

Dietary Regulations of the Intestinal Barrier Function at Weaning**

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ABSTRACT : Weaning is a complex phase when the mammal suffers the action of different stressors that contribute to negatively affect the efficiency of the intestinal mucosa and of the whole local integrated system, that acts as barrier against any nocuous agent. The components of this barrier are mechanical, chemical, and bacteriological; immunological and not. The development of contact with a saprophyte microflora and the maintenance of feed intake after the interruption of motherly nutrition are essential for the maturation of an equilibrated local immune function and for a functional integrity of villi. Opportunities and limits of some dietary strategies that can contribute to reduce negative effects of weaning on health and performance are discussed. Knowledge on the possible mechanism of action of probiotics are upgraded, particularly for their supposed role in the balance between different immune functions (effector/regulatory). Some tools to control pathogen microflora are reviewed (acids, herbs, immunoglobulin sources) and practical feeding systems are proposed. (*Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 4 : 596-608*)

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INTRODUCTION

Of the animal body, the mucosa of the gastrointestinal tract is the largest surface of contact with harmful pathogens and antigenic molecules. For this reason a proper barrier action from this tissue is required to counteract these agents. This function is particularly important when the animals experience sudden changes of diet and/or environment. This is the case of the weaning. In this phase the young mammals are quite immature, the capacity to digest solid diets is reduced and there is a lag time before feed intake recovers to the one before weaning. This affects the trophism of the intestinal mucosa and its morphology.

The components of the gut mucosal barrier in mammals are summarized in table 1 and are more systematically described in the review of Bosi (2000). Here some more relevant and emerging topics will be discussed.

IMPORTANCE OF GUT COMMENSAL MICROFLORA FOR THE DEVELOPMENT OF LOCAL IMMUNE SYSTEM

In the newborn, the gastrointestinal tract need to develop the complex ecosystem observed in the mature animal, that hosts a diverse and highly evolved microbial community composed of hundreds of different microbial species. In the normal "not aseptic" environment the colonization takes part rapidly.

When the environment is very clean the settlement is delayed. Observations on germ free and SPF piglets show that for a maturation of the local immune system a contact with microbial antigens is required.

In pig the local immune system is based on: a) a diffuse system, intraepithelial (IEL) and in the lamina propria, and b) organized compartments: Peyer's patches that are isolated, discrete in jejunum and upper ileum and continuous single in terminal ileum, and mesenteric lymph nodes. The major site of initiation of the local immune response in the gut is in the Peyer's patches, where specialized membranous cells (M cells) localized in the epithelium associated with the follicle continuously transport particles from the lumen to the underlying tissue. Here the antigens are then taken up by macrophages and dendritic cells (low-density cells smaller than macrophages and showing evidence of cytoplasmic protuberances or dendrites), processed and presented to local CD4+ T cells, that provide help to the stimulation of B cells. The B cells can migrate into the lymph cycle. After recirculating, a part of these Ig plasma cell precursors enter the intestinal lamina propria (Rothkötter et al., 1999). In the lamina propria, B cells/plasma cells are preferentially around the crypt and T cells the villi. Here suppressor/cytotoxic CD8+ cells are in and immediately underlying the epithelium, while helper/inducer CD4+ cells lie deeper in the lamina propria. The reasons of this organization are only partially explained, however we know that the complete picture in normally reared pigs is seen after 4 weeks of age. At 1-2 months germ free pigs exposed to normal dietary antigens have a number of intraepithelial lymphocytes similar to newborn pigs and in specific pathogen free the number is intermediate between conventional and germ free pigs (Rothkötter et al., 1999). A lack of increase of CD4+ and CD8+ cells in the lamina propria of 49-day old germ free

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Table 1. Components of the gut mucosal barrier in mammals (Adapted from Bosi, 2000)

Non-immunological	Immunological
Mechanical	Local
Healthy enterocyte	Gut-associated lymphoid tissue
Tight junction	Intra-epithelial lymphocytes
Cell turnover	Aggregates in the <i>lamina propria</i>
Normal motility	Peyer's patches
Chemical	Mesenteric lymph nodes
Antioxidants (urate in saliva)	Secretory IgA: endogenous
Gastric acidity	maternal
Salivary lysozyme	
Other antimicrobial peptides:	
- histatins (salivary glands)	
- defensins (small intestine Paneth cells)	Systemic
- calprotectin (mouth, esophagus)	
Lactoferrin	Circulatory lymphocytes
Mucous secretion	Hepatic Kupffer cells
Bile salts	Bacteriological
Bacteriological	Aerobic and anaerobic micro-organisms
Aerobic and anaerobic micro-organisms	

pigs was observed (Pabst and Rothkötter, 1999). In conventional pigs the majority of lymphocytes present in ileum Peyer's patches at 1.5 months of age are B cells while in germ free many more T cells are found (Pabst and Rothkötter, 1999). These data show that microbial antigen is required to develop active follicles, IEL's, and lymphocytes in the lamina propria.

Invasive bacteria can enter epithelial cells directly or utilize M cells, located over Payer's patches, but a third route has been recently discovered. Dendritic cells extend across the epithelium and uptake by their projections into the intestinal lumen (Rescigno et al., 2001). This pathway can be used by noninvasive bacteria and may allow uptake of soluble antigens and presentation of microbes to intraepithelial lymphocytes or those in aggregates. This action depends on its process into proteolytic peptides, and load onto major histocompatibility complex (MHC) class I and II molecules. However the initiation of an inflammatory response (Th1 type), of an antigen-specific immune responses (Th2 type), or immunological tolerance (Th3 type) depends on the production and balance of different cytokines. Bacteria can also activate intestinal epithelial cells to secrete a variety of cytokines that organize innate immune responses (McCormick et al., 1993). At the contrary, some nonpathogens (nonvirulent *Salmonella* strains) may attenuate synthesis of inflammatory effector molecules elicited by diverse proinflammatory stimuli (Neish et al., 2000).

Cukrowska et al. (2001) associated 8-day old colostrum deprived germ free piglets with non pathogenic *E. coli* O86 *per os* and observed that the subjects after only four days showed high specific IgA titers against the commensal.

However, this local response disappeared rapidly. At the contrary, a presence of specific IgA-secreting cells was observed later in plasma and this was accompanied with a profound polyclonal stimulation of the immune system, resulting in increased levels of so called natural antibodies. The immune system – specifically B cells – must be able to generate enough different antibodies to recognize every possible antigens. After an exposure to an antigen, the variable regions (V) of the antibody genes acquire many changes. So an increase of polyclonal natural antibodies can be favorable as a wide spectrum of specific antibodies are mobilized and the basis for further specific reaction against pathogens is created.

Also in rabbit, diversification of the primary antibody repertoires occurs after birth (1st-2nd month), when the lymphoid tissue is associated with a colonized gut. In fact, by 12 weeks of age, nearly 90 % of the immunoglobulin VDJ genes in peripheral blood lymphocytes were un-diversified when the appendix was legated shortly after birth to prevent microbial colonization and all other gut-associated where surgically removed (Lanning et al., 2000).

By contrast species such as chicken, sheep, and cattle are known to develop primary Ab repertoires through somatic diversification with little contribution from combinatorial rearrangement, and diversification appears to occur independent of exogenous Ag (Lanning et al., 2000).

However a question rises: after the first phases of induction of the local immune system, is there an adaptive stage?

Research mainly on mice demonstrates that immune system is able to down-regulate, i.e. to develop tolerance. This can be maintained: a) by cell apoptosis (deletion of

antigen-specific T cells); b) by anergy (stimulated T cells are refractory in the absence of co-stimulatory signals); by development of regulatory T cells (suppress of antigen-specific responses following antigen rechallenge).

In pigs the researches of the group of the University of Bristol and others strongly support that the immune development in the intestinal mucosa occurs as a balance between the response against antigens (effector function) and regulatory functions (Bailey et al., 2001). A balance between these two functions is important to maintain the intestinal integrity. Furthermore there is some evidence from the observations on the phenotype of mucosal T-cells that in pig this balance is normally set towards regulatory function (Bailey et al., 2001). The weaning can be considered a disturbing factor of this balance.

At this point it can raise a second question: can probiotic bacteria have a role in the balance of the different functions and do the organisms tolerate them? From the survey of literature we find that observations about the effect of probiotics on the specific immunity against themselves are scarce. In human Faure et al. (2001) observed only transient variation in lactobacilli specific IgA in feces of children fed milk fermented by yogurt symbiosis and *Lactobacillus casei* and no variation for the diet only with yogurt.

In any case, the relevance of gut microflora for the development and maintenance of intestinal mucosal barrier is one of the fundamentals of the adoption of probiotics in the animal feeding. On the base of the presented observations we can explain why some probiotic can improve some immune parameters and at the same time maintain favorable growth performances.

For probiotic dietary use, many immune effects are claimed. Some times these effects are conflicting and can depend on different underlying mechanism of actions.

Adjuvanticity

Some non-pathogenic bacteria promote health by inducing a non-specific immune response and enhancing a systemic antibody response against an administered antigen. These effects can be applied for example for the use of vaccines. Maassen et al. (2000) orally dosed mice with eight different *Lactobacillus* species. Of these, strains of *L. reuteri* and *L. brevis* stimulated more cells in gut villi to produce the proinflammatory and/or Th1 cytokines IL-1 β , IL-2 and TNF- α and enhanced the IgG response against parenterally administered haptenated chicken gamma globulin. It is possible that the immune response evoked by the two strains was against themselves. The other species were not immunogenic. Many other examples of an adjuvant effect can be found in the review of McCracken and Gaskins (1999).

Regulatory effects on enterocytes and/or dendritic cells

The adjuvant properties can be ascribed to some products of the bacterial cell wall. Germ line-encoded receptors have been identified for microbial products such as mannan, lipopeptide, peptidoglycan, lipoteichoic acid, lipopolysaccharide, and CpG-DNA. A family of receptors (Toll-like receptors) in mammals cells recognizes these microbial components. Between these cells there are the dendritic cells, that in the immature form are residing in non-lymphoid organs and are characterized by the capacity to uptake antigens and to phagocyte. The bacterial cell wall induce their maturation (Resigno et al., 2001) and initiate to evocate the innate immune responses, and, after this, the development of antigen-specific adaptive immunity. Some of these microbial molecules have been tested *per os* in producing animals, but up to now not enough practical consequences were driven (see Bosi, 2000). A synthetic molecule derived from the muramyl dipeptide, the smallest bioactive unit of bacterial peptidoglycan, has been recently proposed for human application as adjuvant (Vidal et al., 2001).

Different pathways after dendritic cells activation can lead to different immune compartments. This is linked to different cytokines secretions. Christensen et al. (2002) exposed bone marrow derived dendritic cells to different Lactobacilli and tested their capacity to induce cytokines production.

For pro-inflammatory IL-12 and TNF- α , the tested *lactobacilli* species ranked in the following way: *L. casei* >>*L. plantarum* Lb1>*L. fermentum* Lb20>*L. johnsonii* La1 > *L. plantarum* 299v>>*L. reuteri*. The first species induced a response that far exceeded the one by the lipopolysaccharide, used as a control, while the latter in practice did not show any response. Similar but less pronounced patterns were observed for IL-6 and IL-10. It is important to observe that *L. reuteri* markedly inhibited *L. casei*-induced IL-12, IL-6, and TNF- α production. This effect was mediated by an increased production of IL-10 that suppress IL-12 and favors a Th2 or Th3 response. So this species can act to inhibit the action of other Lactobacilli.

Also Von Der Weid et al. (2001) found a strain of *Lactobacillus paracasei* able to suppress the production of many cytokines, maintaining IL-10 production and increasing Transforming Growth Factor- β (TGF- β) by a population of CD4+T cells with low proliferative capacity. This was considered a mechanism implicated in oral tolerance.

Effects in challenged animal, after the local sensitisation with probiotics: enhancement of the innate immune system, particularly for increased macrophage phagocytosis; lymphocyte proliferation (B and T cells); humoral response.

In piglets infected by Rotavirus and *Escherichia coli*, the probiotic use of *Bifidobacterium lactis* HN019 increased blood leukocyte phagocytic response, T-lymphocyte proliferative response, pathogen-specific antibody titers from gastrointestinal tract (Shu et al., 2001). Consequently, the piglets showed reduced fecal shedding of the two pathogens and a better diarrhea score.

PRODUCTS AND AGENTS THAT CAN REDUCE THE NEED FOR ACTIVATION OF LOCAL IMMUNE SYSTEM

In some cases dietary solutions can interfere with settlement and development of pathogens. The dietary action can be directed against the microorganism or addressed to the mucosal receptors that are used by microorganism to adhere to the gut wall. In this case as a result of the contrast against pathogens we do not expect an increased immune response, but, at the contrary a reduced one. Furthermore, if the animal does not need to divert energy to immune activation and inflammation, we expect an increased growth.

Direct action against microorganisms

Many molecules with antimicrobial action are produced by the animals (see Table 1). However, knowledge to improve their action with the diet, is still poor. Furthermore, it is possible that some of these molecules are highly conserved between species and are innate defenses. For example, a porcine defensin, *pBD-1*, is constantly expressed in oral mucosa, but the observations on consensus binding sites for inflammatory molecules can state that it plays a surveillance role maintaining the steady state of microflora on mucosal surface (Zhang et al., 2000). It is possible to hypothesize the use of these molecules in the weaning diets, but due to the likelihood of the composition and action between species, the risk of favoring some bacteria resistance could limit their interest. Otherwise subjects over-expressing these molecules or transgenic bacteria for the genes responsible for the synthesis of these peptides could be produced.

Acidic secretion by the stomach is an important tool to control some gut infections. Zinc deficiency reduces the activity of the enzyme that controls chloric acid (carbonic anhydrase) (Huber and Gershoff, 1973). Diets with high buffering capacity bind free chloric acid and can reduce the chemical control in the stomach (Bolduan et al., 1988).

Organic acids are well known as preservatives. But their properties can be useful to control microflora in the gut when the acid secretion for the stomach is reduced, after the suppression of milk suckling. Their efficacy depends on their ability to diffuse through the membrane of bacteria when they are in undissociated form; then, inside the cell,

the acid dissociates and suppress cells enzymes and nutrient transport systems. Partanen and Mroz (1999), regressed growth performances of piglets from 35 experiments from the literature and observed that formic acid, formate salts, fumaric acid and citric acid improved growth and feed efficiency whatever was the molecule and the dose within the range 80-400 mEq/kg feed. Organic acids are often supplied as salts, because in this form they are odourless, easier to handle, less corrosive and more soluble.

When acidifiers are added to a diet with low acid binding capacity, the intense gastric acidification, can however reduce the secretion of hormone gastrin and, consequently, the hydrochloric acid secretion (Kemmer-Kroonsberg, 1993). Otherwise, highly buffered diets can increase the base excess in the blood, induce a overstimulated bicarbonate secretion from the pancreas (Kemmer-Kroonsberg, 1993) and, possibly, affect digestibility. In both situations, the control of overgrowth of *E. Coli* and other dangerous microbes can be unsuccessful. To overcome these problems it is possible to feed slow release acidifiers that do not affect secretions in stomach and duodenum. In weaning pigs, the addition of protected acids to high or low acid binding diets (with free fumaric acid added to the high buffering one), increased protein digestibility and *Lactobacillus* counts and reduced *E. Coli* counts (Bosi et al., 1999) (Figure 1).

Recently the addition of K-diformate to a starter diet for piglets decreased total anaerobic bacteria, lactic acid bacteria, coliforms, and yeasts in feces and in digesta from various segments of the gastrointestinal tract, without affecting the gastric or intestinal pH (Canibe et al., 2001).

However, in the light of a prolonged use of acidifiers, some caution is required as there are some indications of

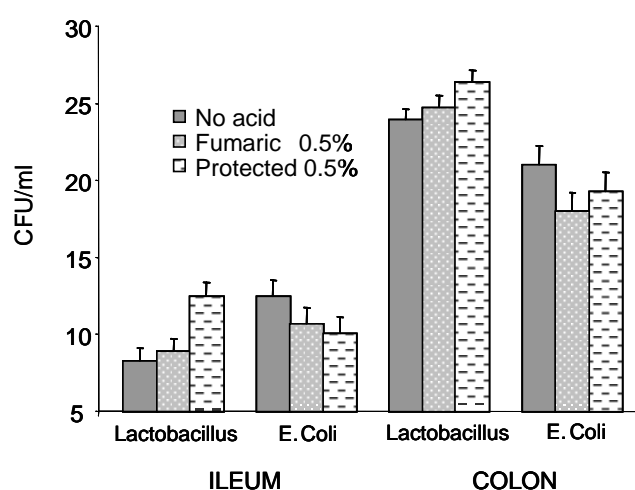


Figure 1. Effect of dietary acid addition on the *Lactobacillus* and *E. Coli* content in ileum and colon of early weaned pigs (Bosi et al., 1999).

development of acidic resistance of *Salmonella typhimurium* and *Escherichia coli* by exposure to short-chain fatty acids (Guilfoyle and Hirshfield, 1996; Kwon and Ricke, 1998). Audia et al. (2001) described some of the mechanisms of acidic resistance even at pH 2 or 3.

Acidic secretions are products of the metabolism of some probiotic bacteria (Lactobacilli, Bifidobacteria). The dietary use of molecules that improve their development (so called Prebiotics) was object of a presentation in the Past Symposium (Mosenthin and Bauer, 2000), where it was however stressed that several research failed to detect major effect on microbial characteristics in small intestine digesta. Bacteria can also produce molecules active against other bacteria (bacteriocins). However of 92 *Enterococcus faecium* and *Enterococcus faecalis* strains isolated from pig feces, only seven produced bacteriocins (Du Toit et al., 2000). Furthermore, Jin et al. (2000a) observed that some Lactobacilli strains maintained the ability to contrast the growth of many strains of Enterotoxigenic *Escherichia coli* when were grown in the presence of proteolytic enzymes (Table 4), but when the pH was set to 6.5 the inhibitory effect disappeared (data not in Table). This could signify that the production of organic acids, and not of bacteriocins, counteracted the growth of pathogen Coli.

The dietary addition of some fermentable substrate can however be useful when probiotics are fed. The effectiveness of *Lactobacillus casei* in the prevention of E-coli induced diarrhea in conventional and gnotobiotic pigs was potentiated by the addition of maltodextrins (Bomba et al., 1999).

Many natural molecules deliverable with the diet can control overgrowth of microbes. Between these, herbs and plant extracts are receiving increasing attention.

In Table 2 bacteriostatic and bacteriocidal concentrations for a range of plant essential oils and essences are presented. Between the products tested thyme and bay showed the action more diffused and at the lowest dilution.

In Table 3 some of the results found on the use of these products in the feeding of young animals are presented. Many cases when the vegetable products were effective can be seen. At the same time we can observe that researches were not officially presented with the products that in table 3 showed the best bacteriostatic or bacteriocidal action.

Competition at the sites of action of pathogens

Many pathogens have developed the ability to adhere to the cells of the gut wall, in order to disrupt its barrier efficiency and translocate, or simply to induce secretions by the enterocyte.

This attachment can be more or less specific. This depends on the molecular recognition that involves the protein (for example the fimbriae) or glucidic structures (other adhesines) of bacteria and the receptors exposed by the epithelial cells (glycoproteins, proteoglycans or glycolipids). In many cases the interaction can be very stable, like in the case of the complex of antigen and antibody or enzyme and substrate. For these examples it is often possible to find a competitor that can impede the formation of the complex. In some cases similar molecules can be find in commensal bacteria.

However the pathogens had the time of evolution to express their avidity for the molecules present in the gut wall. So of a total 43 strains of lactobacilli, showing in some cases the ability to aggregate the pathogenic *E. coli* O129-K88+, none was able to impair the colonization of enterocytes by this *E.coli* (Spenser and Chesson, 1994).

Table 2. Bacteriostatic and bacteriocidal concentrations (%) for a range of plant essential oils and essences (lowest concentration at which bacteria failed to grow in broth, but were cultured when 100 µl samples were plated onto agar (Bacteriostatic=b.stat) or were not cultured (Bacteriocidal=b.cid) (Smith-Palmer et al., 1998)

OIL	<i>Escherichia coli</i>		<i>Staphylococcus aureus</i>		<i>Listeria monocytogenes</i>		<i>Salmonella enteridis</i>		<i>Campilobacter jejuni</i>	
	b.stat.	b.cid.	b.stat.	b.cid.	b.stat.	b.cid.	b.stat.	b.cid.	b.stat.	b.cid.
Basil	0.25	0.5	0.1	0.5	0.05	0.25	0.1	0.5	0.25	>1
Bay	0.05	0.1	0.05	0.075	0.02	0.04	0.05	0.075	0.075	0.5
Clove	0.04	0.1	0.04	0.04	0.03	0.04	0.04	0.075	0.05	1
Cinnamon	0.05	0.1	0.04	0.04	0.03	0.075	0.05	0.1	0.05	>1
Garlic	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
Marjoram	>1	>1	0.05	0.25	0.02	0.25	>1	>1	0.25	>1
Nutmeg	>1	>1	>1	>1	<0.01	0.05	>1	>1	>1	>1
Onion	>1	>1	>1	>1	>1	>1	>1	>1	>1	>1
Peppermint	>1	>1	0.04	0.25	0.03	0.5	>1	>1	0.1	>1
Rosemary	>1	>1	0.04	0.1	0.02	0.1	>1	>1	0.5	>1
Sage	>1	>1	0.075	1	0.02	0.25	>1	>1	>1	>1
Spearmint	0.25	0.5	0.075	>1	0.04	0.25	0.25	0.5	0.25	>1
Thyme	0.05	0.1	0.02	0.03	0.02	0.03	0.04	0.04	0.04	0.05

Table 3. Principal effects of herbs utilized in young animals (From Todesco, 2001, modified)

Animals	Herbs	Purpose	Effects	References (cited by Todesco, 2001, except a,b and c)
Calves Pigs	St. John's wort, wild chamomile, marigold flowers, bearberry leaves	- on fecal samples: influence on Gram-negative bacteria	<ul style="list-style-type: none"> • positive effects: potentially action on virulence properties of some bacteria 	Turi et al. (1997)
Piglets	<i>Echinacea</i> , genzian-root, essential oils of juniper and thyme, tannins and silicic acid	- comparison with Carbadox and Colistin	<ul style="list-style-type: none"> • no different effects • leukocyte count was higher in plant extracts treated piglet group 	Savoini et al. (2000)
Piglets	<i>Origanum</i>	- diarrhea incidence - mortality incidence - productive performance	<ul style="list-style-type: none"> • significantly reduction of diarrhea and mortality incidence • increased weight gain rate, better feed utilisation 	Tsinas et al. (1998)
Piglets	<i>Origanum</i>	- productive performance	<ul style="list-style-type: none"> • increased weight gain rate, • better feed utilization 	Tsinas et al. (1998)
Piglets	<i>Origanum</i>	- controlling colibacillosis	<ul style="list-style-type: none"> • lower diarrhea • lower mortality rate • better growth performance 	Kyriakis et al. (1998)
Piglets	Sweet basil, chamomile flowers, calendula florets, fennel fruits, fenugreek seeds, and/or an aqueous aloe extract	- immunological status	<ul style="list-style-type: none"> • positive effects 	Kolacz et al. (1997)
Piglets	Garlic	- productive performance - comparison with Mecadox	<ul style="list-style-type: none"> • with garlic at 0.05% better feed utilization and ADG, compared to the negative control group, lower when compared to Mecadox group results 	Jost (1997)
Piglets	<i>Echinacea purpurea</i> (purple coneflower)	<i>idem</i>	<ul style="list-style-type: none"> • same growth as mecadox diet (at the max dose of the herb) 	Holden and McKean (2000a)
Piglets	Garlic	<i>idem</i>	<ul style="list-style-type: none"> • performance lower than mecadox diet 	Holden and McKean (2000b)
Piglets	Peppermint	<i>idem</i>	<ul style="list-style-type: none"> • performance lower than mecadox diet 	Holden and McKean (2000c)
Piglets	Herb mixture not specified	- productive performance	<ul style="list-style-type: none"> • better feed utilization, • better ADG 	Wetscherek W. (1998)
Rabbits	<i>Sophora flavescens</i> (SF), <i>Clinopodium Polycephalum</i> (CP) and <i>Polygonum hydropiper</i> (PH)	- antidiarrhoeal effects	<ul style="list-style-type: none"> • beneficial effect on the acute inflammatory exudation • inhibition of the motility of the small intestine in the SF group 	Wu Li Fu et al. (1998)
Rabbits	<i>Geranium</i>	- antagonize the increase in secretion from intestinal mucosa to the lumen and diarrhea	<ul style="list-style-type: none"> • astringent action to support antidiarrheal effect 	Ofuji et al. (1998)

Similar results were obtained by Jin et al. (2000a). In fact some strains of Lactobacilli showed a good inhibitory activity against two strains of *E. coli* K88+ and some could adhere to intestinal cells, but none could successfully impede the adhesion to intestinal mucus by the pathogen (Table 4). Better results were fortunately obtained by Jin et

Table 4. Comparison of different *Lactobacillus* spp ability to oppose to 5 *E. coli* K88+ strain growth, to adhere to porcine enterocytes, and to exclude the adhesion of such *E. coli* to enterocytes (Jin et al., 2000a)

Strain	Antagonist action on 5 <i>E. coli</i> K88 strain growth (inhibition ray, mm)	Adhesion to enterocytes	Aggregation of <i>E. coli</i> in presence or absence of lactobacilli	
			<i>E. coli</i> K88ac	<i>E. coli</i> K88 + MB
<i>Control (bovine seric albumin) -</i>		1,0	100	100
La 141	7,9	2,0	91,3	94,1
La 119	< 4	1,5	94,4	96,5
Ld 134	< 4	1,5	95,0	91,8
Lp 136	< 4	1,8	91,5	84,7
Ld 138	7,9	1,2	nt	nt
Lb 132	< 4	1,1	nt	nt
Ld 118	< 4	1,0	nt	nt
Lp 115	9,4	< 1	nt	nt
Others ¹	< 4	< 1	nt	nt

¹ on 36 strains, in total, isolated from piglet small intestine

nt : - not tested

La = *Lactobacillus acidophilus*, Ld = *L. delbrueckii*, Lb = *L. brevis*, Lf = *L. paracasei*

et al. (2000b) with other bacteria. *Enterococcus faecium* 18C23 (at 10⁹ UFC/ml) in co-culture with *E. coli* on pig intestinal mucus intestinal inhibited at 90% the growth of the second microorganism.

A mucosal competitive exclusion culture was isolated from caecum of healthy pigs. The administration of this culture within the first day of life resulted in significant reductions in mortality, incidence of faecal shedding, and in gut colonization by *E. coli* (Genovese et al., 2000) and in gut colonization by *Salmonella choleraesuis* (Fedorka-Cray et al., 1999) in piglets challenged with the respective pathogens. However in this case it is not clear which mechanism could reduce the growth of pathogens.

The isolation of the structures used by pathogens to adhere to the enterocyte can help in the screening of molecules or products that can interfere with their receptors.

The *E. coli* expressing the K88 fimbrial antigens are between the agents causing the neonatal diarrhoea and post-weaning diarrhoea in pigs. The fimbriae can be separated from the pure culture of *E. coli* and used in an Enzyme-linked immuno-absorbent assay where molecules or products are tested for their ability of blocking the binding of the fimbriae to isolated brush border membranes taken from the intestine of pigs (Miller et al., 2001).

With this test it was observed that the adhesion to the membranes of enterocytes was inhibited by egg powder obtained from hens immunized against the K88 fimbrial antigens (Miller et al., 2001). Furthermore in vitro inhibition of adhesion of enterotoxigenic *Escherichia coli* K88 to piglet intestinal mucus by egg-yolk antibodies was demonstrated by Jin et al. (1998)

In the chicken freshly-laid egg, IgA and IgM are found in the white and IgG are in the yolk. From the yolk of one egg up to 200 mg of antibodies can be harvested. The use of

egg-yolk antibodies from hens immunized with K88 fimbriae, to control intestinal diseases of pigs has recently received much attention. In pigs orally challenged with *E. coli* K88 or 987P the addition of specific egg-yolk antibodies reduced mortality, the incidence of diarrhoea, the faecal shedding of *E. coli*, the number of scours (Yokoyama et al., 1992; Marquardt et al., 1999). Anti *E. coli* and *Rotavirus* hyper-immunised eggs reduced *E. coli* K88 infection in neonate colostrum-deprived and colostrum-fed pigs, but were inefficient against *Rotavirus* infection (Rizvi et al., 2001).

In weaned pigs specific egg-yolk antibodies reduced also intestinal colonization (Zuniga et al., 1997) and cases of diarrhoea and mortality from the agent of edema disease (*E. coli* F18) (Imberechts et al., 1997).

The same strategy can be applied for the protection against some *Salmonella* species. Up to now good survival rates were observed in mice infected with *S. typhimurium* or *S. enteritidis*, supplemented with egg-yolk antibodies obtained immunizing hens with an outer membrane protein (Yokoyama et al., 1998).

Furthermore, in calves the oral supplementation of 10 g/day specific egg yolk antibodies against rotavirus, coronavirus and *Escherichia coli* F5 improved daily live weight gain (Erhard et al., 2000) and a synergic effect was observed with the addition of a probiotic (*Bacillus cereus* var. *toyoi*).

The results obtained with egg-yolk antibodies can at least in part explain the positive performance observed in piglets fed spray dried porcine plasma and spray dried porcine products (SDP) (for reviews, Coffey and Cromwell, 2001; Van Dijk et al., 2001). In some cases the improvement observed depends on the reduction of post-weaning intestinal diseases. SDP contains immunoglobulins

and glycoproteins (Van Dijk et al., 2001); the firsts could counteract specifically and a-specifically the pathogens, whereas the latter could reduce the adhesion of pathogens to specific receptors in the mucosa (Van Dijk et al., 2001).

A proof is that some SDPs could reduce the requirement for a stimulation of immunoglobulin A against *Escherichia coli* K88 (Bosi et al., 2001). Furthermore it was shown that the immune response was only required in receptive subjects. The susceptibility to K88ac *E.coli* is related to the presence of a galactosylated receptor protein complex in the intestine of susceptible animals (Jeyasingham et al., 1999). The binding receptors are present along all the small intestine, but seem to be more concentrated in the mid-small part (Chandler et al., 1994; Jeyasingham et al., 1999). For these reasons it can be hypothesized that SDP products can be substitutes for the use of antibiotics in feed, to control the most import intestinal pathogens. Bosi and Mengheri (not published) found a relevant IgA specific activity only in plasma from weaning pigs receptive to K88 adhesion fed a control diet. At the contrary, notwithstanding the oral challenge with the ETEC strain, scarce anti-K88 IgA were observed for receptive pigs fed the SDP diet or the diets supplemented with a specific antibiotic (Figure 2). SDP also improved growth and reduced salivary Anti K88 IgA as did dietary medication (Figure 3). In addition, respect to fish meal feeding, SDP halved the level of pro-inflammatory interleukin-8 mRNA in the intestine (Mengheri and Bosi, not published) and the reduction was bigger in susceptible pigs. This again confirms that positive growth performances can be achieved when the immune stimulation is reduced.

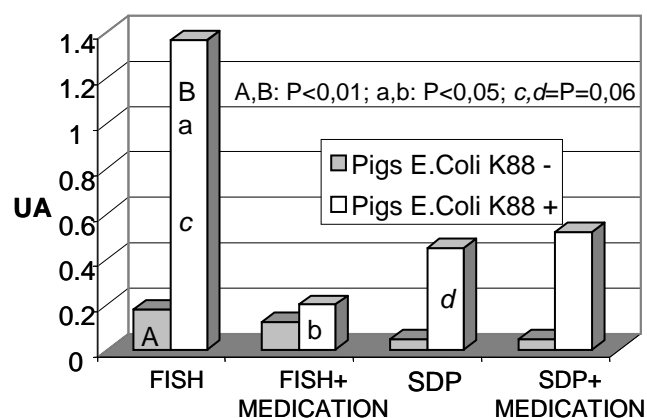


Figure 2. Effect of protein source, diet medication and susceptibility to *E. coli* K88 on plasma IgA specific activity (units/ml) in weaned piglets orally challenged with *E. coli* K88

THE "CASE" OF THE USE OF ZINC OXIDE AS GROWTH PROMOTER AND FOR THE CONTROL OF DIARRHEA

In many experimental condition the supplementation with zinc oxide at levels exceeding the nutritional requirements (3 g/kg) improved growth performance and controlled diarrhea in the weaning pig (Hill et al., 2001). However, in other experiences no effect on growth was recorded (Mavromichalis et al., 2000). In any case the required dose is ten times the maximum concentration permitted in the European Union. In this world area and in other countries, concerns about zinc transfer to the soil are strongly present. Furthermore, in the same subjects showing growth improvement, signs of toxicity in the liver were described (Jensen-Waern et al., 1998).

Consequently, the knowledge of the mechanism of action of ZnO would be important to develop dietary strategies more adequate for the overall health of the animals and for the environment. But from research results, different hypotheses can be proposed. The first question could be: ZnO only or also other zinc sources? No conclusive response can be found in literature. Woodworth et al. (1999a and b) found that 3000 ppm Zn from ZnO stimulated growth of weaning pigs, whereas the same effect was not obtained with the supplementation with zinc sulphate or zinc-aminoacids complex. Only two published research compared the same high zinc dietary additions. In four trials, zinc oxide, zinc sulphate, zinc-methionine or zinc-lysine, did not improve growth, respect the base diet (Schell e Kornegay, 1996). In general, zinc contents in plasma, liver and kidney increased with zinc supplementation. In the second research, growth-promoting efficacy of pharmacological dietary doses of tetrabasic zinc chloride (1500 mg/kg) was at least equal to the one with ZnO (Mavromichalis et al., 2000). Nevertheless, the

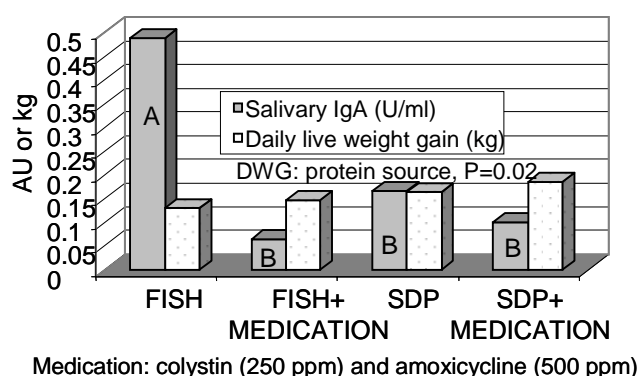


Figure 3. Effect of protein source and feed medication on daily weight gain (g) and saliva IgA specific activity (units/ml) against *E. coli* K88, in weaned piglets orally challenged with *E. coli* K88

injection of zinc acetate in the range of 3 days before and 3 day after weaning could not increase weaning performance (Schell e Kornegay, 1994). This could state that the requirement for circulating zinc is not higher than the amount obtained from the normal dietary supply. However this consideration contrasts with the observation that the addition of zinc in a form strongly chelated was effective in promoting growth at levels lower than that required for ZnO (Paik et al., 2000).

It is well-known in human medicine that ZnO is effective against bacteria, mainly gram positive. High doses of ZnO reduced the passage of bacteria across the intestinal epithelial barrier (Mavromichalis et al., 2000) and stabilized the microflora (Katouli et al., 1999). But Li et al. (2001) did not find any effect on microflora and Mores et al. (1998), while registering a reduction of diarrheas, did not observe any reduction of fecal shedding of *E. Coli* K85 in infected pigs.

For ZnO a reduced availability is often ascribed when it is compared with other zinc sources (Schell and Kornegay, 1996). Hence a delayed permanence in the gut could explain an antimicrobial effect. On the contrary the efficacy of ZnO did not change substantially with the commercial source (Mores et al., 1998) and with the biological availability (Mavromichalis et al., 2000). This can lead to exclude an effect of the quantity of absorbed zinc. Furthermore the effect of ZnO was often additive to the one of the medication in the diet (Chen et al., 1999; Mahan et al., 2000). This is again not in favor of the antimicrobial hypothesis.

A local effect of ZnO can be suspected, considering its wound healing action in the topic use. In pig, with the wound healing effect an increased endogenous gene expression of IGF-1 (Tarnow et al., 1994) was observed. This growth-stimulating factor could favor a better recover of intestinal epithelium after the weaning stress or reduce its negative effects. With 3,000 mg/kg ZnO from weaning, pigs after 11 days had higher villous width and higher villous height to crypt depth ratio all along the small intestine (Li et al., 2001). In piglets fed diets supplemented with 3750 mg/kg ZnO (Carlson et al., 1998) and challenged with TGE virus (Stanger et al., 1998), the morphology of intestinal epithelium was improved at the end of the weaning period. However we did not find any effect on growth and diarrhea intensity in enterotoxigenic *E. coli* - challenged pigs, supplemented with low doses of zinc in different forms (fat-protected or pectin-linked) or with 3,000 mg/kg ZnO (Bosi et al., 2001).

In this trial we observed that immunoglobulin A specific for the *E. coli* K88 of challenge, increased with the zinc supplementation (Figure 4). However this effect was limited to the subjects sensible to the *E. coli* K88 adhesion at the level of the intestinal villi. This could lead to the conclusion

that a high zinc supplementation induced an increased specific immunity, but in other challenge trials we always observed higher plasmatic IgA contents in subjects with impaired health conditions and reduced growth.

From studies on mice nutrition, we know that diarrhea is one of the major symptoms of zinc deficiency and that in this case the expression of the inflammatory molecule Interleuchine-1 β is over-expressed (Vignolini et al., 1998). We are evaluating if different zinc levels over the standard zinc requirements affects positively the expression of this cytokine in the small intestine of piglets. Preliminary results from the trial previously described show that the supplementation with 2,500 mg/kg ZnO or 200 mg/kg ZnO fat-protected reduces the expression of this inflammatory molecule.

In conclusion, researches support different mechanism of growth promoting action of zinc when supplemented at doses over the standard requirements. However, till the base mechanism is not clearly disclosed, it will be impossible to formulate such growth promoting diets in the Countries where regulations limit zinc supplementation.

RELEVANCE OF FEED INTAKE IMMEDIATELY AFTER WEANING TO PRESERVE THE EPITHELIAL BARRIER FUNCTION

In the practice of animal production, the weaning is associated with a variety of stressors. One consequence is the reduced feed intake, which immediately affects the availability of nutrients essential to maintain the integrity of the epithelium. The observations on the structure of the mucosa after weaning of pigs show villous atrophy with a reduction of its height and then a compensatory increased

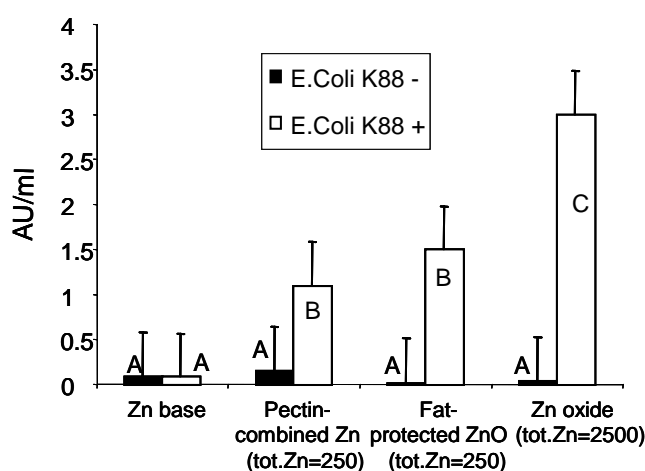


Figure 4. Effect of zinc sources and doses (ppm) and intestinal susceptibility to *E. coli* K88, on plasma IgA specific activity (units/ml) in weaned piglets orally stimulated with *E. coli* K88

crypt cell production and crypt depth. Research data demonstrate that the overall intake is more important than the high quality of the diet (Bosi, 2000, for more information) to maintain an efficient mucosa. Furthermore increased paracellular transport and altered T-cell subsets are observed at low feed intake (Spreeuwenberg et al., 2001). However when the quality of the diet permits a higher intake at weaning, the integrity of villous is improved. This is observed when a liquid diet substitutes a solid diet of the same composition (Hurst et al., 2001). After early investigations on “artificial rearing”, in trials on several experimental farms, liquid diet proved to be more productive than solid diet (Odele and Harrell, 1998). However we need more experience on easy-to-manage feed delivery system and cheaper feeding techniques. Furthermore, in the case of fermented liquid diets, the acidification can contribute to maintain gut health. A controlled development of favorable lactic acid bacteria can then limit the multiplication of Salmonella in the feed through competitive exclusion.

Variably produced fermented liquid feed diets reduced the clinical signs of experimentally induced dysentery and maintained the bacterial community structure of uninfected subjects (Leser et al., 2000) and reduced the levels of coliform bacteria (Mikkelsen and Jensen, 1998) or of *Enterobacteriaceae* (Winsen et al., 2001), or increased yeasts (Mikkelsen and Jensen, 2000) in different parts of gastrointestinal tract. According to Mikkelsen and Jensen (2000), for a good fermentation of the feed in the liquid, 8 hours at 20° C are necessary, and half of the product should remain in the tank for the next fermentation process.

However, in the farm, in the absence of control over what ingredients are fermented, the type of bacteria used, fermentation temperature and the method of storage and delivery to the pig, malfermentation can occur, with feed avoidance and weight loss.

FINAL CONSIDERATIONS

Some examples of dietary tools to improve the gut barrier function were presented. These could contribute to maintain growth and health in weaned mammals, without the use of in feed antibiotic/growth promoter additives. However, to diffuse dietary alternatives more knowledge is required on: a) basal mechanisms of action; b) compounds metabolically active; c) stability in the feed compounds; d) effects of feed presentation; e) effects on healthy subjects or at risk or ill subjects; f) dosages; g) interactions between different alternatives and with base feeds; h) residual and long term effects for the consumer health. Then the adoption of new additives can be limited by the opportunity of preserving these solutions for human nutrition and care. Finally it would be important the transfer and the adaptation

of the experience on gut barrier modulation done essentially in the pig, to other species, such as ruminant species, rabbit, horse and so on.

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