoid combining tools that may act antagonistically.

## **Feed additives:**

A wide range of feed additives, for which an effect is claimed on intestinal zoonotic agents in general and on *Salmonella* carriage in particular, is available on the market. They belong to different groups: antibiotics, prebiotics, probiotics and symbiotics.

### **Antibiotics:**

The prophylactic or curative use of antibiotics for the control *Salmonella* infections in poultry is prohibited under the E.U. regulation N° 2160/2003, amended by E.U. regulation N° 1091/2005.

Antibiotics also have been used in poultry feed since many years for their growth promoting effects. Antibiotic growth promoters mostly have their effect by modifying the intestinal microbiota, targeting mainly gram-positive bacteria which are associated with poor health and performance of the animals (Bedford, 2000). Most of the zoonotic agents found in the chicken gastro-intestinal tract however are gram-negative. Taking into account this typical spectrum of activity of the antibiotics used as growth promoters, and also taking into account that the concentrations used in feed are below the minimum inhibitory concentrations as tested in vitro, one may not expect any beneficial effect of

growth promoters against intestinal carriage of zoonotic agents in poultry. Nevertheless for some growth promoting antibiotics in some studies a reduction of *Salmonella* shedding has been reported (reviewed by Van Immerseel et al., 2002). In contrast some other growth promoting antibiotics seem to induce increased shedding (Teirlynck et al., unpublished data). This discussion has lost its meaning however, since growth promoting antibiotics have been banned from poultry feed in the E.U. since 1 januari 2006.

The ionophore class of anticoccidial drugs has antibiotic activity as a side effect. Salinomycin, monensin and narasin are ionophore antibiotics (polyether antibiotics) with documented activity not only against protozoa (Gumila et al., 1997) but also against gram-positive bacteria (Dutta and Devriese, 1984). Although there is some controversy in the literature concerning the effect of salinomycin on *Salmonella* shedding, in most cases no effect of ionophores on intestinal carriage of zoonotic agents has been reported (reviewed by Van Immerseel et al., 2002).

### **Prebiotics:**

Prebiotics are non-digestible feed ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health (Gibson and Roberfroid, 1995). In this definition it is understood that certain bacterial species multiplying in the colon can have a beneficial effect on host health. These bacterial species can also be administered through the feed. These are then called probiotics (see below).

Most prebiotics are carbohydrates. They can be mono-, di-, oligo- or polysaccharides. Moreover they can be natural or synthetic (Iji and Tivey, 1998). A number of monosaccharides including glucose and fructose are digestible and thus not considered prebiotics according to the standard definition. Mannose is by far the most commonly used monosaccharide feed additive (Allen et al., 1997). Mannose is the saccharide ligand for bacterial type 1 (sef17) fimbriae. These type 1 fimbriae are common surface projections of *Salmonella*, which the bacteria use to attach to the intestinal mucosa. Supplementing 1 or 2% mannose in the feed has been shown to reduce *Salmonella* colonization. Mannose binds type 1 fimbriae and thereby blocks adhesion of type 1 fimbriae bearing bacteria to epithelial cells (Finucane et al., 1999). *Salmonella* bacteria however have several alternative adhesion mechanisms available. Moreover supplementing such high amounts of mannose (or synthetic oligosaccharides containing mannose) may have certain disadvantages both technical and economical.

Amongst many available disaccharides, lactose, lactulose and lactosucrose have documented prebiotic effects, conferring protection against *Salmonella* organ invasion (Corrier et al., 1993; Iji and Tivey, 1998; Terada et al., 1994). Lactose is not absorbed or digested, passing down the intestinal tract, where it is used by the microbiota (Tellez et al., 1993). This however may lead to mild scouring (Corrier et al., 1993).

Oligosaccharides usually are obtained through enzymatic synthesis or through hydrolysis of polysaccharides. Fructo-oligosaccharides (FOS) are short-chain polymers of beta 1-2 linked fructose units, produced mostly by hydrolysis of inulin (usually from chicory). Their use in broiler feed leads to a reduction of colonization of the intestine by *Salmonella* (Bailey et al., 1991). FOS are able to selectively stimulate *Bifidobacterium* spp. within the gut microbiota (Roberfroid et al., 1998). When FOS were fed (15g/day, 15 days) to healthy adult human volunteers, numbers of *Clostridium* spp. in stool samples decreased (Gibson et al., 1995). Galacto-oligosaccharides (GOS) are  $\text{Glu} \square 1-4 [\square \text{Gal} 1-6]_n$  are usually produced synthetically from lactose syrup. They are not broken down in the stomach or small intestine. GOS also have been shown to selectively stimulate bifidobacteria (Holma et al., 2002). Mannan-oligosaccharides (MOS) derived from the yeast cell wall fragments of *Saccharomyces cerevisiae* are commercially available for use in poultry feed. Mixing 4000 ppm in the feed reduced caecal *Salmonella* Typhimurium concentrations after experimental infection in broiler chicks (Spring et al., 2000). Caecal contents from hens fed MOS protected chicks from colonization with *Salmonella* Enteritidis (Fernandez et al., 2000). The mechanism of action may be through blockage of type 1 fimbriae mediated *Salmonella* adhesion to the mucosa. MOS supplementation induced alterations in numbers of lactobacilli, enterococci or anaerobes in the caeca (Spring et al., 2000). More recently a wide range of other oligosaccharides, including isomalto-oligosaccharides, soy-oligosaccharides and xylo-oligosaccharides currently are being investigated for their prebiotic and health protecting effects

in laboratory animal models and human volunteers (reviewed by Tuohy et al., 2005). Little is known however regarding their potential for protection against zoonotic agents in poultry.

The most common polysaccharide prebiotics in chicken feed probably are guar gum (from the seeds of *Cyamopsis tetragonolobus*) and partially hydrolyzed guar gum (PHGG). Feed containing 250 ppm PHGG protected the chicks against *Salmonella* Enteritidis from 14d onwards. This is thought to be due to improvements in the balance of the intestinal microbiota (Ishihara et al., 2000).

Taken together, most prebiotics (apart from MOS) with a proven protective activity against zoonotic pathogen carriage, act through a shift in the composition of the intestinal microbiota, or else through a modification of the metabolic activity of some indigenous intestinal microorganisms. An alternative approach thus can be to directly administer these so-called beneficial microorganisms. These feed additives containing the microorganisms are called probiotics.

### **Probiotics:**

Probiotics by definition are live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance (Fuller, 1989). The original idea was coined over 30 years ago, when Nurmi and Rantala (1973) administered a suspension of gut contents derived from healthy adult chickens to newly hatched chicks, thereby protecting the chicks against *Salmonella* Enteritidis gut colonization. This concept is called competitive exclusion. Commercial products based on this concept have been on the market for a number of years. One major handicap of this concept however is that the microbiota, which are included in these products, are not identified (undefined). Moreover these competitive exclusion products hitherto are not commonly included in feed. Therefore over the years considerable efforts have been made to identify specific microorganisms that confer a similar protection.

Already at the beginning of the previous century it was found that drinking milk improved health and longevity of men, with lactobacilli playing an important role in this phenomenon (Metchnikoff, 1907). More recently lactobacilli have been isolated from chicken intestines and tested for their probiotic activity (Gusils et al., 1999). When a *Lactobacillus salivarius* strain was inoculated directly into the proventriculus of day-old chicks at the same time as a *Salmonella* enteritidis strain, the *Salmonella* bacteria had completely been cleared by day 21. Moreover it was shown that this *Lactobacillus salivarius* strain could be included in a commercial feed mixture (Pascual et al., 1999). Certain lactobacilli isolated from the chicken gastro-intestinal tract can adhere to chicken intestinal mucins and may produce bacteriocins (Kizerwetter-Swida and Binek, 2006). This probably explains the reported (Jin et al., 1996). growth inhibiting effects of Lactobacilli against *Salmonella* Enteritidis in vitro. In *Lactobacillus reuteri* the antimicrobial metabolite that inhibits growth of *Salmonella* and *Campylobacter* has been identified and called reuterin (Mulder et al., 1997). It is unclear however to what extent the antibacterial metabolites also play a significant role in vivo. Other mechanisms also are involved: *Lactobacillus plantarum* has mannose-sensitive receptors, which may allow this strain to compete for the same adhesions sites in the intestine as *Salmonella* (Bengmark, 1998). *Lactobacillus lactis lactis* and *Lactobacillus acidophilus* isolates upregulate oxidative burst and degranulation in chicken heterophilic granulocytes both in vitro and in vivo (Farnell et al., 2006). We previously showed that massive influx of heterophilic granulocytes in the propria mucosae of the caeca plays a role in early protection against *Salmonella* colonization (Van Immerseel et al., 2003a), which underlines the possible significance of this mechanism in vivo.

The many reports on the successful application of lactobacilli as probiotics, beneficial not only to the growth and performance, but also to the resistance of broilers against *Salmonella* infections, suggest a profound effect of these microorganisms on the intestinal ecosystem. In humans, favourable effects on gut health have been reported not only with Lactobacilli, but also with bifidobacteria, enterococci and other lactic acid bacteria. All these bacterial species are abundantly present in the gastrointestinal tract. Their major product in vitro is lactate (Macfarlane and Gibson, 1996). Despite this, lactate is seldom detected as a major fermentation product in gut contents. This is assumed to reflect lactate utilization by other bacterial species. In the bovine rumen and the pig gastrointestinal tract, it is known that *Veillonella* and a number of other species utilize lactate and convert it to acetate and propionate. Also lactate-utilizing bacteria, producing butyrate as a major fermentation product, have recently been isolated from human faeces (Duncan et al., 2004). These butyrate producing

bacteria are strict anaerobes which are difficult to grow in vitro and therefore cannot be used as feed additives at present. Nevertheless, short chain fatty acids seem to play a crucial role in gut health.

### **Short chain fatty acids**

Short chain fatty acids are the major bacterial fermentation products in the large intestine (Tuohy et al., 2005). Short chain fatty acids are also commonly added to feed and drinking water. The short chain fatty acids however cannot be classified as prebiotics *sensu stricto*, since they are rapidly absorbed and utilized by the host tissues. At high concentrations (1%) these products have an antimicrobial effect in moist environment. This microbial growth inhibition is traditionally explained by the ability of these acids to pass across the bacterial cell membrane in undissociated form, dissociate in the more alkaline bacterial cell interior and thereby acidify the bacterial cell cytoplasm (Kashket, 1987). When the acid-treated feed is eaten by the chickens, it is both warmed and moistened, and thus the activity of the short chain fatty acids should increase. It has long been known however that up to 95% of the short chain fatty acids produced during carbohydrate fermentation may be taken up and utilized by the host (Cummings et al., 1987). Thus antimicrobial activity in the lumen of the gastrointestinal tract is largely limited to the crop and gizzard (Thompson and Hinton, 1997).

Among the different short chain fatty acids, butyrate is of particular importance, as it provides 50% of the daily energy requirements of the gastrointestinal mucosa, playing an important role in proliferation and differentiation of the epithelial cells (Macfarlane et al., 1997). Butyrate upregulates the expression of tight junction proteins, thereby enhancing the barrier function of the intestinal epithelium (Bordin et al., 2004). Sodium butyrate administered in feed in concentrations up to 0.2% increases feed conversion ratio, daily weight gain and intestinal villus length in broilers (Hu and Guo, 2007). We showed that butyrate and propionate (as opposed to acetate and formiate), at concentrations similar to those naturally produced in the lower gastrointestinal tract of the chicken, reduce invasiveness of *Salmonella* in intestinal epithelial cells in vitro (Van Immerseel et al., 2003b). This phenomenon of reduced invasion by butyrate is mediated by a specific down-regulation of the genes encoded on the *Salmonella* pathogenicity island 1 (Gantois et al., 2006a). Coating butyric acid on a carrier protects the acid from absorption in the upper digestive tract, transporting the acid down to the caeca, where *Salmonella* bacteria are known to colonize and invade the mucosa (Desmidt et al., 1997). As expected, coated butyric acid in feed protects chickens from caecal colonization of *Salmonella* (Van Immerseel et al., 2005).

### **Dietary formulations that influence intestinal carriage of zoonotic agents**

Industrial poultry feed commonly is formulated combining a number of raw materials and additives using computerized linear programming. The criteria set in the program are essentially the nutrient requirements of the animals for maintenance and growth, the price of the raw materials, and a maximum level for certain components that may have negative effects on the animals or on the environment when incorporated in the feed at too high concentration. Although certain raw materials are known to constitute a risk for the introduction of zoonotic agents, this is not always taken into account during formulation. Nevertheless recent experimental studies have generated indications that diet formulations excluding animal proteins and fat may help to reduce colonization of *Campylobacter jejuni* in the ceca (reviewed by Hariharan et al., 2004). Ongoing studies in our lab also suggest that different formulations of feeds may render broiler chicks more or less susceptible to infection with *Salmonella* Enteritidis. There are indications that certain feed ingredients might induce a shift in the intestinal microbiota (Apajalahti et al., 2001; Engberg et al., 2004).

### **Conclusion**

A wide range of feed additives are presently available on the market for which a protective effect against intestinal carriage of zoonotic pathogens is claimed. For most of these the claims are merely

derived from empirical trials. Insight in the intestinal ecosystem and the mechanisms rendering the birds more or less susceptible to the zoonotic agents however is growing. It is clear that short chain fatty acids may influence gut health and carriage of zoonotic pathogens in the gut through specific mechanisms. Understanding the phenomenon of cross-feeding between bacterial species in the intestine probably is the way to move forward in improving gut health and resistance against zoonotic pathogens. Although little is known so far on the influence of the different feed ingredients, the long term perspective probably is that feed ingredients will be formulated to steer the balance and the cross-feeding between the bacterial species to the benefit of the host.

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