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A new nutritional approach for promoting gut health and animal performance

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Summary

The use of antibiotics as growth promoters has characterized the development of livestock productions for more than 50 years. Consumer and political opinions, and a scientific concern that resistance to antibiotics selected in animals might be transmitted to human pathogens, affecting the efficacy of the pharmacological treatment, created a great debate in Europe which led to a complete ban of AGP in 2006. However, the correlation between the development of antibiotic resistance in bacteria and the agricultural usage of antibiotics has never been clearly demonstrated or supported by evidence.

In pig production systems, the main consequences of the ban were a transient impairment of animal welfare and growth performance, particularly after weaning, and economic losses related to increased production cycles length and veterinary expenses.

As reported by Lallès *et al.* (2009), various nutritional approaches aimed to the optimization of the weaning transition and reduction of the GIT diseases have been proposed, but the success rates are really low compared to antimicrobials. Nutritional strategies and adoption of breeding management focused on disease prevention are fundamental to counteract the spread of infectious disease in food animals, especially in intensive farming. However, the search for successful alternatives is hampered by a lack of knowledge about the mechanisms of the antibiotics-mediated growth enhancement. AGP have been considered for a long time as acting essentially on the gut microbiota, by reducing the potentially harmful effects of the intestinal microbiota. A recent theory suggested an anti-inflammatory role of AGP compounds, which caused a down regulation of the immune response, thus allowing a larger amount of energy available for the animal growth. Immunomodulating compounds are gaining interest, since the correct development of both innate and acquired immune system of the piglets is a key factor in determining the outcomes of a large exposure to antigens and stress conditions, typical at weaning.

Moreover, nutrition has become a tool to improve health and wellness in both humans and animals, due to the health promoting activities of particular compounds, e.g. β-glucans, lycopene, ω3 or conjugated linoleic acid, present in food and feed.

The antisecretory factor protein (AF) is secreted in plasma and other tissue fluids in mammalians. It was shown to be a potent inhibitor of intestinal fluid secretion and inflammation; its immunohistological distribution suggested a role in the immune

system. AF content in sows' milk appears to be a protection factor against neonatal diarrhoea in suckling piglets. Exposure to bacterial toxins induces secretion of AF in plasma, probably reflecting a natural defence mechanism to agents causing diarrhoea, thereby contributing to a favourable clinical outcome and disease termination. Increase of AF level in plasma by dietary means, such as feeding hydro-thermally processed cereals (HPC), has been demonstrated in human and animals. Nevertheless, the mechanism of action behind its biological activity has not yet been clarified and, so far, few data are available on pig field trials. Furthermore, compared to its use in human nutrition, the proper level of HPC diet supplementation has not been defined.

A first purpose of this thesis has been to summarize the knowledge on the antisecretory factor and its derivates, evidencing the potentially beneficial effects of AF-inducing diets on both human and animal health and welfare.

In the field trial we tested the effects of two HPC level of diet supplementation (3% *vs* 6%) on weaned piglets growth performance and intestinal mucosa health status. The results confirmed the efficacy of HPC as growth promoters in piglets: compared to the 3% supplemented diet and the control group, 6% of HPC supplementation improved ADG and feed conversion rate. *In vitro* digestion assays of the diets evidenced relevant simple sugars content and a higher rate of starch hydrolysation in the HPC diets compared to control diet. The difference in growth performance observed between groups might be related to a balanced supply of energy-yielding nutrients and amino acids in the diets. Indeed the first week post weaning is characterized by reduced voluntary feed ingestion and reduced pancreatic enzymes activity, therefore feeding a diet with highly digestible starch guarantees a correct amount of energy, limiting the amount of starch available for microbial fermentation in caecum and colon. In the current study the concentration of I-FABP in plasma were low in all groups and not affected by the dietary treatments, suggesting that, in good hygienic conditions, the dietary treatments did not affect small intestinal integrity.

In vitro test were realized to investigate the antisecretory and the anti-inflammatory properties of the AF protein and its mechanism of action by using AF-16, a 16-meric peptide which contains the active domain of the protein.

The Ussing chamber experiments were performed on polarized IPEC-J2 cells to investigate the antisecretory activity of AF-16. The peptide did not inhibit the CT-induced Isc increase as did DPC, a voltage-dependent CFTR/Cl⁻ blocker. A slower rate of Isc increase was observed during the simultaneous administration with AF-16 and

CT. A recent study by Johansson et al. (2008) hypothesized that AF binding to the integral membrane protein flotillin-1, localized in the lipid rafts, causes an alteration of ion channels and receptors localization. It is possible to speculate that the effects observed on CT-induced Isc might be due to a kind of "disturbing effect" on the toxin binding to GM1, which is also localized in the lipid rafts.

To evaluate the anti-inflammatory activity of AF, as a possible immunomodulating feed additive, two kind of *in vitro* assays were made. High dosages of AF-16 (1×10^{-4} M) were found to reduce the LPS-stimulated NO production in RAW264.7 cell, thus supporting the hypothesis of an anti-inflammatory action. The effects observed could be due to the blockage of LPS activity and consequently the iNOS inhibition. Moreover, the presence in the peptide sequence of cysteine, which contains a SH group, and proline, which is a well known free-radicals scavenger, may act as a scavenger for unpaired electrons, thus reducing free radicals generation. On the contrary, no significant results were obtained on PMA-stimulated ROS generation in pig alveolar macrophages.

The literature review showed how the AF-inducing diets and the AF-therapy is an interesting approach to improve welfare in humans affected by several disease

Cereals might also be helpful for people subjected to the risk of intracranial pressure increase, i.e. patients affected by brain pathologies like tumors in conditions of sudden pressure changes, as during long flies, or exposed to overpressure as divers. AF-rich egg yolk powder gives the opportunity of a direct and fast AF administration to young children and to people with impaired food consumption.

It is remarkable that the hydrothermally processed cereals, being simple cereals kernels, may have a positive image towards the modern European consumers, who are increasingly sensitive to ethical considerations and whose opinion has a great influence on legislation. Compared to other possible alternatives to antimicrobial growth promoters, these processed cereals do not contain any active compound that can be lost in the upper gut, since their action is to stimulate the animal capability to produce it. In addition, we found that HPC supplementation improved the feed conversion rate: a more efficient use of nutrients present in the diet allow decreased nutrition costs and reduced nitrogen and phosphate excretion in manure and their accumulation in the soil.

List of abbreviations

5-HT	5-hydroxytryptamine
ADFI	average daily feed intake g/day
ADG	average daily gain g/day
AF	antisecretory factor
AF-16	antisecretory factor peptide (16-mer)
AGA	Amylogucosidase test
AAO-HNS	American Academy of Otolaryngology-Head and Neck Surgery
BW	body weight
cAMP	cyclic adenosine monophosphate
CD	Crohn Disease
CDA	Clostridium difficile Toxin A
CFTR	Cystic Fibrosis Transport Regulator
CNS	Central Nervous System
CT	cholera toxin
CP	crude protein
DHR 123	dihydrorhodamine 123
DPC	diphenylamine 2-carboxylic acid
EAE	Experimental autoimmune encephalomyelitis
EEA	Essential amino acids
FCR	feed conversion rate
GABA	gamma-Aminobutyric acid
GIT	gastrointestinal tract
GM1	Monosialotetrahexosylganglioside
HPC	hydrothermally processed cereals
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
ICP	intracranial pressure
I-FABP	intestinal fatty acid binding protein
IL	Interleukin
iNOS	inducible nitric oxide synthase
Isc	short circuite current

LPS	Lipopolysaccharide
LT	labile toxin
MHC II	major histocompatibility complex class II
MTC	Medullary Thyroid Tumor
NO	nitric oxide
PMA	phorbol myristate acetate
PWDS	post weaning diarrhoea sindrome
rAF	recombinant antisecretory factor
ROS	reactive oxygen species
SBS	short bowel syndrome
SD	standard error
SDP	spray dried plasma
SEM	mean square error
SGLT 1	sodium-dependent glucose transporter 1
TEER	transepithelial electrical resi stance
TNF-α	tumor necrosis factor alpha
UC	Ulcerative Colitis
VAS	visual analogue scale
VIP	vasointestinal peptide
VRE	vancomycin-resistant enterococci
vWm	von Willebrand motif

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Antibiotic Growth Promoters in animal husbandry

Introduction

The introduction of antibacterial agents 70 years ago led to a revolution in the management of bacterial infections. The use of antimicrobials (commonly referred to as antibiotics), as growth promoters has characterized the development of livestock productions for more than 50 years.

The growth promoting effect of antibiotics was casually discovered in the United States where dried mycelia of S*treptomyces aureofaciens,* containing tetracycline, added to poultry diet with the aim to increase vitamin B_{12} intake, improved the growth performances (Moore *et al.*, 1946; Stokestad and Jukes, 1950).

In 1951, the United States Food and Drug Administration authorized the use of antibiotics as feed additives; during the 1950s and '60s each European Country approved its own regulation about the non therapeutic use of antibiotic growth promoters (AGP) (Jones and Ricke, 2003; Castanon, 2007).

From that time on, AGP became extensively used worldwide in livestock production, often being relied upon not only to enhance growth but also to substitute in part poor husbandry conditions (Soulsby, 2008). Added to the animal diets at sub therapeutic doses, AGP proved to be effective in improving feed efficiency and growth performance. The use of AGP prompted the intensive livestock productions and became fundamental to control and counteract the spread of infectious disease in farm animals.

FAO considered that the sub-therapeutic employ of AGP significantly contributed to reduce animal protein deficiency in the world (Piva and Rossi, 1999).

Animal breeding has undoubtedly drawn great advantages from the sub-therapeutic use of AGP mainly in young animals. Improvement of weight gain, feed efficiency and health status, reduction of environmental pollution, have been the most important results obtained.

However, concerns about antibiotic resistance development which might be transferred to human pathogens, affecting the efficacy of the pharmacological treatment, created a great debate in Europe, though this correlation has never been clearly demonstrated or

supported by evidence, their use became increasingly controversial until the complete European ban (Pradella, 2006).

European regulation on AGPs

During the '50s, resistance to tetracycline used at growth-promoting levels was observed (Dibner and Richards, 2005).

The problem of antibiotic resistance linked to the use of AGP was dealt for the first time in the United Kingdom in the Swann Report in 1969. In order to reduce the risks of diffusion of antibiotic resistance between bacteria strains, the Swann report recommended a distinction between antibiotics used in human and veterinary medicine and those used as AGP (Swann, 1969). The debate created by the Swann Report led to a change in the European regulation on feed additives, with the adoption of the Council Directive on Additives 70/524. Since national regulations of each member State differed as regards basic principles, Dir 70/524 aimed at an harmonization of laws concerning additives in feeding stuffs, to establish and regulate the common market for animal feeds. Moreover, Dir 70/524 defined a list of antibiotic molecules authorized as additives in animal feeding and established a clear distinction between antibiotics for human and animal therapy and those used as growth promoters.

In 1986, Sweden prohibited the use of additives belonging to the groups of antibiotics in animal feeding (Aarestrup, 2003). In 1995, when Sweden accessed as a member of the European Union, it was authorized to keep in force its legislation on feed additives before accession, until 1998. However, Sweden submitted applications, accompanied by detailed scientific grounds, asking for regulation for the antibiotics authorized in the Community.

Following the ban on all growth-promoting antibiotics in Sweden, other member states prohibited on their territories the use of some AGP. The driving forces behind the national bans were consumer and political opinions, and a scientific concern that antibiotic resistance selected in animals might be transmitted to humans (Casewell *et al.*, 2003)

As results of this national initiatives, and in spite of negative opinion of the Scientific Committee of Animal Nutrition (SCAN), the European Union banned the use of avoparcin in 1997; the four remaining antibiotics (bacitracin, spiramycin and tylosin, and virginiamycin) used for growth promotion were banned in 1999, on the basis of the Precautionary Principle (Casewell *et al.*, 2003).

In 1997, the World Health Organization published a report on the medical impact of the use of antibiotics as growth promoters in food animals, suggesting a link between the

two on an epidemiological basis and advised the national governments to establish a surveillance of the antimicrobial usage and that animal health management should be routinely practiced to avoid the prophylactic use of antibiotics (Dibner and Richards, 2005).

Regulation 1831/2003 of the European Parliament established the European ban of AGP since 1st January 2006, while anticoccidial substances, such as antibiotics ionophores, will be banned as feed additives before 2013. After this date, medical substances in animal feeds are limited to therapeutic use by veterinary prescription.

European ban on AGP: consequences on animal welfare

The Nordic Countries experience had anticipated that the antimicrobials bans might have adverse consequences on animal health and welfare, and economic consequences for farmers. The most common consequences reported were increased diarrhoea in young piglets, higher incidence of necrotic enteritis in poultry, increased coccidiosis and metabolic disorders in cattle (Pradella, 2006).

New nutritional strategies and adoption of breeding management focused on disease prevention, together with therapeutic treatments using antibiotics with broad spectrum of activity, which belonged to the same classes of antibiotics used in human and animal medicine, were fundamental in counteracting the spread of infectious disease in food animals (Wierup, 2001; Wegener, 2003; Grave *et al.*, 2006).

While in slaughter pig production no negative clinical or economic effects were observed, and the growing rate was at least as good as in Countries using antimicrobials, most serious consequences were observed in piglet production where significant problems due to increased incidence of intestinal and respiratory infections, particularly at the weaning. The higher incidence of infectious disease often required antibiotic-medicated feed at therapeutic dosage before prophylactic actions could decrease the use (Wierup, 2001). This unexpected occurrence evidenced that antimicrobial growth promoters also had a previously unrecognized health promoting and disease-preventing effects.

Wierup (2001) reported that the use of antibiotic therapies in Sweden increased during the following 4-year after the ban, involving up to 75% of the pigs. Nonetheless, the overall use of antimicrobials in food animals was reduced: it decreased of 55% in the

13-years after the antimicrobials withdrawal. However, the production losses in Swedish pig husbandry were non fully recovered 20 years after the ban (Casewell, 2003; Wierup, 2001).

Afterwards, the withdrawal of antibiotics caused a transient impairment of animal welfare and despite the efforts to improve other aspects of husbandry, the veterinary use of therapeutic antibiotics, identical to those used in human medicine, has increased, and this constitutes a hypothetical hazard to human health in relation to resistance in *Salmonellae* spp, *Campylobacters* and zoonotic strains of *E. coli* (Casewell, 2003).

Trends of antibiotic resistance development after AGP ban

Antibiotic resistance is a natural feature of antibiotic-producing organisms as a protection from their own products, and of other originally susceptible organisms which became resistant to their competitive attack (Philips *et al.*, 2004). Already in 1944 Fleming found that some strains of *Staphylococcus aureus* developed antibiotic resistance and gave the first warnings (Economic and Social Committee, 98/C/ 407/02).

Resistance to antibiotics was first identified in human hospitalized patients, where penicillin-resistant *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) were dominant.

Since antimicrobial resistance is an inevitable consequence of antibiotics use, a reduced usage should determine a progressive reduction in the acquired antibiotics resistance. Nonetheless, even low-level resistance can lead to a reduced antibiotic efficacy within the clinically susceptible range.

The debate on the use of AGP was based on the assumption that all such use might be an hazard for human health since it might act as an important source of resistance genes in bacteria that could be transferred to human pathogens via the food chain (Phillips *et al.*, 2004).

One of the main expectations of the European AGP withdrawal was a diminished antibiotic resistance but, so far, the results obtained are contradictory.

Salmonellae spp. are Gram-negatives organisms responsible for the major zoonoses in Europe. Tetracyclines as AGP were banned in EU in 1970 because of the appearance of multiresistant *Salmonella typhimurium* strains during the '60s. It seems that the ban has

not achieve its objective if we consider that the percentage of tetracycline resistance in *Salmonella* spp. isolated from retail chicken in the United Kingdom (23%; Food Standards Agency, 2001) were found to be higher than in the United States (19%; NARMS, 2000), where tetracyclines are still allowed as growth promoters (Bywater, 2005). Moreover, tetracyclines are used in Europe for disease treatment, making it difficult to discriminate any selection pressure resulting from the former growth promotion use as opposed to therapeutic one (Bywater, 2005).

In Europe, after the avoparcin ban, reduced carriage of vancomycin resistance in enterococci (VRE) in animal and human faeces was reported (van den Bogaard, 1998; Klare *et al.*, 1999; DANMAP, 2003). On the contrary, in the US the VRE incidence was found higher despite the fact that avoparcin has never been used there in food animals (CDC 1999).

A recent European survey on antimicrobial susceptibility among zoonotic and commensal bacteria in food producing animals has shown a very low presence of resistance towards drugs used in humans. Indeed, some VRE was detected but in a very low number (de Jong *et al.*, 2009).

The prevalence of *Salmonellae* in Europe after the ban decreased and the few cases of human infection are related to consume of imported products and travellers (Aarestrup and Pires, 2009).

In the meantime, the pattern development of resistance among human pathogens has not changed, being more correlated with antimicrobial use in human hospitals, general practice, incorrect waste disposal, than to veterinary use.

Economic and environmental implications

Post weaning period in pig production has been the most critical phase during which are experimented the detrimental effects caused by the elimination of AGP (Göransson, 1997, Lallès *et al.*, 2007). Lallès *et al.* (2004) reported that the mortality of piglets in Europe amounted to 17% of the all pig born and great part of this loss was due to infectious diseases.

The ban of AGP had an economic impact on farms, due to increased costs for veterinary prescription and therapies, lost of feed use efficiency, prolongation of the production cycle.

It was reported that in Sweden the time necessary to reach 25 kg of piglet live weight increased of 5.2 days and the production costs increased of 1-3 $ head (Robertsson, 1994; Kjeldsen, 2005; Jensen, 2006).

Estimates of the financial impact of antimicrobial withdrawal in United States on consumers were evaluated to be in a range between 5 and 40 $ per capita per annum (NRC, 1999; Mathews, 2001).

By improvement of feed conversion, AGPs had a favourable effect on manure production: less feed was required to achieve a given amount of growth in an animal, as a result of which nitrogen and phosphate excretion was reduced in proportion to manure production (Gropp and Schumacher, 1997; Roth and Kirchgesser, 1993). Thus, the termination of AGP use could cause an increased N and P excretion.

Moreover, decreased feed efficiency is likely to cause an increased demand for cropland, with consequences on the land prices and a possible increased grazing pressure in fragile landscape (Phillips *et al.*, 2004; Montesissa and Calini, 2006).

Weaning piglets without AGPs: what a stress!

Producing pigs without the use of in feed antimicrobials is a great challenge for the European swine industry, especially at the weaning.

Weaning is a critical period for all mammalians and particularly for piglets in intensive production systems where, in order to maximize the annual sow production, the suckling period has been reduced from 5-6 to 3-4 weeks of age. The employ of AGPs has been strategic in reducing the effects of premature weaning on piglets health and growth performance. Indeed, early weaning practices expose piglets to nutritional, social and environmental stresses when the immune and the gastrointestinal systems have not completely reached the functional development (Partanen and Mroz, 1999).

Several stresses take place at the same moment: switch from liquid and highly digestible milk-based diet to a less digestible more complex cereal-based dry feed, separation from the sow, mixing of the litters, change of the environment (Hiss and Sauerwein, 2003). This situation often results in low voluntary feed intake, severe growth check and lead to an impaired health status and to a major susceptibility towards infections and gut disorders (Lallès *et al.*, 2004, 2007, 2009). Indeed, weaned piglets frequently show malabsorption syndrome, characterized by increased excretion of carbohydrates and

fatty acids in faeces, watery stools and degeneration of the intestinal epithelium. Opportunistic pathogens such as enterotoxigenic *E. coli* can take advantage from the poor health condition of piglets causing the post weaning diarrhoea syndrome (PWDS) (Kyriakis, 1989).

Recent review articles have focused on morphological and functional changes (Pluske, 2001), physiological changes (Lallès *et al.*, 2004), host-microbe interactions (Gaskins, 2003) and on the effects of dietary ingredients on the gut integrity and performance in weaned pigs (Vente-Spreeuwenberg and Beynen, 2003; Verdonk *et al.*, 2005; Lallès *et al.*, 2007; Lallès *et al.*, 2009).

Stress may be an initiating factor for weaning-associated alterations in the gastrointestinal tract resulting in altered gastric motility and emptying, mucus secretion, permeability and water absorption (Fieramonti, 2003). At least the increase in intestinal permeability involves the hypothalamo-pituitary-adrenal axis and endogenous glucocorticoids. Acute and chronic stressors, alone or in combination with starvation, have been shown to induce intestinal mucosal damage and increased permeability in rats (Saunders *et al.,* 1994; Spitz *et al.,* 1996; Kiliaan *et al.,* 1998; Bagchi *et al.,* 1999; Wirén *et al.,* 1999; Santos *et al.,* 2000; Saunders *et al.,* 2002).

Moeser *et al.* (2006) investigated the effect of weaning-associated stresses on piglets gastrointestinal health and found that corticotrophin release factor (CRF) and cortisol level in serum during increased during the first week after weaning. The higher CRF level was associated to an injury of the intestinal barrier function, evidenced by reduced jejunal and colonic transepithelial electrical resistance (TEER) and increased short circuit current (Isc) measured *in vitro*. Furthermore, the blockade of the CRF receptors prevented the intestinal disturbances (Moeser *et al.*, 2006).

The intestinal epithelium is composed by a single layer of columnar cells and it is the organism's first line of defence against harmful microorganisms and antigens within the intestinal lumen (Gewirtz *et al.*, 2002). It appears that the activation of the central stress pathways is the main cause of the alterations of the intestinal mucosal physiology, as the increased secretion and barrier disruption observed at weaning. The intestinal barrier break-down leads to increased intestinal permeability to diet antigens, bacteria and toxins, resulting in mucosal inflammation and release of cytokines and chemokines.

Mc Cracken *et al.* (2001) found a transient increase of IL-1 concentration in piglets blood during the first 2 days post weaning. Piè *et al.* (2004) demonstrated that weaning is associated to a transient up-regulation of intestinal expression of pro-inflammatory

cytokines IL-1β, IL-6 and TNF-α, that varied in the different regions of the gut. The over expression of pro-inflammatory cytokines mRNA was rapidly down-regulated within 2d post weaning, likely to avoid the tissue damage with the exception of TNF-α, which remained high in both ileum and colon. The pro-inflammatory cytokines release leads to modifications of the intestinal physiology, mainly affecting the permeability capacities of the intestinal mucosa, the enzymes activity, and it determines loss of appetite and stimulation of general catabolic activity. When stimulated by enteric pathogens, intestinal epithelial cells release a range of inflammatory mediators other than cytokines, such as cyclooxygenase (COX) 2 and nitric oxide (NO) and reactive oxygen species (ROS) (Verdonk, 2005). Reactive oxygen species and nitric oxide are known to play a double role in biological systems, since they can be either harmful or beneficial to the organism (Valko *et al.*, 2006). Expression of cyclooxygenase (COX) 2 and increased production of prostaglandin E2 in intestinal epithelial cells leads to increased chloride secretion (Eckmann and Kagnoff, 2005).

Feed intake determines performance and gut morphology in weaned pigs (Pluske *et al.*, 1997; Spreeuwenberg, 2002). It was estimated that at weaning only 50% of piglets consume their meal within 24h and it take 3 days to meet the requirement of energy and food intake for maintenance (Le Dividich and Sève, 2000). The dramatic reduction of nutrient intake and the diet changes lead to adverse alterations of intestinal morphology, including villous atrophy and crypt hyperplasia (Pluske *et al.*, 1997) caused by an increased rate of cell loss and a decreased rate of cell renewal, reduced absorptive capacity and brush border enzyme activity (Hampson 1986, Kelly *et al.*, 1991a and 1991b; Miller *et al.*, 1984a,). The increased rate of cell loss is likely due to apoptosis or programmed cell death. Under normal conditions apoptosis of differentiated enterocytes on the villous is rare but it increases in response to pathological and stressful conditions such as lack of luminal nutrition (Fukuyama *et al.*, 2001).

The weaning process, characterized by a shift of the diet, feed restriction and stress, leads to instability of the intestinal microflora, with reduction of the lactic acid bacteria community (Kostantinov *et al.*, 2003, Lallès *et al.*, 2007). At birth the gut is colonised by microorganisms deriving from the mother and the environment, starting from lactic acid bacteria, enterobacteria and streptococci. The intestinal microflora is a complex and dynamic ecosystem which interact with the host. Intestinal microbiota is considered an essential for the development of the animal immune system and physiology (Hooper, 2006). Epithelial cells recognize the colonising bacteria by the virtue of cell-surface

pattern of recognition receptors such as Toll-like receptors (TLR) (Kelly, 2001). The development of the intestinal microbiota communities is affected by the diet components and changes in the amount of nutrients available for microflora fermentation determines the type and the metabolic activity of the intestinal microflora.

Looking for an alternative

The 1999 partial ban of antibiotics as in-feed additives has awakened the interest for feed additives and led to intensive research for valid alternatives to AGP, able to counteract health problems in farm animals, and particularly in weaned piglets. Indeed, AGP were more effective in young animals such as in piglets after weaning, specially raised in poor sanitary conditions (Anderson *et al.*, 1999).

So far, various nutritional approaches aimed to the optimization of the weaning transition and reduction of the GIT disease have been proposed, but the success rates were really low compared to antimicrobials (Lallès *et al.*, 2009).

According to Niewold (2007), the search for effective alternatives is hampered by a lack of knowledge about the mechanisms of the antibiotics-mediated growth enhancement.

In-feed antibiotic growth promoters have been considered for a long time as acting essentially on the gut microbiota, by decreasing the potentially harmful effects of pathogenic bacteria (Dibner and Richards, 2005). The major theories proposed to explain the mechanism of action of the AGP recognize in the intestinal microflora the main target:

- ✓ AGP inhibited endemic subclinical infections, thereby reducing the energy cost of the innate immune system;
- ✓ the use of AGP resulted in a decrease in bacterial population, therefore lowering the accumulation growth-depressing metabolites production by intestinal;
- ✓ AGP reduced microbial use of nutrients;
- ✓ AGP improved the ingestion and use of nutrients due to a thinner of the intestinal wall.

All these theories share the hypothesis that the intestinal microflora reduces, either directly or indirectly, the animals growth and that the AGP-mediated growth

enhancement is due to the antimicrobic properties of these molecules. The limitations and contradiction of these theories were reviewed and analyzed by Niewold (2007), who suggested an anti-inflammatory role of the AGPs. According to the author, it is unlikely that antibiotics, administrated at doses lower than minimum inhibitory concentration (sub-MIC), might have an inhibitory effect on the intestinal pathogens or a modulate the selection of a "good microflora" (Niewold, 2007). Moreover, the chronic use of antibiotics should have led resistance in the target microorganisms and thus affected their efficacy. As remarked by Gaskins (2002), the use of probiotics as growth promoters is a paradox, since the gut microbiota is supposed to produce growth depressing metabolites such as ammonia and bile degradation products. It is also curious that AGP had similar growth promoting effects on animals of different species and at different stage of development, which undoubtedly have a different gut microflora (Niewold, 2007).

Stress conditions, as transport or weaning, were demonstrated to enhance the inflammatory state of the intestinal mucosa and its permeability (Mc Cracken *et al.*, 1999; Niewold, 2000; Bailey *et al.*, 2005). Permeability of the intestinal epithelium to macromolecules is increased in presence of inflammatory conditions as in the gut mucosa, which is considered an organ in a state of constant and controlled inflammation (MacDonald and Monteleone, 2005). A side effect of the antibiotic molecules is that they can accumulate inside the inflammatory cells, enhancing the destruction of intracellular pathogens (Labro, 2000; Niewold, 2007). The accumulation of antibiotics within the phagocytes leads to down regulation of the innate immune response with a sensitive reduction of the production of pro-inflammatory cytokines that results in lower catabolic stimulus (Schoevers *et al.*, 1999; Labro 2000, Niewold 2007). Furthermore, they can inhibit or decrease the innate immune response such as production of reactive oxygen metabolite and pro-inflammatory cytokines by macrophages. According to Niewold (2007), the energy saved by the down regulation of the immune response (due to the anti-inflammatory effect of AGP) is thus available for the enhancement of the animal growth. This theory suggested that the inhibitory/modulator effects observed on the microflora of animals fed AGPs are secondary ones, likely due to a healthier intestinal mucosa (Niewold, 2007). Therefore, the alternatives to in-feed antibiotics useful should be substances capable to down regulate the inflammatory response at the intestinal mucosa level, which is a physiological effect of weaning.

Spray dried plasma (SDP)

SDP is a by-product obtained from animal blood after exclusion of cells, concentration and spray drying. Three types of SDP products, from porcine (SDPP), bovine (SDBP) and unknown or mixed animal origin (SDAP) are available commercially (Lallès *et al.*, 2009). Feeding SDP was temporary banned in Europe as a consequence of the bovine spongiform encephalopathy crisis, but is now allowed for pigs. Hygienic collection and processing of the blood are essential to avoid the transmission of infectious disease (DeRouchey *et al.*, 2004; van Dijk *et al.*, 2001).

It was reported that SDP has beneficial effects on weaning piglets weight daily gain, feed intake and feed efficiency (Coffey and Cromwell, 2001; van Dijk, 2001; Moretò and Pèrez-Bosque, 2009). In an epidemiological study of Canadian swine farms, feeding SDP was associated with reduced mortality due to porcine reproductive and respiratory syndrome and porcine circovirus type 2 (Dewey *et al.*, 2006).

The origin of the plasma has great influence on the growth promoting properties of SDP, porcine plasma was reported to be more effective (Owusu-Asiedu *et al.*, 2002). Reduced extent and severity of diarrhoea, decreased ETEC shedding by feeding SDPP to piglets challenged with pathogens was reported (Owusu-Asiedu *et al.*, 2003).Dietary inclusion of SDPP (6%) improved ADG and ADFI during the first 10 d after weaning while no significant effect were seen on G:F rate and on intestinal morphology (Zhao *et al.*, 2007).

Improved feed intake, due to high palatability of the diet and high digestibility of the protein, was considered the major contributing factor for the enhanced growth in animals fed SDP. Actually, since SDP contains from 15 to 20% of immunoglobulin, great part of the beneficial effects observed on growth performance are related to its immunological properties (Thomson, 1994; Pierce, 2005; Lallès *et al.*, 2007 and 2009). SDP was reported to lower pro-inflammatory cytokine expression in many organs and to decrease the immune cell density in the intestinal mucosa (Touchette *et al.*, 2002, Jiang *et al.*, 2000, Bosi *et al.*, 2004, Moretó *et al.*, 2008). SDPP fed to early-weaned piglets challenged with ETEC K88 enhanced growth performance, decreased intestinal mucosal damage and inflammation, reduced IgA anti-K88 secretion and pro-inflammatory cytokine expression in the gut (Bosi *et al.*, 2004).

Porcine plasma was found to contain specific *E. coli* F18 antibodies, and when tested in *E. coli* F18-challenged piglets, it improved growth and feed intake (Owusu-Asiedu *et al.*, 2002). In contrast, other studies on piglets fed a plasma source without specific

immunoglobulin against *E. coli* F18 showed that SDP impedes *E. coli* F18 binding to the enterocytes by receptor competition through a non-specific protection mechanism (Nollet *et al.*, 1999). SDP from pigs vaccinated against specific pathogens resulted in modest production advantages, compared to conventional SDPP, in piglets challenged with the same pathogen (Niewold *et al.*, 2007). This could suggest that non-specific mechanisms might play a role or that pre-existing antibodies might already be present in conventional SDPP as a consequence of 'natural' challenge (Lallès *et al.*, 2009).

The enhancement of growth performance is mainly attributed to the reduced inflammatory response in the intestine and to the maintenance of mucosal integrity that limited the pathogen colonization, due to its immunoglobulin fraction (Bosi *et al.*, 2004; Lallès *et al.*, 2009). The mechanisms by which SDP might counteract pathogen infectivity may include improvement of immunocompetence or reduction of pathogen adhesion to the mucosa by the immunoglobulins and glycoproteins present in plasma (Sanchez *et al.*, 1993; Van Dijk *et al.*2001).

Spray-dried plasma appears to have good characteristics to be a valid alternative to AGP: it reduces the overstimulation of the immune response allowing more of the available energy and nutrients to be used for growth and other productive functions rather than being diverted to support the immune response (Moretò and Perez-Bosque, 2009).

Organic acids

Potential alternative to antibiotic growth promoters are certainly organic acid and their salts. Organic acids were traditionally used as food preservatives to prevent, through pH depression, microbial contamination of raw materials (Partanen and Mroz, 1999; Canibe *et al.*, 2005). The antimicrobial activity of organic acids is due to their ability to change from undissociated to dissociated form, depending on the environmental pH. Undissociated acid can freely diffuse through the membrane of microorganisms into their cell cytoplasm, where the acid dissociation, due to the neutral pH, leads to a suppression of cell enzymes (decarboxylases and catalases) and nutrient transport systems (Lueck 1980). The efficacy of an acid in inhibiting microbes depends on its pKa value, which is the pH at which 50% of the acid is dissociated. Organic acids with higher pKa values are more effective preservatives and their antimicrobial efficacy is generally improved with increasing chain length and degree of unsaturation (Foegeding

and Busta, 1991). Limited digestive and absorptive capacity due to inadequate HCl production, insufficient pancreatic and intestinal enzymes activities, sudden changes in feed consistency and intake are involved in the post weaning gut disorders (Aumaître *et al.*, 1995; Cranwell, 1995). The stomach pH plays an important role in limiting the entry of bacteria into the gut. Furthermore, low gastric pH reduces the gastric emptying rate, allowing more time for protein hydrolysis in the stomach (Mayer, 1994). Lowering dietary pH by weak organic acids, such as benzoic, formic, fumaric, lactic, propionic, butyric acid and their salts was reported to improve growth performance, reducing intraluminal concentration of coliform bacteria and diarrhoea incidence in piglets (Partanen and Mroz, 1999; Canibe *et al.*, 2005; Lallès *et al.*, 2009). However, it was reported great variability on piglets growth performance response to organic acids, likely related to the type and dose used (Partanen and Mroz, 1999, Lallès *et al.*, 2009). A combination of 1% lactic acid and 1% formic acid added to a piglet's diet reduced gastric pH and the concentration of lactic acid bacteria and enterobacteria (Hansen *et al.*, 2007). Inclusion of 1.8% of K-diformate to a piglets starter diet had no effect on growth parameters and on gastric and intestinal pH, meanwhile a decrease of total anaerobic bacteria, lactic bacteria and coliforms was found in digesta and faeces (Canibe *et al.*, 2001). The efficacy of 6 organic acids in counteracting post-weaning diarrhoea syndrome was evaluated by Tsyloyannins *et al.*(2001), who found improved growth performance, reduced diarrhoea severity and K88 *E. coli* excretion in faeces, though mortality rate was not affected. Free Ca-formate supplementation in weaning diet fed to ETEC-challenged piglets improved post-challenge growth, feed intake, gain to feed ratio, reduced the days of diarrhoea and faecal excretion of total (but not K88-specific) *E. coli* and increased villous height in the small intestine (Bosi *et al.*, 2007). Torrallardona *et al.* (2007b) found that Ca-formate, fed to piglets challenged with *E. coli* K99, did not improve neither growth performance nor total *E. coli* count in ileum, but jejunal villous height was reduced.

Reduced feed intake of organic acid supplemented diets was reported (Eisemann and van Heugten, 2007). Microencapsulation is an important tool to improve acidified diets consumption, to avoid gastric absorption of organic acids, delivering them to the small intestine where they can exert the antimicrobial activity. A great advantage of this technique is to reduce the amount of acid use (Piva *et al.* 2007).

The beneficial effects of butyrate and its precursors on colon mucosa morphology were reviewed by Hammer *et al.* (2008). Sodium butyrate (SB) included (0.3%) to a starter

diet, fed to piglets for 2 weeks after weaning, improved feed to gain ratio, increased gastric dry matter percentage and induced large changes in microbial community composition of caecum and distal colon, depressing amilolytic bacteria growth (Castillo *et al.*, 2006; Manzanilla *et al.*, 2006).

Combined supplementation of tributyrin and lactitol to piglets diets increased growth performance and villous height in the jejunum, reduced histamine levels in jejunal and caecal tissues and caecal crypt depth (Piva *et al.*, 2002). Tributyrin alone had adverse effects on growth performance, with no influence on jejunal villus height, even if it reduced drastically caecal crypts depth; lactitol alone stimulated jejunal villous height and lactic acid level in the caecum while reducing caecal crypt depth (Piva *et al.*, 2002). However, the results obtained so far with oral supplementation of SB or its precursors, indicate that the outcomes are influenced by various factors such as dose, supplementation length, site of observation and age of the animals (Lallès *et al.*, 2009).

Phytogenic feed additives

Essential oils and oleoresins are claimed to exert antioxidative, antimicrobial and growth-promoting effects in farm animals (Windish *et al.* 2008). The mechanism of action of essential oils has not been clearly identified but it appears that the antimicrobial activity is related to changes in lipid solubility of bacteria cell wall (Stein, 2006). Essential oils appear to act synergistically with organic acids (Burt, 2004; Manzanilla *et al.*, 2009; van Dijk *et al.*, 2009).

Michiels *et al.* (2007) assayed the effects of thymol, eugenol, carvacrol and trans-cinnemaldehyde, alone or in combination, in *in vitro* simulations of the fermentation in the GIT. Trans-cinnemaldehyde was found to be the most successful against coliform bacteria, even at low doses, while carvacrol and thymol showed to have broad spectrum of activity and to act synergistically by reducing the lactobacilli population, whose development was not inhibited by trans-cinnemaldehyde. Si *et al.* (2006) demonstrated the high efficacy of carvacrol, thymol and eugenol against *Salmonella typhimurium* DT104, *E. coli* O157:H7 and *E. coli* K88, while little inhibition was found towards beneficial lactobacilli and bifidobacteria when they were incubated with pig caecal digesta.

However, data on swine evidenced a high variability of phytogenic additives on growth performance and intestinal morphology (Namkung *et al.*, 2004; Demir *et al.*, 2005; Jamroz *et al.*, 2006; Nofrarias *et al.*, 2006; Oetting *et al.*, 2006). A mixture of carvacrol,

cinnemaldehyde and *Capsicum* oleoresin, given at increasing doses to weaned piglets, reduced diarrhoea incidence, increased the intestinal lactobacilli and improved the lactobacilli/enterococci rate, while no significative influence was observed on growth parameters (Manzanilla *et al.*, 2004, Castillo *et al.*, 2006, Nofrarias *et al.*, 2006). Walter and Bilkei (2004) reported beneficial effects of carvacrol (180 ppm) on piglets weight daily gain and feed conversion rate. Furthermore, the percentage of CD4$^+$, CD8$^+$ and MHC-II$^+$ was increased in peripheral blood after carvacrol feeding. These findings suggested a regulatory role of essential oils in the immune response.

In vitro studies revealed that carvacrol can protect human lymphocytes and other cells from oxidative stress, even if it is cytotossic at high concentration (Aydin *et al.*, 2005; Horvathova *et al.*, 2006). Treatment of porcine intestinal epithelial cells (IPEC-1) with >5 mM of carvacrol caused disruption of the epithelial barrier function (Roselli *et al.*, 2007). Bimczok *et al.* (2008) reported a reduced *in vitro* pig lymphocyte proliferation after exposure to carvacrol, likely due to apoptosis. Manzanilla *et al.* (2006, 2009) and Nofrarias *et al.* (2006) found a diminished number of intraepithelial lymphocytes in the jejunum of pigs fed a diet containing phytogenic feed additives.

According to Michiels *et al.* (2008), the absence or the reduction of the antimicrobial properties of essential oils in *in vivo* trials might be due to the low doses used and to the early absorption in the upper part of the GIT. Moreover, diet composition, including fat, protein, carbohydrates and water play a key role in the antimicrobial activity of essential oils (Si *et al.*, 2006, Manzanilla, 2009).

Prebiotics and Probiotics

A probiotic is defined a non-pathogen microorganism able to colonize the gut and prevent the outbreak of intestinal disorder. According to Gibson *et al.* (2004), a prebiotic is "a selectively fermented ingredient that allows specific changes both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host wellbeing and health". Inclusion of fermentable carbohydrates and/or probiotics to the diet of weaning piglets is a way to improve the microbial balance in both the small and large intestines (Williams *et al.*, 2001; Bauer *et al.*, 2006). The addition of sugar beet pulp, inulin, lactulose and wheat starch, was found to significantly affect microflora composition in the gastrointestinal tract of piglets (Konstantinov *et al.*, 2004a). As reported by Konstantinov *et al.* (2004a, 2006a), inclusion of fermentable carbohydrates in the diet could enhance colonic microbial stability and diversity, while

stimulating the growth of *Lactobacillus sobrius*, a beneficial member of the porcine commensal microbiota. Using chaperonin-60 gene clone libraries, it was shown that *L. amylovorus*-related populations, likely *L. sobrius*, are highly abundant in the ileum of weaned piglets fed either corn–barley or wheat-based diets, even if with high individual variation (Hill *et al.*, 2005). Loh *et al.* (2006) found that the inclusion of inulin to different basal diets significantly affected the proportion of piglets with detectable levels of bifidobacteria, while the lactobacilli population was not affected.

After weaning, since the lactase activity is reduced, lactose can act as prebiotic for microbial fermentation. High lactose level in weaner diets improved bifidobacteria and lactobacilli populations while decreasing *E. coli*, increased volatile fatty acids (VFA) and butyric acid and reduced branched VFA (Pierce *et al.*, 2006, 2007). However, the beneficial effects of lactose are influenced by the diet protein level and inulin supplementation.

Van Nevel *et al.* (2005) found that the addition of galactomannans to piglets diet caused decreased numbers of lactobacilli in stomach and jejunum feeding guar gum, while carob tree seeds increased *E. coli* counts in distal jejunum and in jejunal mucosa.

Fructo-oligosaccharides (FOS) are oligosaccharides, mainly of plant origin, that have been shown to resist to endogenous glycolytic enzymes of the host and to arrive unaltered to the colon (Oku *et al.*, 1984). FOS can significantly modulate the colonic microbiota by increasing the number of specific bacteria and thus changing the composition of the microbiota. With the combination of an oligosaccharide and a probiotic, the benefits include improved survival of the probiotic bacteria during passage through the upper intestine and a more efficient colonization in the colon, together with a stimulating effect of the oligosaccharide on growth and activities of both the probiotic and endogenous bacteria (Roberfroid, 1998).

At weaning, the attitude of bacteria to produce a range of compounds is useful to inhibit the pathogenic bacteria growth through the competitive exclusion (Diez-Gonzalez, 2007). Probiotics are claimed to enhance the population of beneficial microorganisms, by enhancing microbial enzyme activity and by improving diet digestibility and nutrients utilisation (Burgstaller *et al.*, 1984). Probiotics can positively affect gut microbiota balance, intestinal epithelium integrity, proper maturation of the gut associated tissue and the neuro-endocrine system function (Metzler *et al.*, 2005). A mix of four lactobacilli isolated from weaning pigs decreased diarrhoea, *E. coli* presence and anaerobes counts in the gut (Huang *et al.*, 2004). Dietary supplementation of *L. sobrius*

strain 001T to a diet based on fermentable fibre improved the body weight gain of weaned pigs orally challenged with ETEC K88, reduced ileal ETEC, but it had not effects on diarrhoea severity (Konstantinov *et al.*, 2005).

Supplementation with *Lactobacillus rhamnosus* GG, a "human" well-known probiotic, to weaning piglets challenged with ETEC, resulted in reduced growth and a trend to more ETEC excretion in faeces was observed (Trevisi, 2005). An interesting study on the interactions between intestinal physiology, diet supplementation with the probiotic *E. coli* strain Nissle 1917 and ETEC challenge has been conducted by Schroeder *et al.* (2006). Piglets were creep-fed during the suckling period, with a diet supplemented (or not) with *E. coli* strain Nissle 1917 for 10 d, starting from d 7 of age, weaned at 21 d of age and challenged with ETEC at 4 and 24 h post weaning. The probiotic used abolished diarrhoea, reduced jejunal secretagogue-induced chloride secretion and, compared to the non supplemented piglets, suppressed the decreased paracellular permeability usually seen after ETEC challenge (Schroeder *et al.*, 2006). High doses of *E. coli* strain Nissle 1917 increased the density of CD8$^+$ cells in the ascending colon while at low dosage there were no influence on the number and neither distribution of intestinal immune cells nor that of antimicrobial peptides (Duncker *et al.*, 2006).

Supplementation of piglets with *Enterococcus faecium* tended to decrease serum IgG levels after 20 d of treatment (Broom *et al.*, 2006). Interestingly, supplementing the sows with *E. faecium* strongly decreased the incidence of diarrhoea in piglets the first week post weaning (Taras *et al.*, 2006). It reduced the level of cytotoxic (CD8$^+$) T-cells in the jejunal epithelium of their piglets, probably in relation with the lower frequency of β-haemolytic and O141 serovars of *E. coli* (Scharek *et al.*, 2005).

Many mechanisms are involved in the probiotics-mediated intestinal epithelial cell protection against ETEC, including competitive exclusion, reduced ETEC adhesion, maintenance of epithelial tight junction integrity, reduced neutrophil transmigration and increased mucin gene expression, with great variability depending on the probiotic strain used (Roselli *et al.*, 2005a).

However, it is noteworthy that the data concerning the efficacy of probiotics are often contradictory and that bacteria strains that were supposed to be host competitor for nutrients are used as animal growth promoters.

Amino acids

Peptides and proteins are major constituents of body tissues. Several essential and non-essential amino acids are considered to have therapeutic effects on the GIT and on the whole organism (Kim *et al.*, 2007). Specific amino acids can promote health by improving (gut) tissue anabolism, by reducing the impact of stress and by modulating local immunology (Lallès *et al.*, 2009). Experimental results of administration of amino acids with biological activity to diseased animals suggested that the immune system requires a specific amino acid profile, different from that for growth (Reeds *et al.* 1994). From literature, the amino acids that proved to affect physiology, immunology and metabolism of the animals are glutamine, tryptophan, arginine, cysteine and threonine (Lallès *et al.* 2009). Glutamine and glutamate are known to be a major energy source for intestinal epithelial cells and, when added to weaner diets, preserved the enterocytes turnover rate, due to improved mitotic processes and decreased apoptosis that resulted in higher villi (Domeneghini *et al.*, 2004, 2006). Glutamine was proved to stimulate the innate and the adaptive immune system in early weaned piglet, as shown by increased densities of macrophages and lymphocytes in the ileal mucosa (Domeneghini *et al.*, 2004). Glutamine supplementation resulted in lower blood cortisol on the first day after weaning (Zhou *et al.*, 2006). Koopmans *et al.* (2006) found that tryptophan reduced the weaning-associated damage of the intestinal mucosa, reduced blood cortisol and noradrenaline levels and preserved growth in weaned piglets submitted to immune stress. A standard weaning diet supplemented with 100 mg/kg L-tryptophan improved daily weight gain and feed intake of piglets during the first 4 days after *E. coli* K88 challenge (Trevisi *et al.*, 2008).

Alanine and glycine have been shown to stimulate the production of the so-called antisecretory factor protein (AF), improved growth performance and reduced the incidence of diarrhoea (Göransson *et al.*, 1993; Göransson, 1997). Cereals kernels submitted to a patented hydrothermal process were to stimulate the AF endogenous secretion and improve animals growth performance (Lange and Lönnroth, 2001).

The AF protein, derived from different sources, inhibited intestinal fluid secretion and inflammation in enterotoxin-challenged rat and pig intestinal loops (Lange and Lönnroth, 2001). The antisecretory factor is present in sows' colostrum and milk is transferred from the sow to the foetus across the placenta (Sigfridsson *et al.*, 1995). According to Lönnroth *et al.* (1988), there is a correlation between AF content in milk, its level in piglet's blood and incidence of diarrhoea in the litter, it was hypothesised

that AF could be a protection factor against diarrhoea in suckling piglets. In piglets plasma, AF activity has a cyclic variation and is sensitive to stress: at the weaning it declines, reaching the lowest level the third day after weaning (Lönnroth and Lange, 1988; Lange *et al.*, 1993). The onset of diarrheal disease is significantly correlated with the low plasma AF activity (Lange and Lönnroth, 2001).

Antisecretory factor as a potential health-promoting molecule in humans and animals

Abstract

The antisecretory factor (AF) is a protein secreted in plasma and other tissue fluids in mammalians with proved antisecretory and anti-inflammatory activity, its immunohistological distribution suggests a role in the immune system. The expression level and the distribution of AF protein are altered during an immunological response. Exposure to bacterial toxins induces secretion of AF in plasma, probably reflecting a natural defence mechanism to agents causing diarrhoea, thereby contributing to a favourable clinical outcome and disease termination. Increase of AF level in plasma by dietary means, such as hydro-thermally processed cereals (HPC), has been demonstrated in human and animals. Administration of HPC to patients affected by inflammatory bowel disease, gastroenteritis and Ménière's disease relieved symptoms and improved quality of life. A recent study showed the positive effect of HPC diet supplementation on prevention of the effects of exposure to low levels of blast overpressure in rats, reducing the extent of intracranial pressure increase and cognitive function impairment. AF-rich egg yolk powder improved health status in children suffering acute and chronic diarrhoea, reducing the frequency and increasing the consistency of stools. This kind of functional food could be used for prophylaxis in populations exposed to a high risk of morbidity and mortality caused by diarrhoea and as a complementary therapy in patients affected by chronic intestinal inflammatory disease to improve well-being. In pig husbandry AF-inducing diets, owing to their antisecretory activity and anti-inflammatory action, are suitable option as alternative to antibiotic growth promoters to counteract post-weaning diarrhoea.

Introduction

Antisecretory factor (AF) is a 41 kDa endogenous protein which was originally purified in human pituitary gland by Lönnroth and co-workers (1988) and its gene has been cloned and sequenced. It has been established that AF is synthesized in most tissues of mammalians and can materialize linked to the 26 S proteasome or free in the cytoplasm and/or nucleus (Davidson and Hickey, 2004; Lange, 1982; Lange *et al.*, 1999). AF was shown to inhibit enterotoxin-induced intestinal hyper secretion in both human and animal subjects. In a rat intestinal loop model, one picomole of purified AF caused a significant reduction of cholera toxin and *E. coli* toxin-induced secretion (Lange *et al.*, 1987). Rapallino *et al.* (2003) hypothesized that AF may act at the level of intestinal epithelial cells, by regulating the chloride homeostasis, thereby possibly counteracting excessive secretion of fluids in the intestinal lumen. The immunohistological distribution of AF suggests that this protein may play a role in the immune system as an anti-inflammatory agent. In rat jejunal loops, AF inhibited the inflammatory response caused by toxin A from *Clostridium difficile* (Björck *et al.*, 2000). Intestinal exposure to bacterial toxins or intake of specific dietary compounds has proved to enhance the antisecretory and anti-inflammatory activity of endogenous AF (Johannson *et al.*, 2008). Nowadays, diarrhoeal diseases remain a major global health concern, especially in the developing countries where the diffusion of infectious gastrointestinal pathogens is favoured by the poor hygienic conditions. The 4th Millennium Goal of the UN stated that between the years 1990 and 2015 childhood diarrhoea should be reduced by two thirds (United Nations, 2008). Recent reports state that childhood diarrhoea is responsible for 18% of all child deaths, it is estimated that 2 million children die each year due to excessive fluid loss, despite the introduction of oral re-hydration therapy (Grøndhal *et al.*, 2002; Bryce *et al.*, 2005).

Not only is diarrhoeal disease a problem in developing countries, but it remains an issue of wealthy countries, due to a high incidence of chronic gastrointestinal diseases.

Induction of endogenous AF through nutritional means, in addition to rehydration therapy and/or the pharmacological therapies, was proved to noticeably reduce fluid loss and bowel movements, resulting in an improvement of the patient's well-being (Bjork *et al.*, 2000; Zaman *et al.*, 2007). A patented hydrothermal process, similar to malting, allows the kernels, mainly wheat and barley, to reach an optimal concentration of specific amino acids and oligosaccharides, able to induce endogenous AF secretion

(Laurenius *et al.*, 2003). Included in the diet of patients affected by intestinal chronic inflammatory diseases the hydrothermally processed cereals (HPC) improved the illness symptoms, reducing daily bowel movements and tissue damage (Laurenius *et al.*, 2003). In livestock production, the efficacy of AF-inducing diets could be of great interest, especially as a tool to counteract post weaning diarrhoea syndrome in piglets. Indeed, wweaning is the most stressful event in piglets life, bred in intensive piggeries. Weaning is frequently associated with a reduced nutrient intake, severe growth check, impairment of the health status and to a higher susceptibility towards infections and gut disorders (Lallès *et al.*, 2009; Berkeveld *et al.*, 2009).

For more than 50 years, the use of antimicrobial growth promoters (AGP) in animal diets improved growth performances and prevented the side effects of early weaning practice. Increasing concerns about the risk of antibiotic resistance diffusion, associated to the large agricultural use of antimicrobials, led the European Union to a complete ban of AGP since January 1st 2006. As experienced by the Nordic countries, which banned the AGP during the '80s and the '90s, the main consequences of the elimination of AGP was an increased morbidity and mortality of animals, due to an increased incidence of infectious gastrointestinal and respiratory diseases.

This has led to intensify the research to find alternatives to in-feed antibiotics.

AF-inducing diets seem to be interesting alternatives to AGP and a useful tool to improve livestock efficiency, animal health and welfare.

The aim of this review was to summarize the information about the antisecretory factor protein and its derivatives, evidencing the beneficial effects of AF-inducing diets on both human and animal health and welfare.

The antisecretory factor molecule

Genetics

The AF protein was first purified in rodents extracts of the pituitary gland and intestinal mucosa and later in pig blood.

AF protein is evolutionary highly conserved and it appears to be a unique protein since no family of AF-like proteins has been identified (Lange and Lönnroth, 2001).

Genes coding for AF protein are present in all mammalians and homologous of AF genes were found only in eukaryotic organisms. In the human genome, potential AF

genes are expressed in the chromosomes 1, 19 and 23, with pseudogenes located on chromosomes 10 and 15.

The sequence responsible for the antisecretory activity is present only in mammalian proteins while in others eukaryotic organisms the proteins codified by the AF genes act as regulators of the proteolytic degradation in the proteasome (Lange and Lönnroth 2001).

AF protein from plasma is structurally different from the one obtained by the pituitary gland, Lange and Lönnroth (2001) showed the first is polymeric, while the second is monomeric.

Active site

Post translational modifications of the AF, such as proteolysis, glycosylation, phosphorilation, or conformational changes, could result in exposure or hiding of active sites, essential for the exertion of the biological activity (Jennische *et al.*, 2003).

Trypsin digestion of the AF protein enhanced two fold the capability to counteract the cholera toxin-induced intestinal secretion, indicating that *in vivo* AF might be activated by hydrolysis (Johansson *et al.*, 1997b). The amino acids sequences derived from recombinant human AF trypsin digestion, were tested in a rat intestinal loop model in order to identify the active domain of the protein. The antisecretory activity of the truncated forms was related to the presence of a specific amino acid sequence between residues 35' and 50' in the N terminal part, the antisecretive potency was found to be dose dependent. According to Johansson *et al.* (1997b), the active region of mammalian AF is likely exerted by a small peptide of 8 amino acids (35-IVCHSKTR-42). Compared to the other peptides with antisecretory activity, AF appears to be the most potent, since somatostatin is active at nanomole and enkephalines at micromole concentrations, while AF is active at picomole dosages. Indeed, only 10^{-12} mol of AF are needed to revert cholera toxin-induced hypersecretion in a rat intestinal loop model.

The smallest peptide with antisecretory and anti-inflammatory properties is the AF-16, a 16 amino acid-long comprising the sequence VCHSK TRSNP ENNVG L, synthesized with solid phase technique (Johansson *et al.*, 1997b).

Receptors

AF is present in most body tissues, it can be found in free form in the cytoplasm and/or nucleus or linked to the 26 S proteasome (Lange *et al.*, 1999).

Bioinformatic models suggest that the AF protein has a von Willenbrand-like motif (vWm) in the N terminal part, between residues 5-188, which contains the antisecretory sequence (Johansson *et al.*, 2008). The vWm proteins are protein binders, Johansson *et al.* (2008) hypothesized that AF might exert its biological activities by binding to the receptor with its N terminal part.

With the C terminal part, AF binds to polyubiquitin, an intracellular protein, via 2 sites localized between residues 211-230 and 282-301, without involving the vWm. This interaction is probably involved in the regulation of the proteolytic processes in the proteasome.

Sections from rat brain, double labelled with antibodies against AF and flotillin-1, showed that there is a high degree of co-localization between AF and flotillin-1 in neurons and plexus choroideus (Johansson *et al.*, 2008). Dot-blot analysis evidenced that AF interacts with flotillin-1 by the means of its N terminus, carrying the anti-inflammatory and anti-secretory active site.

The flotillin protein family is phylogenetically well preserved and consists of two proteins, flotillin-1 and flotillin-2 (Vassileva *et al.*, 2008). Flotillins are ubiquitarious and are known to have a role in endocytosis, participate in signaling, regulation of the actin cytoskeleton reorganizations and actin-dependent cell adhesion and motility (Langhorst *et al.*, 2005; Babuke and Tikkanen, 2007).

Flotillin-1 is an integral membrane protein component of the lipid rafts, specialized cell membrane micro-domains enriched in cholesterol, sphyngolipids and gangliosides (Johansson *et al.*, 2008; Allen *et al.*, 2007; Rossy *et al.*, 2009). In the lipid rafts there are proteins of key importance for cohesion and signal transduction such as receptors, cytoskeletal contacts and ion channel complexes (Johansson *et al.*, 2008).

The lipid rafts appear to play a key role in the assembly of signaling processes (Katoh *et al.*, 2009) and to mediate many crucial cellular processes as endocytosis, oxidative stress, apoptosis, ion homeostasis and protein membrane trafficking and turnover (Morgan *et al.*, 2007; Allen *et al.*, 2007). Though lipid rafts make up a small percentage of the cell membrane, their high concentration of molecules involved in cellular signaling makes them a natural target for bacteria and viruses (Van der Goot and Harder, 2001). Cholera toxin attaches the intestinal epithelium by binding to ganglioside GM1, which is localized in the lipid rafts as flotillin-1 (Li *et al.*, 2003). Johansson at al. (2008) suggested that the interaction between AF and Flotillin-1 could affect secretory

processes by regulating the localization of signal proteins, such as receptors, in the lipid rafts.

AF and flotillin-1 are also co-localized in the central nervous system (CNS) and their interaction might affect the AF modulation of glutamatergic and GABAergic synaptic transmissions (Johansson *et al.*, 2008).

Role of AF in the immune system

In situ hybridization and immunohistochemical analysis demonstrated that AF is expressed in various mammalian organs (Lange *et al.*, 1999).

Monoclonal TLD antibodies, obtained from rats immunized with cultured microglial cells, were screened by Davidson and Hickey (2004a) with the aim to investigate the roles of perivascular and microglia cells in the inflammatory processes in the CNS.

TLD-1A8A is a monoclonal antibody that appears to specifically recognize the AF molecule cloned from rat microglia; administration of TLD-1A8A in T-cell proliferation or mixed-leukocyte response assays increased T cells proliferation (Davidson and Hickey, 2004b).

T-cell proliferation assays performed with rat serum containing AF showed that the antisecretory factor blocked the TLD-1A8A effect on T-cells, suggesting a role for AF in the regulation of T cell responses (Davidson and Hickey, 2004b).

However, the effects on the immunological response observed *in vitro* did not clarify the nature of the interaction between AF and its antibody. Davidson and Hickey (2004b) hypothesized the existence of a receptor for AF on T cells surface which might be disrupt by the antibody, blocking the normal suppressing signal. Alternatively, the binding of T cells membrane-associated AF to the antibody might activate the antigen presenting cells determining T cells proliferation (Davidson and Hickey, 2004b). Immunohistochemical analysis performed on rat tissue sections, treated with TLD-1A8A, evidenced that in lymphoid organs such as spleen, thymus and lymph nodes, AF was highly expressed by macrophages and cells with dendritic morphology. In the gut, AF was expressed by scattered macrophages in the connective tissue of the villi and particularly in the Peyer's patch, important in the immune surveillance of the intestinal tract. In the CNS, AF was found in blood vessels and perivascular cells, a bone marrow derived cell population important initiator and regulator of inflammatory reactions

(Hickey, 2001; Davidson and Hickey, 2004a). Davidson and Hickey (2004b) observed that macrophages expressed AF even in absence of a direct immunological stimulus, even if the activation of T-cells induced an up-regulation of AF expression in macrophages.

The immunological stimulus, due to experimentally induced autoimmune encephalomyelitis (EAE) in rats, modified the AF expression level and distribution in the inflamed nervous system, where the expression was enhanced in parenchymal microglia and infiltrating macrophages (Davidson and Hickey 2004b). In inflamed spinal cords, the mRNA expression showed a particular kinetics, with a drop at the onset of the clinical symptoms, a peak in coincidence with the highest disease severity and a decrease at baseline levels in correspondence with the complete recovery of the rats (Davidson and Hickey 2004b). Despite the accumulation of AF-positive leukocytes in the CNS during the course of the disease, no overt changes were apparent in either expression levels or distribution of AF in peripheral organs, such as spleen and lymph nodes. Since AF levels are at almost undetectable levels on circulating monocytes, Davidson and Hickey (2004b) suggested a mechanism whereby AF is upregulated on these cells at the area of inflammation upon macrophage activation and/or as a result of interaction with activated T lymphocytes.

Pro-inflammatory factors such as LPS, IFN-γ up-regulated AF expression in rat alveolar macrophages only 72-96h after the treatment. The delayed kinetics was attributed by the Authors to the anti-inflammatory effect of AF, the AF up-regulation was associated with a redistribution of the protein within the cell, mainly in the cytoplasm and in the cell surface.

Administration of the anti-AF antibody TLD-1A8A to rats with induced EAE increased the severity of clinical symptoms and the duration of the disease, with up-regulation of pro-inflammatory cytokines expression as IL-18 and IL-6 and decreased anti-inflammatory IL-10 level.

Davidson and Hickey (2004a, 2004b) hypothesized that the increased AF expression during the course of EAE could be a means of counteracting the pro-inflammatory environment and limiting the tissue damage.

The effects of AF on the nervous system

The amino acids γ-aminobutyric acid (GABA) and glutamate are, respectively, the most common inhibitory and excitatory neurotransmitters in the CNS (Galofrè *et al.*, 2009). Once in the synapse, they act on ionotropic receptors of GABA ($GABA_A$) and glutamate (Galofrè *et al.*, 2009). In mammals, overstimulation of $GABA_A$ activity results in central depression, whereas inhibition of $GABA_A$ receptor activity leads to general excitatory symptoms and convulsions (Madsen *et al.*, 2008; Medina and Chudotvorova, 2006). Excitotoxicity occurs when ionotropic glutamate receptors are excessively activated and it is characterized by excitatory symptoms and degeneration of neurons (Leist and Nicotera, 1998; Onley, 2002).

GABA exerts its effect on both CNS and enteric nervous system by increasing the Cl^- conductance of the cell membrane.

AF is expressed in the CNS and repeated challenges of rat intestine with cholera toxin increased the AF expression (Lange *et al.*, 1985).

In vitro studies, with isolated Deiters'cell membrane from rabbit's glia, advanced the knowledge about the effects of AF and its mechanism of action on the nervous system.

AF, purified from pig pituitary gland and its peptides, were shown to be potent blockers of Cl^- and GABA permeation across isolated rabbit Deiters'cell membrane in a dose-dependent fashion (Lange *et al.*, 1985 and 1987; Rapallino *et al.*, 2003). The AF concentration causing the half maximal inhibition was found to be $3 \cdot 10^{-10}$ M, more than a thousand time lower than the met-5enkephalin, an opioid messenger considered one of the most potent inhibitor of GABA membrane permeation *in vitro* (Lange and Lönnroth, 2001).

AF might exerts its effect on the post synaptic stage since i*n vitro* studies evidenced that AF binding to the Deiters cell membrane is reversible while AF had no effect on ^{3}H-GABA transport in integral cells (Lange *et al.*, 1985).

Nipecotic acid and bicuculline, respectively blocker of carrier mediated transport of GABA and selective blocker of $GABA_A$ receptor, abolished the AF effects on the neuronal membrane, while picrotoxin improved the AF inhibitory effect on Cl^- permeation (Lange *et al.*, 1987).

Kim *et al.* (2005) suggested that AF acts as a regulator of the chloride homeostasis, thus possibly counteracting an excessive secretion of fluids in the intestinal lumen.

AF protein is expressed in rat hippocampus and can pass the blood-brain barrier (Kim *et al.* 2005). Extra-cellular application of exogenous AF peptide to hippocampal slices resulted in a 40% reduction of GABAergic transmission while glutamatergic one was not affected (Kim *et al.*, 2005). The same effect was also observed in hippocampal slices from rats immunized with cholera toxin administration or fed a diet containing hydrothermally processed cereals. To explain the AF mediated effect on GABA transmission, Kim at al. (2005) hypothesized that AF might decrease the postsynaptic response by an internalization of $GABA_A$ receptors and subsequent degradation in the proteasome.

The enteric nervous system, enclosed in the wall of the gastrointestinal tract, regulates the motility and fluid transport, mainly without conscious control (Gwynne and Bornstein, 2007). Intestinal secretion is controlled by local reflexes which likely involve GABAergic neurons. When binds to $GABA_A$ receptors, GABA has a depolarizing and exciting effects on the enteric nervous system (Krantis, 2000). *In vitro* studies evidenced an inhibitory effect of GABA on motor activity of the guinea pig distal colon, inducing a relaxation in smooth muscle cells (Minocha and Galligan, 1993; Bayer *et al.*, 2002). Two AF peptides, differing in lengths and molecular weight and containing the same antisecretory domain, were tested to investigate the role of AF on colon contractions in guinea pig. Both peptides increased the contractions frequency, though at different levels, likely due to conformational differences that might have influenced the interaction with $GABA_A$ receptors (Harrison *et al.*, 2004). The Authors suggested that the positive effect of AF on the colon contraction frequencies during pathological conditions could be a way to protect the mucosa from noxious agents (Harrison *et al.*, 2004).

If the antisecretory factor acts as a negative modulator of GABAergic transmission also in the gut, it may counteract hypersecretion by decreasing the activity in secretory reflexes (Kim at al., 2005).

AF-inducing Feed in Animal Nutrition

Intestinal fluid accumulation can be caused not only by bacterial enterotoxins or neurohormones but even by hyperosmotic solutions. Intestinal secretion induced by the intake of solutions enriched with particular sugars, such as mannose and sorbitol, and

amino acids as glycine and alanine, triggered AF production in rat and pig pituitary glands (Lönnroth and Lange, 1987). The concentration of monosaccharides and amino acids used were low, not able to induce clinical and subclinical diarrhoea.

Cereals such as wheat, barley, rye, oats, rice, corn, millet, durra and sorghum, subjected to a patented hydrothermal process, similar to malting, achieve a determined content of sugars and amino acids that, once fed to animals or humans, triggers AF endogenous secretion. According to the patent (IPC8 Class: AA61K4700FI; USPC Class: 424439) the hydrothermally processed cereals can be defined as healthy and fresh kernels that has been subjected to malting. The hydrothermal process means that the grain kernels are steeped and thereafter are allowed to germinate at a carefully controlled water content and temperature until its sprout germs have developed. The germinated kernels are dried and desprouted. The drying can be driven so that the enzyme activity is changed to a more or less extent. The product then obtained is malt. The nutritive substances of the kernel have then, to a restricted extent, been hydrolysed and the enzymes of the sprout have been activated. This partial hydrolysis also facilitates the attack of the endogenous enzymes of the digestive system on the nutritive substances. It is obvious that a certain precooking or heat treatment also can increase the hydrolysis rate.

As shown in Table 1, the hydrothermal process increases the glucose level from 0.3 mg/g to up to 5.3 mg/g while for fructose, sucrose and maltose the concentration values are respectively raised up from 0.3, 8.4 and 0 to 3.1, 65.7 and 9.0 mg/g. The concentration of specific amino acids such as lysine, histidine, glutamic acid, tryptophan and isoleucine were also increased by the hydrothermal process (Bjorck *et al.*, 2000). Since the European ban of in-feed antimicrobials the reduction of post-weaning diarrhoea (PWD) incidence is a major goal for the pig industry (Castillo *et al.*, 2008).

The Swedish government ban of AGP in 1986 prompted the research for the development of postweaning feeds antibiotics-free for piglet nutrition, that were economically and ecologically competitive (Lange and Lönnroth, 2001). According to Göransson *et al.* (1993, 1997), AF-inducing diets could be an important alternative to AGP and a useful tool to improve livestock efficiency and animal health and welfare.

AF is present in sows colostrum and milk and there is a correlation between AF content in milk, its level in piglet's blood and the incidence of diarrhoea in the litter (Lange *et al.*, 1988). AF is transferred from the sow to the foetus across the placenta (Sigfridsson

et al. 1995). In piglets AF activity in plasma has a cyclic variation, it declines at weaning, reaching the lowest level the third day post weaning (Lange *et al.*, 1993). The onset of diarrheal disease is significantly correlated to the low plasma AF activity (Lange and Lönnroth, 2001).

Table 1. Content of sugars and aminoacids in cereals before and after the hydrothermal process. From Björck *et al.* 2000).

Content	Before process (mg/g DM)	After process (mg/g DM)
Glucose	0.3-0.4	0.6-5.3
Fructose	0.3-0.4	0.6-3.1
Sucrose	8.4-14.4	36.8-65.7
Maltose	0.0	4.0-9.0
Histidine	0.0	0.06-0.25
Glutamic Acid	0.12-0.20	0.42-0.44
Lysine	0.03-0.06	0.15-0.29
Tryptophan	0.09-0.22	0.28-0.45
Isoleucine	0.0	0.05-0.30

Lönnroth and Lange (1988) found that the AF production is stress sensitive. In rats, experimentally induced stress or injection of ACTH caused a rapid decrease of AF content in both plasma and pituitary gland. The physiological drop of AF activity at the weaning can be inhibited if piglets are fed an AF inducing diet (Lange and Lönnroth, 2001). As shown in Table 1, piglets fed a diet with optimised sugars and amino acids content had increased AF activity in plasma, reduced diarrhoea incidence and improved growth performance: daily weight gain increased from 11.3 to 33.8%, meanwhile post-weaning diarrhoea incidence decrease of 59.09% at least (Göransson *et al.*, 1993).

Table 2. Effects of AF-inducing diet on piglets growth performance, plasma AF content and post-weaning diarrhoea (PWD) incidence. Farm 1-5 from Göransson *et al.*, 1993; farm 6 from Ulgheri *et al.*, 2009.

Farm	n° pigs	ADG, g 0-35d		AF, units/ml plasma		PWD, %	
		C	AF	C	AF	C	AF
1	303			0.3	0.92	60	17
2	169			0.31	0.91	66	27
3	40	222	247	0.42	0.87	35	10
4	54	266	325	0.79	1.05	15	4
5	325	284	380	0.76	0.94	31	2
6	144	351	378	-	-	-	-

An AF-inducing diet, made up with a milk substitute enriched with sugars and amino acids, was shown to be an effective growth promoter in calves, but only if reared in poor hygienic conditions (Lange and Lönnroth, 2001).

Eggs are rich of AF, localized mainly in the yolk (Lange *et al.*, 1994). According to Lange et al. (1994) the large amount of AF in the yolk could provide the chicken a kind of protection against gastrointestinal diseases until the animal capability to actively ecrete endogenous AF is restored, indeed the blood concentration of AF in chickens decreased from the hatching and start increasing at 21 days of age (Lange *et al.*, 1994). As in pigs, the blood AF concentration in chicken is stress sensitive. The egg yolk powder from hens fed HPC is rich of the antisecretory factor protein that has proved in clinical studies to abolish diarrhoea in children affected by both acute and prolonged diarrhoea and to improve the symptoms in patients affected by irritable bowel diseases (Zaman *et al.*, 2007; Eriksson *et al.*, 2003a and 2003b).

The exact mechanism of action of AF at the cellular level remains unclear. Laurenius et al. (2003) suggested that the hydrothermal processing of the cereals might causes an un-masking of epitopes, which are not exposed in non-processed cereals and which are possible ligands for binding sites in the intestinal mucosa (Laurenius et al., 2003).

AF and gut health

In healthy small intestine villi absorb nutrients, while crypts secrete water and electrolytes. The main constituents of intestinal secretion are mucus, enzymes, water, Na^+, Cl^-, HCO_3^- and immunoglobulins A.

Intestinal secretion is necessary to obtain a chyme with concentration of nutrients, water and electrolytes optimal for absorption processes and it also might have a function in the homeostatic mechanism as the control of fluid and acid/base balance in the body (Wapnir and Teichberg, 2002). In pathophysiological conditions intestinal secretion protects the mucosa by washing away and diluting the harmful substances (Lundgren, 2002).

During gastrointestinal diseases, regulatory mechanisms of secretion are altered and inflammation and diarrhoea, caused by either infectious or non infectious etiologies, are the result of changes in fluid and electrolytes transport in the small and/ or large intestine (Fordtran, 1967).

Enteric pathogens that cause the most severe diarrhoea diseases are rotavirus, *Vibrio cholera*, *Shigella* spp, *Salmonella* spp, and enterotoxigenic *Escherichia Coli* (Petri *et al.* 2008). The intestinal hypersecretion caused by *V. cholera* toxin and *E. coli* labile toxin (LT) has been deeply studied in experimental animals. Cholera toxin (CT) induced diarrhoea has been considered a prototype of enterotoxin-caused diarrhoea, because it does not cause histological changes in the intestine despite substantial rates of net fluid secretion (Field *et al.*, 1972). Both CT and LT are heterohexameric molecules which consist of a single A-subunit and five identical B-subunits (Fan *et al.*, 2004). Despite their similarities, CT and LT induce secretion by different mechanisms at level of receptors and transductional pathways involved in the secretory response (Griffiths and Critchley, 1991; Mourad *et al.*, 1995).

Both cholera toxin and *E. coli* LT attach to GM1-ganglioside via the B-subunit and the binding itself triggers the toxin internalization. Inside the cell, A-subunit catalyses ADP-ribosylation of the Gs-subunit of adenylate cyclase, increasing mucosal cAMP level (Moss *et al.*, 1981). CT and cAMP stimulate active Cl^- secretion by activating or inserting Cl^- channels into the apical membrane of the crypt cells and inhibit electroneutral NaCl absorption by decreasing the activity of Na^+/H^+ and Cl^-/HCO_3^- exchange in villous cells, without interfering with the glucose stimulated Na absorption (Petri *et al.*, 2008). Studies with fish TTX (tetrodotoxin) showed that CT interacts with the enteric nervous system, inducing the secretion of neurohormones as 5-hydroxytryptamine (5-HT) and vasoactive intestinal peptide (VIP) (Cooke *et al.*, 1994; Cassuto *et al.*, 1981).

LT is the major virulent factor of ETEC (Holmgren and Svennerholm, 1992); it was found that LT binds to several brush border galactoproteins only weakly recognized by CT, and was suggested that binding of LT to this additional receptors might have a role in the intestinal secretion pathophysiology (Mourad *et al.* 1995). Recent studies demonstrated that, beyond its ability to bind to different glycolipids on enterocytes surface, LT binds to lipopolysaccharides (LPS) on the surface of *E.coli* (Horstman and Kuehn, 2000). As a result of the LT-LPS surface interaction, more than 95% of secreted LT is found associated with *E. coli* outer membrane vesicles, rather than being secreted in a soluble form (Horstman and Kuehn, 2000). LT is able to mediate the internalization of intact outer membrane vesicles into host cells (Kuehn and Kesty, 2004). In addition, competitive binding assays and microscopy with fluorescently labeled ETEC vesicles revealed that GM1 and LPS binding sites are distinct since binding process can occur in

the same time (Horstman and Kuehn, 2000). In cultured human intestinal cells, a third binding substrate for LT are the sugar residues of the receptor molecule of human blood group A antigen (Holmner *et al.*, 2007).

According to Mourad *et al.* (1995), LT induces secretion without involving 5-HT, whose receptors antagonist had no effect on LT-induced fluid and electrolyte secretion.

Clostridium difficile Toxin A (CDA) has a marked pro-inflammatory effect on the intestinal mucosa causing fluid accumulation, inflammation and tissue damage. It binds to glycosphingolipids receptors and is internalized in the enterocytes (Lönnroth *et al.*, 2003). Inside the cell, toxin A exerts enzymatic activity, by catalysing glucosydation, triggering the secretion of multiple inflammatory mediators as substance P, histamine, nitrogen oxide and prostaglandin (Kelly *et al.*, 1998).

Rotavirus infection is one of the leading causes of infantile gastroenteritis. The virus infects the mature enterocytes that line the small intestine and, consequently, it impairs activities of intestinal disaccharides and Na^+-solute symports coupled with water transport. Rotavirus' protein NSP4 induces a calcium ion-dependent chloride secretion, disrupts SGLT1 transporter-mediated reabsorption of water, causes maldigestion of carbohydrates and likely activates the calcium ion-dependent secretory reflexes of the enteric nervous system. The malabsorption of nutrients in the intestine is associated with inhibited water reabsorption and can lead to diarrhoea and malnutrition (Lorrot and Vasseur, 2007).

Shigellosis is one of the major causes of diarrhoea-related morbidity and mortality in developing countries, transmitted through contaminated food and water as well person to person contact (Kotloff, 1999, Baldi *et al.*, 2009). Effects of *Shigella spp.* infection are inflammation, mucosal ulceration and bleeding of the gastrointestinal tract (Baldi *et al.*, 2009).

AF and enteropathogenic bacteria

The AF protein and its derivatives seem to be the most potent inhibitor of intestinal hypersecretion of various aetiologies. Indeed, less than a picomole of human recombinant AF (rAF) was shown to inhibit cholera toxin induced fluid secretion in a rat model (Johansson *et al.* 1997). Torres *et al.* (1991) suggested that AF is released as a response to the hypersecretion itself, rather than to the causative agents. Grøndal and colleagues (2002) tested different AF derived peptides on pig intestinal loops challenged with different secretagogues including CT, LT and 5-HT and found that AF

inhibited intestinal fluid accumulation in the proximal small intestine of CT-treated loops while there was no effect in the distal part.

In vivo experiments conducted with rats demonstrated that repeated exposition to CT causes a long-lasting over-expression of AF in the intestinal mucosa able to counteract the CDA-induced signal, thereby preventing intestinal inflammation and fluid secretion (Lönnroth *et al.*, 2003). The CT-induced tolerance to CDA might be mediated by the enteric nervous system (Lönnroth *et al.*, 2003). CDA causes the secretion of substance P that triggers the release of a wide range of chemical mediators by the mucosal enterocytes (Goyal *et al.*, 1996). In the gut, AF is highly expressed in the submucosal T-lymphocytes, suggesting a sort of immunological memory. The antisecretory activity of the AF protein and its derived peptides is usually estimated by *in vivo* assay based on graded inhibition of CT-induced secretion in intestinal loop model (Lange 1982; Lange and Lönnroth 2001).

In toxin challenged experimental animals, the AF antisecretory effect differs with the type of toxin and AF administration route. Human rAF administrated to rat, via penis dorsal vein or directly into the lumen of intestinal loop, just before or one hour after the *Cl. difficile* Toxin A challenge, reduced mucosal damage and fluid accumulation by 60-70% (Johansson *et al.*, 1997). Intravenous injection of rAF counteracted the intestinal hypersecretion but not the intestinal epithelial damage caused by okadaic acid (Johansson *et al.*, 1997).

The *in vivo* assay used to evaluate the AF activity in plasma is based on graded inhibition of CT-induced hypersecretion in ligated loops of the rat small intestine (Lange, 1982). As observed by Johansson *et al.* (2009), this method is reliable and reproducible, it is labor intensive, technically difficult, and furthermore, it requires a large number of experimental animals.

Polyclonal antibodies, raised against linear epitopes of AF proteins and its derived peptides, were used to investigate the possible changes in the protein structure responsible for its biological activities (Jennische *et al.*, 2006). These antibodies were used to screen the presence of conformational variants of the antisecretory protein in the pig central nervous system, though they were not able to discriminate between active and inactive AF form, a great inconvenient since most of the AF present in plasma is in an antisecretory inactive state (Jennische *et al.*, 2006).

A first step in the development of an *in vitro* assay, able to detect the AF active forms, has recently been made with the realization of an ELISA test. This *in vitro* assay has

been developed using monoclonal antibodies raised against human placenta AF (Johansson *et al.*, 2009). The efficacy of the ELISA test, compared with *in vivo* assay (ligated rat intestinal loop), was assessed by analysing AF activity in plasma samples from volunteers which ate the hydrothermally processed cereals. The plasmatic-AF values obtained by ELISA determination well correlated with the AF-activity as measured by bioassay with the intestinal loops ($r = 0.85$) (Johansson *et al.*, 2009).

AF and Irritable Bowel Disease

Inflammatory bowel disease (IBD) is a complex disorder, characterized by chronic inflammation of the gastrointestinal tract, abdominal pain or discomfort, associated with altered bowel habits (Ferguson *et al.*, 2007). The two main types of IBD are ulcerative colitis (UC) and Crohn disease (CD). UC is characterized by chronic mucosal inflammation and epithelial dysfunction in the large intestine, which causes open sores, ulcers and bleeding. The main symptom of UC is frequent bloody diarrhoea (Eriksson *et al.*, 2003a). UC causes inflammation and ulcers in the top layer lining of the large intestine while Crohn's disease can affect all layers of the intestine. CD can affect any area of the gastrointestinal tract, from the mouth to the anus, but it most commonly affects the ileum. The swelling extends deep into the lining of the affected organ, causing pain and frequent bowel movement.

IBD is currently considered as a bio-psychosocial disorder with disturbances of motor function, heighted gut sensitivity, and possibly central nervous system disturbances (Levy *et al.*, 2006). There is a genetic component in the incidence of IBD, since approximately 20% of people with one form of IBD have a blood relative with IBD (Ferguson *et al.*, 2007). Studies with twins showed that 36% of monozygotic twins share the disease, compared to only 4% of dizygotic twins (Jess *et al.*, 2005). The highest incidence rates and prevalence of IBD have been reported from northern Europe, the UK, and North America, though the rates are beginning to stabilize. On the contrary, rates continue to rise in low-incidence areas such as southern Europe, Asia and in most developing countries (Loftus *et al.*, 1998). Curative treatment for IBD has yet to be established, up until now therapeutic focus is on alleviating symptoms (Eriksson *et al.*, 2003a).

Bjorck *et al.* (2000) evaluated the effect of an increased endogenous AF secretion on the clinical outcome in patients suffering IBD. Patients with long lasting UC or CD received a diet containing either placebo cereals or HPC, without changing food habits

and conventional medical treatment. The outcomes were evaluated by analysing objective and subjective data. Every patient kept a diary to daily record number of bowel movements and stools consistency, clinical improvement or clinical deterioration was plotted as percentages on a visual analogue scale (VAS score) and expressed as percentage of change. The study demonstrated that a diet supplemented with HPC was able to induce an increase of AF in plasma of patients suffering irritable bowel syndrome (IBS). Increased AF level in plasma was associated to an improvement of patient's welfare, reduced frequency of bowel movement and solid stools. Rectal biopsies analysed by immunohystochemistry showed that HPC supplementation induced a larger number of AF positive cells compared to biopsies from the patients receiving the placebo cereals. However, a correlation was found between AF content in plasma and biopsies cells staining. No effects were observed on C reactive protein in plasma. The AF values in plasma declined slowly after the end of the experiment.

The influence of orally administrated AF, provided by egg yolk with a high AF content, was evaluated in a randomized double blind study which involved 20 patients suffering from acute onset of UC (Eriksson *et al.*, 2003). The study concluded that AF, used as a supplement to conventional pharmacological and nutrition therapies, can help to alleviate inflammation as shown by rectal biopsies. Passive administration of AF by the means of AF-rich egg yolk powder, followed by active induction through a diet supplemented with HPC, induced the regression of Crohn disease in a patient with long standing disease. The patient had a resected colon and was not responsive to conventional pharmacological treatment (Eriksson *et al.* 2003b). Another study by Eriksson *et al.* (2003a) was conducted on patients with acute attack of ulcerative colitis receiving AF or placebo treatment as a supplement to the conventional pharmacological and nutritional therapy usually performed. The AF therapy showed to have a positive effect on the inflammatory reaction of colon mucosa. Rectal biopsy analysis evidenced a reduction in the relative number of neutrophils and eosinophils granulocytes in the AF group but no effect on the intestinal secretion was observed.

However, Ekesbo *et al.* (2008) reported no additional beneficial effects of HPC diet supplementation in a placebo-controlled diet study with irritable bowel syndrome (IBS) patients that received placebo-cereals or active AF inducing cereals. It was noted that both groups' general conditions, measured by a VAS score, improved regardless of which diet was consumed, likely due to a good fibre amount of the diets, that may have had a bulking effect and reduce diarrhoea.

AF and short bowel syndrome disease

Short bowel syndrome (SBS) can appear after intestinal surgery, radiation injury or from loss of function due to vascular insufficiency. It can result in malnutrition and dehydration due to malabsorption of fluid, electrolytes, minerals and nutrients (Tilg, 2008). It is often associated with intestinal failure requiring parenteral nutrition, impairment of health related quality of life (HRQoL) and morbidity (Messing *et al.*, 1999; Nightingale, 2001).

Lange *et al.* (2003) performed a study on patients with resected intestine and found that the extent of AF activity and the ability to maintain a high level of plasmatic AF after HPC treatment is significantly correlated to the length of the remaining small intestine. The study found that a length of at least 100 cm of small intestine is required to induce an improvement of AF-activity by dietary means and the presence or absence of the large intestine did not appear to affect AF activity.

Pagoldh and colleagues (2008) conducted a double blind trial to investigate the effects of diet supplemented with HPC or with non processed cereals (NPC) on HRQoL in a heterogeneous group of patients affected by SBS of various etiologies, with different short bowel length (which had been subjected to different intestinal surgical techniques). NPC were more effective in reducing the daily faecal volume, especially in patients with ulcerative colitis, probably because of the high content in gel forming dietary fibre that improved the fluid absorption. The surgical techniques seemed to influence the clinical effects of the cereals supplementation (Pagoldh at al., 2008). Moreover, the effects of the diet supplementation were more evident in the last part of the trial, likely due to sensitization processes of the mucosa, which was independent from the type of cereals used in the first part of the trial.

According to the authors, an interesting approach to SBS would be a supplementary diet targeting increased viscosity of the intestinal content that could prolong the transit time and, as a consequence, improve the nutrient uptake (Pagoldh at al., 2008).

AF and endocrine diarrhoea

Endocrine diarrhoea is caused by an imbalance of specific hormones which inhibit water and electrolyte absorption and/or increase fluid secretion or stimulate motility. The most important hormones involved in the regulation of intestinal secretion are VIP, serotonin, substance P, prostaglandin E2, calcitonin, thyroxin, pancreatic polypeptide hormone (Dodi *et al.*, 1996).

Carcinoid syndrome and medullary thyroid carcinoma (MTC) can lead to an alteration of the hormones balance. Carcinoid syndrome refers to a range of symptoms that occur as a result of carcinoid tumours. Carcinoid tumours originate in the cells of the neuroendocrine system, could occur anywhere along the gastrointestinal tract and in the lungs and are characterized by production of serotonin (5-HT) (O'Toole *et al.*, 1999). The syndrome is characterized by endocrine induced diarrhoea with flushing and bronchospasm, caused by the tumours ability to secrete vasoactive peptides (Dodi *et al.*, 1996). MTC is a neuroendocrine tumor that develops in the thyroid C cells and occurs either as an erratic event or secondary to a germline mutation with an autosomal dominant pattern of inheritance (Kaltsas *et al.*, 2004). Thyroid C cells are responsible for production of both calcitonin and carcinoembryonic antigen (CEA) (Flemming et al,. 1999). Neuroendocrine tumours may induce severe persistent diarrhoea, sometimes therapy-resistant (Waangberg *et al.*, 1996). Laurenius *et al.* (2003) investigated the effects the AF therapy, AF-rich egg yolk powder or HPC, on patients suffering from endocrine diarrhoea due to midgut carcinoid syndrome or metastasizing MTC. This study showed that even though the severely ill patients were optimally medicated, several of them responded positively to the AF-therapy. Passive intake of AF had positive effects on the frequency of bowel movements, indicating that AF was still active after passing the upper gastro-intestinal tract. Compared to the passive AF-therapy, the HPC therapy required a larger intake volume, which was difficult to reach in severely ill patients (Laurenius *et al.*, 2003). Thus AF-rich egg yolk powder could be the best option for patients who are not able to ingest large amounts of food.

AF therapy: a tool to prevent child diarrhoea in developing countries?

As previously stated, diarrhoea remains a problem amongst children especially in developing countries, in some cases even life threatening. Severe malnutrition and

infections are the main cause of the children's premature and avertable deaths (Brown, 1990). Diarrhoea can be prevented by improving the hygienic conditions, through vaccination and encouraging mothers to breastfeed their infant (Mihrshahi *et al.*, 2008). Breast milk is easily digestible and contains antibodies that can protect the infant from bacterial and viral infections. Furthermore, it aids the development of the child's immune system. According to Ziyane (1999), exclusively breastfed infants under two months are 25 times less likely to die of diarrhoea related illness than non-breastfed infants. However, in many diarrhoea cases, breastfeeding alone does not suffice (Saleemi *et al.*, 2004).

In a study conducted in Pakistan on children suffering from acute and prolonged diarrhoea episodes, was found that when given B 221, a medical food based on AF-rich egg yolk powder, the incidence of diarrhoea subsided (Zaman *et al.*, 2007). The study, which lasted 3 days, involved 240 children from 6 to 24 months; every 5 hours they were randomly given 2 g of B 221 or placebo (regular egg yolk powder), dissolved in rehydratation solution. In children suffering from acute diarrhoea (less than 7 days of diarrhoea illness), the AF administration improved frequency and consistency of the stools, the clinical outcome after 3 days was positive for 83% of the children fed B 221, while the response to placebo was positive in only 54% of children. In children with prolonged diarrhoea, B 221 treatment resulted in a 91% successful clinical outcome, compared to the placebo group which only had a 63% success rate.

As evidenced by Zaman *et al.* (2007), it was of note that the medical food containing AF counteracted diarrhoea of different etiologies, since it was unlikely that the children involved in the study were infected by the same agent. Dosing and mode of administration of B 221 are still yet to be understood, but according to authors, higher dosage might be useful in the management of acute diarrhoea in children, specially in developing countries, avoiding the appearance of prolonged diarrhoea, with its threatening consequences.

Svensson *et al.* (2004) found that the AF can be induced in maternal milk by hydrothermally processed cereals intake. Furthermore, the AF-inducing diet prevented the mastitis, a painful infection and inflammation of the breast tissue, likely due to the anti-inflammatory properties of the antisecretory factor. Thus, HPC intake in lactating women might achieve the double goal to prevent mastitis in the mothers and diarrhoea in children.

AF therapy in Ménière's Disease

An increase in AF blood plasma has been found to reduce symptoms in patients suffering from Ménière's disease, a disturbance of the inner ear that can affect hearing and balance, characterized by episodes of vertigo, tinnitus and progressive hearing loss (Hanner *et al.*, 2004). It was first hypothesized that endolymphatic hydrops cause dilation of the cochlear duct affecting the endolymph drainage process. Recent findings suggest that impaired production or resorption of endolymph and/or transport across membranes, appear to be major factors in the pathophysiology of Ménière's disease (Hanner *et al.*, 2004, 2009). Dysfunctions of many structures of the inner ear and of the sympathetic nervous system are likely to be involved in creating the symptoms of this disease (Hanner *et al.*, 2004, 2009).

Experimental evidence about the role of the antisecretory factor as a regulator of fluid transport across the mucosal membranes and its anti-inflammatory properties, induced Hanner and colleagues (2004) to perform a pilot study on patients suffering Ménière's disease, in order to investigate the effect of increased AF plasma concentration on the frequency and extent of vertigo, which are the main symptoms of this disease. The study required that the patients received 1g/kg bw/day of HPC, divided into two to three dosages over 2-4 weeks. Patients were instructed to keep a diary in regards to their subjective symptoms, including frequency, duration of vertigo, fluctuations of hearing. Although the study was performed on a small number of patients (24), the results were interesting since in about 50% of patients the attacks of rotatory vertigo diminished or completely disappeared, with reduced final AAO-HNS functional level scale (America Academy of Otolaryngology-Head Neck Surgery, 1995; range 1 to 6) (Hanner *et al.*, 2004). Even if there was a positive correlation between plasma AF activity after the treatment and the reduction of vertigo (r=0.65, P< 0.001), the response to the diet was individual and not related to the severity of the symptoms (Hanner *et al.*, 2004). The findings of the study confirmed that AF-inducing diet could be useful in some Ménière's disease patients and also reinforced authors' idea that AF could be involved in the inner ear homeostasis. Following the success of the pilot study, Hanner *et al.* (2009), performed a double-blind study on a larger number of patients (n=51) for a longer period of time (3 months). Patients were randomly assigned to two groups: a control which received placebo cereals and the HPC group. In the HPC group, 14 of the 27 patients experienced a decrease in the symptoms, while in the control group only two

participants reported decreased vertigo severity (Hanner *et al.*, 2009). Once the double blind arm of study was concluded, the participants of the control group were offered the option to receive the active cereal treatment for further three months: 22 patients accepted, of these 17 had alleviated symptoms. According to Hanner *et al.* (2009), the results from the open arm of the study might have been influenced by the expectations of the patients. In both trials the feeding treatment had no effects on clinical otoneurological status of patients, assessed before and after the treatment by pure tone average and speech audiometry tests. Therefore, the HPC treatment exerted its effect only on the vertigo component of the disease. A clear explanation of the mechanism of action of AF remains unknown, however many experiments and clinical studies indicate that AF can normalize water and ion transport. It has been shown that AF interacts with the lipid raft protein flotillin-1, and they are both expressed in the inner ear of the rat (Hanner *et al.*, 2004), as well as the human cochlea and endolymphatic saccus (Jennische *et al.*, 2003). Hanner *et al.* (2009) suggested that the interaction between AF and flotillin-1 might cause a down-regulation or a re-organization of aquaporin channels, thus regulating the homeostasis of endolymph.

New insight on AF properties

Brain edema, haemorrhage, obstruction or derangement of the blood-brain barrier, can lead to increased intracranial pressure (ICP) that can damage brain function resulting in inflammation, with possible persistent neurological and psychiatric malfunctions (Vink *et al.*, 2003; Hoane *et al.*, 2006; Säljö *et al.*, 2009a and b). The extent of brain damage is related to the severity and length of ICP increase (Jennische *et al.*, 2008).
In human, blast related brain injuries are known to cause brain edema, vasospasm and intracranial haemorrhage (Armonda *et al.*, 2006). The current therapeutic options applied in counteracting elevated ICP are not indicated at pressure levels lower than 25mm Hg. However, less is known about the medium and long term effects of levels of ICP lower than the treatment threshold on physical, psychological function of patients (Jantzen, 2007). The effects of exposure to low and mild blast levels on brain injury is increasing of interest due to the involvement of large numbers of troops in the Afghani war, where soldiers are often subjected to traumatic brain injury without damage to the head (Säljö *et al.*, 2009). It was recently reported that in a rat model low blast

overpressure in a shock tube (10-30-60 kPa) impaired cognitive functions, assessed by the Morris Water Maze, and caused an elevated intracranial pressure in a dose dependent manner (Säljö *et al.*, 2009a). The same experiment was repeated with rats fed or not a diet supplemented with 20% of hydrothermally processed barley (Säljö *et al.*, 2009b). The study showed that the inclusion of hydrothermally processed barley in the rats diet prevented the impairment of cognitive function and drastically reduced the ICP elevation. The peak of intracranial pressure was reached 10 hours after rats exposure to both 30 and 60 kPa, though in rats exposed to 60 kPa there was a first smaller peak after 10 minutes. At 10 hours from the overpressure exposure, the mean ICP in rats of the control group subjected to 30kPa and 60 kPa raised respectively of 90 and 145%, while in rats fed hydrothermally processed barley the ICP increased of 60 and 50%, respectively. The feed treatments had no effect on the initial peak, recorded after 10 minutes from the exposure at 60 kPa. Säljö *et al.* (299b) suggested that the diet supplementation with hydrothermally processed barley reduced brain edema but the mechanisms behind this protective effect remain unknown.

In a rat experimental model of herpes simplex encephalitis, induced by type 1 virus (HSV-1), AF-16 peptide was used to suppress the raised ICP (Jennische *et al.*, 2008). The intranasal instillation of AF-16 peptide granted a complete rescue of rats receiving 25μg twice daily from the forth day after infection. Prevention of neurological malfunctions, usually associated with encephalitis, was found to be dose dependent. In rats receiving 1μg of AF-16 twice daily, starting immediately after the infection, there was a moderate mortality, while in animals of the untreated group the mortality was about 90% (Jennische *et al.*, 2008). The AF-16 peptide reduced the ICP increase and fluctuations, and also abolished the pressure peaks in treated rats. Jennische *et al.* (2008) suggested that the AF-16 mediated suppression of increased intracranial pressure in infected rats to non-injurious level is might be the cause of abrogated neurological malfunctions.

Table 3. A summary of the clinical outcomes obtained with the "AF-therapy".

Study	Study Population	Treatments	Results
<td colspan="4" align="center">**Diarrhoea of different etiology**</td>
Laurenius A *et al.* **2003**	Patients affected by midgut carcinoid syndrome or metasizing medullary thyroid carcinoma (MTC) Participants (n): 8	1st period: AF-rich egg yolk for four weeks 2nd period: Double-blind crossover period with HPC and control cereals (CC) for six weeks	1st period: 7 patients experienced about 25% decrease in number of bowel movements 2nd period: patients experienced increase solidity of stools.
Zaman S *et al.* **2007**	Children 6-24 months of age affected by acute diarrhoea and prolonged diarrhoea	Each patient was randomly given 2 g of either regular egg yolk or AF containing egg yolk (B221) every 5 hrs for 3 days along with an oral rehydration salt solution.	Administration of B 221 improved health in both groups but no statistical significance was found, most likely due to short experimental period.
<td colspan="4" align="center">**IBD-IBS**</td>
Erikkson A *et al.* **2003**	Patients suffering from acute ulcerative colitis Participants (n): 20	2g of AF-rich egg yolk drinks 4x daily for 14 days or placebo treatment in addiction to the standard pharmacological therapy	Results were not statistically different
Finkel Y *et al.* **2004**	Patient 1 (P1): 16 y old male prolonged tiredness, anemia, abdominal pain; Patient 2 (P2): 16 y old male diagnosed a left sided ulcerative colitis	54 g/d of HPC for a 2 week period in addition to medical treatment	P1 experienced an increase in hemoglobin, decrease of intestinal permeability and improvement of clinical symptoms P2 had a decrease in loose stools and improvement of general well being
Erikkson A *et al.* **2003**	1 patient suffering from Crohn's Disease	As supplement to the conventional medical therapy: AF-rich egg yolk powder 4x/d for 14 days. In addition on day 4 of treatment finely ground HPC administered (0.5g/kg bw/d) by day 10 ungrounded HPC given to patient (1g/kg bw/d)	Approximately 80% decrease of average daily stools
Ekesbo R *et al.* **2006**	Patients affected by IBS Participants (n): 62	100 g of HPC or placebo cereal a day	Overall improvement in symptoms irrespective of diet
<td colspan="4" align="center">**Short Bowel Syndrome**</td>
Pagoldh M *et al.* **2008**	Short Bowel Syndrome patients Participants (n): 26	Randomized double-blind, cross-over multicentre prospective study. Patients received HPC or placebo cereals then treatment was switched between group	Faecal volume significantly decreased after regular cereal treatment

Aim of the thesis

Nowadays nutrition is considered a possible tool to improve health and wellness in both human and animals. Foods like grains, tomatoes, milk, are gaining interest for the health promoting activity of their components such as β-glucans, lignans, lycopene or conjugated linoleic acid.

In pig intensive farming, stressful events related to premature weaning, have a negative impact on animals health and welfare. Indeed early weaning exposes the piglets to nutritional, social and environmental stresses when piglets immune and gastrointestinal systems are not fully developed (Partanen and Mroz, 1999).

For a long time, the employ of in-feed antimicrobials has been strategic in animal nutrition by reducing the detrimental effects of premature weaning on piglets health and growth performance.

Consumer and political opinions, and a scientific concern that resistance selected in animals might be transmitted to humans to the detriment of their health led to a complete ban of in-feed antimicrobials in 2006.

Several nutritional approaches and alternative substances have been proposed but, so far, none of these showed to be as effective as antibiotics on growth promotion and disease prevention (Lallès *et al.*, 2007, 2009). Among the alternatives so far proposed, the immuno-modulating substances are gaining interest, since the correct development of both innate and acquired immune system of the piglets is strategic in determining the outcomes of a large exposure to antigens and weaning-associated stress (Gallois *et al.*, 2009).

The antisecretory factor is present in sows' milk and colostrum, a protective effect of milk AF on offspring gastrointestinal disease has been hypothesized (Lange and Lönnroth, 2001). *In vitro* and clinical studies evidenced the AF (potent) capability to counteract intestinal fluid imbalance of various aetiologies, meanwhile recent reports focused on its immunomodulatory and anti-inflammatory properties.

AF-inducing diets, such as the hydrothermally processed cereals, appear to be possible candidates as replacers of antibiotic growth promoters.

However, the mechanism of action behind its biological activity has not yet been clarified and, so far, few data are available on pig field trials. Furthermore, compared to its use in human nutrition, the proper level of diet supplementation has not been defined.

A first goal of this thesis has been to summarize the information about the antisecretory factor protein and its derivates, evidencing the potential beneficial effects of AF-inducing diets on both human and animal health and welfare.

The main aim of this work has been to verify the efficacy of HPC diet supplementation as alternative to AGP in weaned piglets and evaluate the anti-secretory and anti-inflammatory activities of AF-16 peptide in *in vitro* models.

Effect of hydrothermally processed cereals (HPC) on the performance of weaned piglets.

Abstract

Antisecretory factor (AF) is an endogenous protein that has shown to be a potent inhibitor of intestinal fluid secretion and inflammation. AF content in sows' milk is important for protection against neonatal diarrhoea in suckling piglets. Feeding specific hydrothermally processed cereals (HPC) has proven to increase the plasma level of AF and to be helpful in counteracting diarrhoea in domestic animals. The aim of the study was to investigate the effects of an AF-inducing diet on piglets growth performance and intestinal mucosa.

144 weaned piglets with a body weight (BW) of 6.35 ± 0.52 kg were randomized for sex and weight and allotted to 3 groups fed ad libitum: C) control diet; T1) control diet with 3% supplemental HPC; T2) control diet with 6% supplemental HPC. On day 0, 14, and 42, animals were weighted, feed consumption and feed:gain ratio (FCR) were determined. Blood samples were collected (n=6 animals per treatment group) to determine the effect of HPC on the intestinal enterocytes. None of the piglet showed diarrhoea during the study.

Piglets fed the diet supplemented with 6% of HPC had higher (P<0.05) final BW compared to piglets fed the control diet. ADG was higher for the piglets fed the diet supplemented with 6% HPC 14 days after weaning (P<0.05) and during the whole experimental period (P<0.05). During the second growing phase (14-42 d) animals fed 6% HPC grew more than piglets receiving 3% HPC supplemented diet Piglets fed T2 had a lower FCR (P= 0.05) than piglets fed C. No differences were detected on feed intake and blood parameters. Plasma levels of I-FABP, a parameter of the intestinal health status, were low for all animals and did not significantly differ between treatment groups. *In vitro* digestion assays evidenced a relevant simple sugars content and higher rate of starch hydrolysation in the HPC diets compared to control diet. The difference in growth performance observed between groups might be related to a balanced supply of energy-yielding nutrients and amino acids in the diets. The findings of this experiment support the use of HPC as a natural alternative to AGP.

Introduction

The reduction of post-weaning diarrhoea is one of the main challenges for the pig industry after the ban on antibiotic growth promoters (AGP) in the EU (Castillo *et al.*, 2008).

Antisecretory factor (AF) is an endogenous protein that has shown to be a potent inhibitor of intestinal fluid secretion and inflammation and thereby counteracting dehydration induced by enterotoxins associated with post-weaning diarrhoea.

AF is transferred from the sow to the foetus across the placenta and is present in colostrum and milk. According to Lange *et al.* (1988) there is a correlation between AF content in milk, its level in piglet's blood and the incidence of diarrhoea in the litter (Lange *et al.*, 1988). In piglets, the AF activity in plasma has a cyclic variation and at the weaning it declines, reaching the lowest level the third day of weaning (Lange *et al.*, 1993). Lönnroth and Lange (1988) found that the AF production is sensitive to stress. According to Lange and Lönnroth (2001), the onset of diarrhoeal disease is significantly correlated with the low plasma AF activity and the physiological drop AF activity at the weaning moment can be inhibited, provided that the piglets eat an AF inducing diet.

Swedish researchers optimized a hydrothermal process, similar to malting, that modifies the availability of sugars and amino acids of cereals, able to stimulate AF endogenous secretion.

AF content in sows' milk could be a protection factor against diarrhoea in suckling piglets (Lönnroth *et al.*, 1988). Several clinical studies on humans and pigs have shown that the hydrothermally processed cereals improved the intestinal fluid balance and general health status (Lange and Lönnroth, 2001). In animal husbandry AF-inducing diets, owing to their antisecretory activity and anti-inflammatory action on intestinal mucosa, could be a valid alternative to antibiotic growth promoters, especially in swine production, to counteract post-weaning diarrhoea. Furthermore the higher availability of highly digestible starch is an important energy source for the animals, in a critical period characterized by low voluntary feed ingestion.

The aim of the study was to investigate the effect of an AF-inducing diet on piglets growth performance and intestinal mucosa.

Materials and Methods

Animals and diets

The trial was carried out at the Cerzoo experimental farm. 144 weaned piglets (Large White) were selected in a commercial herd. The animals were weaned at 4 weeks of age with an average initial BW of 6.35 ± 0.52 kg. All piglets were healthy at the beginning of the study. The piglets were randomized for sex and body weight and housed in 36 pens (4 pigs/pen). The hydrothermally processed wheat (HPC) was provided by Viking Malt (Lantmännen, Sweden), while wheat (W) was acquired from the market.

The feeding treatments were: C) control diet (Luigi Ferrari Mangimi, Sarmato, Italy); T1) control diet plus 3% HPC (Varian System Plus I, Lantmännen, SW); T2) control diet plus 6% HPC.

The basal diets for the first and second growing periods were prepared by single mixing in Luigi Ferrari feed mill (Sarmato, Italy). The complete diets of the first and second growing periods with the inclusion of the hydrothermally processed wheat were prepared in CERZOO feed mill, according to the experimental scheme. HPC was included in the basal diet as partial substitute of the wheat meal. The hydrothermally processed cereals were included as partial substitute of the wheat meal used in the control diet. No medication or AGP were included in the diets.

The 6-wk trial included a prestarter period (2 wk) and a starter period (4 wk). Diets (Table 2) were slightly modified according to the requirements of the animals, but maintained constant levels of HPC. Animals had free access to feed and water.

Piglets BW and feed intake were determined at 14 and 42 days after weaning to calculate ADG, ADFI, and F:G. Daily inspections were carried out by qualified personnel to check the general health status of piglets and presence of diarrhoea.

Blood sampling

On day 0, 14, and 42, six randomly-selected piglets for each pen (18 piglets in total, 3 castrated male and 3 female per each treatment) were bled via vena cava puncture to obtain plasma samples for routine haematology and biochemistry analysis. According to

the study protocol the blood samples were analysed also for haptoglobin. At the same time plasma EDTA samples were collected to determine intestinal fatty acid-binding protein (I-FABP) concentration, a parameter used to assess damage of the intestinal mucosa in pigs, which has been determined by using a human commercial ELISA test kit (HyCult Biotechnology BV, Uden, the Netherlands) as described by Niewold *et al.* (2004).

Feed analysis

Both processed and unprocessed wheat and complete diets were analytically characterised. HPC and W were assayed in duplicated according to AOAC (1990) for dry matter (procedure 930.15), crude protein (procedure 975.06), ash (procedure 942.05) and acid detergent fibre exclusive of residual ash (procedure 973.18).

Crude fat content was measured according to the indications of the Direction EEC n. 84/4/EEC of 20.12.83 (G.U. EC L15 18/01/84), crude fiber was determined by the method of Wende (AOAC, 1990). Neutral detergent fibre was assayed with heat stable amylase with correction for residual ash according to Mertens (2002), without sodium sulphite and using Ankom device (Ankom 220, USA) for extraction and filtering. Samples starch content was determined by AOAC Polarimetric method (2000). Soluble sugars were determined according to AOAC method (1999). Soluble protein was quantified following the method described in Licitra *et al.* (1996), which gives soluble protein as the difference between the total content and the insoluble fraction.

Digestible Energy was calculated according to Whittemore's (1987) equation and Net Energy according to the equation of Noblet *et al.* (1994).

Table 1. Analytical characteristics of the HPC *vs* commercial wheat (W), % as dry matter.

	HPC	W
Dry matter	94.61	87.45
Crude Protein	11.48	13.95
Crude fat	1.24	1.31
Crude fiber	1.83	3.25
Ash	1.48	1.44
NDF	12.81	17.60
ADF	3.44	3.46
Starch	63.90	56.92
Total Sugars	7.36	1.92

Table 2. Diet composition (mash feeds).

		Prestarter	Starter
		0-14 days	14-42 days
Barley meal	%	35.00	35.00
Wheat meal	%	36.16	37.52
Soybean meal 46%	%	6.88	11.75
Forcital omega [1]	%	5.00	5.00
Corn meal	%	5.00	5.00
Milk whey, sweet dried	%	5.00	-
Potato protein	%	2.00	-
Soybean oil	%	1.36	1.98
Calcium formate	%	0.94	0.40
Calcium carbonate	%	-	0.30
Monocalcium phosphate	%	0.65	1.37
Fermix[2]	%	0.50	0.50
Sodium chloride	%	0.47	0.46
L - Lysine HCL	%	0.61	0.45
L – Threonine	%	0.20	0.15
DL - Methionine	%	0.18	0.10
L – Tryptophan	%	0.05	0.01

Notes: 1 – Forcital Omega is a combination of highly digestible vegetable protein sources processed with extrusion technology, and high quality salmon oil

Notes: 2 - Vitamin and mineral premix (Adisseo Filozoo, Carpi, Italy) composition per kg of premix: vit. A: 4,166,500 IU; vit. D3: 479,190 IU, vit. E: 9,523 mg; vit. B1: 595 mg; vit. B2: 1,190 mg; vit. B6: 714 mg; vit. B12: 7 mg; D-pantothenic acid: 2,975 mg; vit. H: 35 mg; vit. K3: 952 mg; vit. PP: 4,760 mg; Folic acid: 119 mg; Co: 95 mg; Fe: 7,428 mg; I: 357 mg; Mn: 16,666 mg; Cu: 39,285 mg; Se: 47 mg; Zn: 59,523 mg; carbonate of Ca and Mg to 1,000 g as excipient.

Table 3. Analytical characteristics of the diets (% as dry matter).

	0-14 d			14-42 d		
	CCTRL	T2	T3	1CTRL	T2	T3
Digestible energy[1], MJ/kg	16.06	16.06	16.07	16.01	16.00	16.03
Net energy[2], MJ/kg	11.02	11.92	11.93	11.78	11.79	11.80
Dry matter, %	90.69	90.76	91.05	90.56	90.69	90.73
Crude protein, %	19.18	19.22	19.28	19.41	19.15	18.94
Crude fibre, %	4.24	4.59	4.25	3.86	4.20	3.45
Crude fat, %	4.55	5.11	4.58	3.30	3.34	3.70
Ash, %	5.78	6.06	5.75	9.96	10.02	9.62
Starch, %	45.54	44.31	45.96	38.06	38.87	39.33

Note: 1- According to Whittemore (1987); Note: 2- According to Noblet *et al.* (1994)

<u>Hydrolysis kinetics of cereals and complete diets</u>

Enzymatic starch digestibility with amylase was assessed following the modified method of Mercier and Guilbot (1974). Briefly, 2.5 g of feed samples, ground with 1mm screen, were put into 250 ml glass batches, and 97.5 ml of buffer solution (NaH_2PO_4 0.2 M and Na_2HPO_4 0.2 M) were added in each samples flask. The samples solutions were kept in the heat bath gently shaken for few minutes. At the scheduled time (0', 30', 90', 120' minutes from the beginning of the incubation) 3 ml of samples diluted in buffer enzymatic solution were collected. Samples were then transferred to centrifuge tubes with 13.5 ml of alcoholic-acetic solution and 2.5 ml of buffer solution containing 0.3% of amylase from porcine pancreas (Sigma A3176). The test tubes were frozen for 3 hours and then centrifuged. The supernatant (200 µl) was transferred into a glass vial and dried in a vacuum stove at 40°C.

The pellet was resolved with 2 ml of phenol (2.5%) and 5 ml of concentrate sulphuric acid, shaken in order to homogenise the sample and boiled for 8 min. Once cooled, the glucose content was measured with spectrophotometer at 510 nm wave length.

Amyloglucosidase test (AGA test) was performed to evaluate samples starch degradability due to amyloglucosidase activity. Dried and fine ground (1 mm screen) samples were weighted (1 g) into a 50 ml Erlenmeyer flask, and 25 ml of distilled water and 2.5 ml of acetate buffer pH 4.8 (16.4 g of sodium acetate and 12 ml of acetic acid dissolved in 100 ml of distilled water, pH adjusted with NaOH) were added to each sample, ensuring a good dispersion of the sample. After that 5 ml of amyloglucosidase (from *Aspergillus niger*; Fluka, 70 U/mg) distilled water solution (1%) were added to the sample' solutions, which were then placed in 60ºC water bath for 1 hour. Samples were mixed once in a while during the incubation time. After incubation, 0.5 ml of suspension was diluted in 9.5 ml of TCA 5% (trichloroacetic acid dissolved in distilled water), to allow protein precipitation and to block the enzyme activity.

Samples were centrifuged and analysed for glucose content with a colorimetric method. A 50 µl of sample were collected in a test tube and diluted in 3 ml of GODPOD (colorant reagent). At the same time a standard as well as a blank sample were prepared, as reference value and to correct the instrument readings for the analysis. Samples diluted in GODPOD were kept in the dark for 30 minutes, to allow the colorimetric reaction.

Glucose content was measured with spectrophotometer at 510nm wave length. Digested starch was calculated following the formulas described below.

(Sample Abs/STD Abs) x (6501/ Sample g) = mg of glucose/ g of sample

650= (32.5 dilution x 20 (10 ml/0.5))

In vitro digestibility starch and proteins of diets

Diets samples were digested *in vitro* to investigate the kinetics of diets starch degradation and the amount of free amino acids released after 6 and 24 hours of digestion.

In vitro digestibility of the diets was assessed following the Bedford and Classen method (1993) as described by Stevnebø *et al.* (2006). To simulate gastric conditions, 500 mg of dried ground feed were pre-incubated in 5 ml of HCl 1M and pepsine (50 g/l, pH 1.5; Merck, Darmstadt, Germany) in a heat bath with a swirly system at 39°C for 30 minutes.

After the pre-incubation, 5 ml of NaOH 1N and 5 ml of buffer solution (NaHCO$_3$, adjusted to pH 6-6.5 with H$_3$PO$_4$), 25 ml of enzymatic buffer containing 25mg of pancreatin (300 U α-amylase/mg porcine pancreatin; Sigma Aldrich Co, St. Louis, USA) and 25 mg amyloglucosidase (from *Aspergillus niger;* Sigma Aldrich, 70 U/mg), dissolved in distilled water, were added to each sample to obtain a final volume of 40 ml.

Following the experimental protocol, at the end of the incubation time, 1 ml of the solutions was transferred into an eppendorf tube and placed in a boiling water bath for 2 min to stop the enzymatic reactions. After centrifugation, 50 μl of sample were collected and diluted in 3 ml of colorant reagent. At the same time were prepared a standard as well as a blank sample, as reference value and to correct the instrument readings for the analysis. Glucose concentration and digested starch were determined as previously described in the text.

Free amino acids were determined at 6 and 24 h from the beginning of the *in vitro* degradation.

Amino acids were first extracted with dilute hydrochloric acid (0.1 N). The co-extracted nitrogen molecules were precipitated with picric acid and removed by filtration through a resin column. The filtered solutions were adjusted to pH = 2.20. The amino acids were separated by ion exchange chromatography and determined by reaction with ninhydrin, with photometric detection at 570 nm (G. U. L272/14 19/09/98).

Statistical analysis

Average daily gain, average daily feed intake and F:G were analyzed by the Tukey-Kramer test using the GLM procedure of SAS 9.1 (SAS Inst. Inc., Cary, NC). The pen was used as the experimental unit and initial BW as a covariate. The linear or quadratic effects were not tested due to the low number of treatments. Blood parameters were analysed by the Tukey-Kramer test and the GLM procedure of SAS 9.1 (SAS Inst. Inc., Cary, NC), using the initial values as covariate.

Results

Growth performance

During the trial there were no mortalities and none of the piglets showed signs of diarrhoea or needed to be removed for poor performance or health reasons. Results for BW, ADG, ADFI, and F:G ratio are shown in Table 4.

Significant differences were found among feeding treatments for final body weight and average daily gain. In piglets fed the diet with 6% of HPC supplementation there was a significative improvement in ADG (P= 0.014) during both growth phases. Average daily feed intake was not affected (P> 0.05), even if it tended to be higher during the second growing period and in the whole experiment in T2 and T3 groups. Feed conversion rate improved in T3 during the 0-14 days period and the whole experimental period (P= 0.051 and P= 0.053).

Final BW was higher in T2 and T3 groups compared to T1 (P< 0.05). Pigs fed the diet with the highest HPC supplementation had lower F:G (P< 0.05) during the prestarter period and during the whole experimental period.

Blood parameters

As shown in the Tables (6-9) there were no main effects of feed treatments on haematological and biochemical parameters. A significant difference was found only in blood calcium level at 14 and 42 days, where the values were higher in the control group piglets (P= 0.026 and P= 0.021, respectively).

At 42 days, bilirubin tended to be lower (P= 0.062) while concentration of uric acid increased in pigs fed the diet with HPC supplementation (P= 0.065). Uric acid is a

Table 4. Effect of specially processed cereals on growth of weaned piglets**.**

	Level of hydrothermally processed wheat			SEM	Effect of		
	0%	**3%**	**6%**		**Treat**	**Sex**	**Sex * Treat**
Initial BW, Kg	6,35	6,35	6,36	0,13	0,997	0,075	0,778
Final BW, Kg	21,08 [b]	21,52 [ab]	22,22 [a]	0,32	0,018	0,082	0,332
ADG, g/d							
0-14 d	310 [b]	320 [ab]	339 [a]	8,16	0,014	0,685	0,286
14-42 d	372 [ab]	370 [b]	397 [a]	7,37	0,026	0,014	0,501
0-42 d	351 [b]	353 [b]	378 [a]	6,82	0,003	0,097	0,400
ADFI, g/d							
0-14 d	452	450	450	4,58	0,937	0,680	0,276
14-42 d	552	587	582	10,93	0,063	0,175	0,820
0-42 d	519	540	538	7,15	0,079	0,208	0,602
Feed:Gain							
0-14 d	1,47 [a]	1,42 [ab]	1,34 [b]	0,04	0,051	0,741	0,139
14-42 d	1,50	1,59	1,47	0,04	0,147	0,471	0,681
0-42 d	1,48 [ab]	1,58 [a]	1,43 [b]	0,04	0,053	0,656	0,606

;ADG: average daily gain; ADFI: average daily feed intake

[a,b] Means with different superscript significantly differ (P< 0.05).

product of the metabolism of purine bases, high values in plasma might be related to a higher protein turnover in animal with a faster growth.

Plasma I-FABP was monitored to assess the health status of the intestinal epithelium during the post-weaning period. Feeding treatments did not influence the I-FABP plasma concentration and, as expected by the good health of the animals, the values were low, in the range 61.6-77.1 pg/ml (Table 5).

Plasma I-FABP concentration decreased during the trial in all groups, with significative differences between groups. It is noteworthy that in T2, plasma I-FABP decreased significantly in the first 2 weeks after weaning. At the end of the trial, the lower values were detected in the HPC fed animals.

Table 5. Blood levels of I-FABP in piglets fed diets supplemented with increasing amount of specially processed cereals.

Treatment	Days of growth			Time Effect
	0	**14**	**42**	
Control diet	77.10^b	68.58^{ab}	63.61^a	0.0163
3% HPC	75.49^c	61.59^b	63.80^a	0.0210
6% HPC	77.05^b	67.47^{ab}	62.14^a	0.0308
SEM	3.448	3.009	1.421	
Treatment	0.932	0.301	0.684	
Sex	0.461	0.975	0.108	
T * S	0.085	0.062	0.342	

^{a,b,c} $P<0.05$

Table 6. Effect of hydrothermally processed cereals on blood parameters in weaned piglets after 14 days of growth.

Blood parameters		Level of treated cereals.				Effect of:		
		0%	3%	6%	SEM	Treatment	Sex	S * T
Haematocrit	l/l	0.343	0.334	0.313	0.0101	0.130	0.518	0.939
Glucose	mmol/l	6.13	5.84	5.42	0.2239	0.134	0.837	0.267
Urea	mmol/l	5.87	5.78	5.82	0.3478	0.319	0.915	0.896
Cholesterol	mmol/l	2.16	1.97	2.30	0.1580	0.375	0.520	0.910
Triglycerides	mmol /l	0.39	0.36	0.32	0.0408	0.209	0.615	0.228
Phospholipides	mg/100ml	109.17	104.00	108.45	7.1764	0.860	0.666	0.720
Creatinine	μmol/l	88.62	83.17	91.73	3.2767	0.215	0.556	0.028
Uric acid	mg/dl	0.082	0.098	0.072	0.0154	0.501	0.168	0.876
Bilirubin	μmol/l	1.52	1.02	1.24	0.2163	0.297	0.491	0.858
LDH	U/l	1323.94	1246.71	1369.02	56.35	0.334	0.963	0.279
Aptoglobine	g/l	2.38	2.60	2.12	0.1997	0.662	0.527	0.177
GOT	U/l	46.78	50.94	49.46	5.2492	0.853	0.808	0.547
GPT	U/l	54.50	52.06	46.74	4.6925	0.509	0.325	0.933
Alkaline phospatase	U/l	330.25	33.59	292.11	25.2088	0.457	0.622	0.305
Ca	mmol /l	2.70^b	2.56^a	2.64^{ab}	0.0288	0.026	0.831	0.510
P	mmol /l	3.34	3.31	3.23	0.0859	0.668	0.154	0.534

SEM = Standard error of means.
[a,b] Means with different superscript significantly differ ($P<0.05$).

Table 7. Effect of hydrothermally processed cereals on blood parameters in weaned piglets after 42 days of growth.

Blood parameters		Level of treated cereals.			SEM	Effect of:		
		0%	3%	6%		Treatment	Sex	S * T
Haematocrit	l/l	0.333	0.30	0.23	0.0085	0.535	0.065	0.292
Glucose	mmol/l	6.30	6.45	5.64	0.4871	0.481	0.632	0.272
Urea	mmol/l	6.69	6.84	6.23	0.3599	0.594	0.881	0.929
Cholesterol	mmol/l	2.37	2.43	2.37	0.1209	0.913	0.799	0.983
Triglycerides	mmol /l	0.309	0.314	0.284	0.0416	0.825	0.772	0.964
Phospholipides	mg/100ml	130.24	130.44	123.76	5.8176	0.662	0.522	0.258
Creatinine	μmol/l	89.89	88.55	86.16	2.9967	0.951	0.616	0.043
Uric acid	mg/dl	0.0833	0.1062	0.1699	0.0241	0.065	0.544	0.664
Bilirubin	μmol/l	0.862	0.496	0.286	0.1552	0.0621	0.293	0.875
LDH	U/l	1294.04	1343.22	1471.27	62.359	0.159	0.908	0.277
Aptoglobine	g/l	1.33	1.48	2.22	0.2584	0.605	0.685	0.721
GOT	U/l	50.34	50.92	62.79	6.8580	0.380	0.392	0.278
GPT	U/l	57.64	70.42	65.29	5.389	0.279	0.873	0.777
Alkaline phospatase	U/l	180.94	213.92	180.79	19.329	0.405	0.279	0.296
Ca	mmol /l	2.90[b]	2.77[ab]	2.75[a]	0.0352	0.021	0.842	0.235
P	mmol /l	3.72	3.68	3.57	0.1039	0.582	0.863	0.206

SEM = Standard error of means

Table 8. Blood parameters: haematology after 14 days from the start of the study.

		TCTRL	T2	T3	Effect of treatment	SEM
WBC – leucocytes	K/Ul	16.20	11.64	14.82	0.145	1.5771
RBC – erythrocytes	M/Ul	5.45	5.28	5.47	0.512	0.1242
HGB – Haemoglobin	g/dl	10.62	10.27	10.54	0.650	0.2743
HCT – Haematocrit	%	32.00	31.03	32.58	0.515	0.9401
MCV [1]	fL	58.97	58.80	59.47	0.942	1.4162
MCH [2]	pg	19.58	19.42	19.30	0.901	0.4404
MCH [3]	g/dl	33.22	33.07	32.43	0.465	0.4633
RDW	%	26.63	23.58	26.22	0.268	1.3791
PLT – platelets	K/Ul	582.50	589.83	498.22	0.693	83.1422
Neutrophilis	%	48.80	63.00	54.82	0.237	5.6590
Lymphocytes	%	45.00	31.00	37.42	0.287	6.0128
Monocytes	%	4.36	4.38	5.53	0.569	0.8762
Eosinophilis	%	1.60	1.50	2.04	0.591	0.3866
Basophils	%	0.22	0.13	0.21	0.598	0.0651

Table 9. Blood parameters: haematology after 42 days start of the study.

		CTRL	T2	T3	Effect of treatment	SEM
WBC – leucocytes	K/Ul	20.67	20.87	20.45	0.9894	2.0156
RBC – erythrocytes	M/Ul	6.00	5.73	5.90	0.6805	0.2196
HGB – Haemoglobin	g/dl	10.30	10.06	9.98	0.6734	0.2593
HCT – Haematocrit	%	31.57	30.77	31.52	0.7734	0.8765
MCV [1]	fL	52.65	53.72	53.65	0.6364	0.8755
MCH [2]	pg	17.20	17.57	17.00	0.4247	0.3017
MCH [3]	g/dl	32.70	32.70	31.70	0.1988	0.4300
RDW	%	21.88 b	20.62 a	22.55 b	0.0087	0.3817
PLT – platelets	K/Ul	437.50	406.17	476.67	0.7433	64.2149
Neutrophilis	%	37.22	32.45	42.78	0.3998	5.2375
Lymphocytes	%	56.20	59.55	50.03	0.5227	5.8639
Monocytes	%	4.75	5.83	4.97	0.6767	0.8985
Eosinophilis	%	1.48	1.42	1.69	0.8718	0.3826
Basophils	%	0.33	0.76	0.54	0.1315	0.1422

MCV[1] : mean corpuscular volume
MCH[2] : mean content of haemoglobin / erythrocytes
MCHC[3] : mean globular concentration of haemoglobin

In vitro hydrolysis kinetics

Total sugars determination and *in vitro* enzymatic hydrolysis evidenced the higher availability of sugars in HPC compared to unprocessed wheat, likely due to the hydrothermal process.

Determination of soluble sugars content of the samples by HPLC, showed that HPC contained 6.96% of sugars compared to 1.92% of control wheat (Table 11).

In vitro rate of starch hydrolysis by α-amylase (from porcine pancreas), performed on the wheat kernels (HPC and W), measured after 0, 30, 90 and 120 minutes of incubation, was higher in the hydrothermally processed wheat, during all the time course of the experiment (*Figure*1). Indeed at the beginning of the incubation 11.74% of HPC starch was already hydrolysed to mono and oligosaccharides.

The kinetics of *in vitro* hydrolysis by α-amylase of experimental diets is shown in Figure 2. At time 0, the degree of hydrolysed starch was similar between diets. During the first 30 minutes of enzyme treatment, the rate of starch degradation was similar for T1 and T2, while T3 diet, which had the highest HPC level of inclusion, showed a higher rate. The same trend was observed after 90 minutes of incubation. Between 90' and 120', the degree of starch hydrolysis was improved in both T2 and T3 diets (Figure 2).

Table 10. Soluble sugars determination in hydrothermally processed wheat (HPC) and in commercial wheat (W) assessed by HPLC.

Sugars %	HPC	W
Fructose	0.24	0.11
Glucose	2.70	0.62
Sucrose	0.73	0.57
Maltose	4.86	1.19
Lactose	0.23	0.05

In vitro starch degradation by amyloglucosidase (AGA test) showed that after 1 hour of incubation more than 60% of starch contained in HPC was degraded, compared to 25% of the control wheat (*Figure*3). Hydrolysis of diets by AGA test showed that 13.82% of T1 starch was degraded after 60', compared with 23.39% and 23.72% of T2 and T3.

Figure 1. In vitro rate of starch hydrolysis by α-amylase in HPC and W wheat kernels measured at incubation time 0, 30, 90 and 120 min.
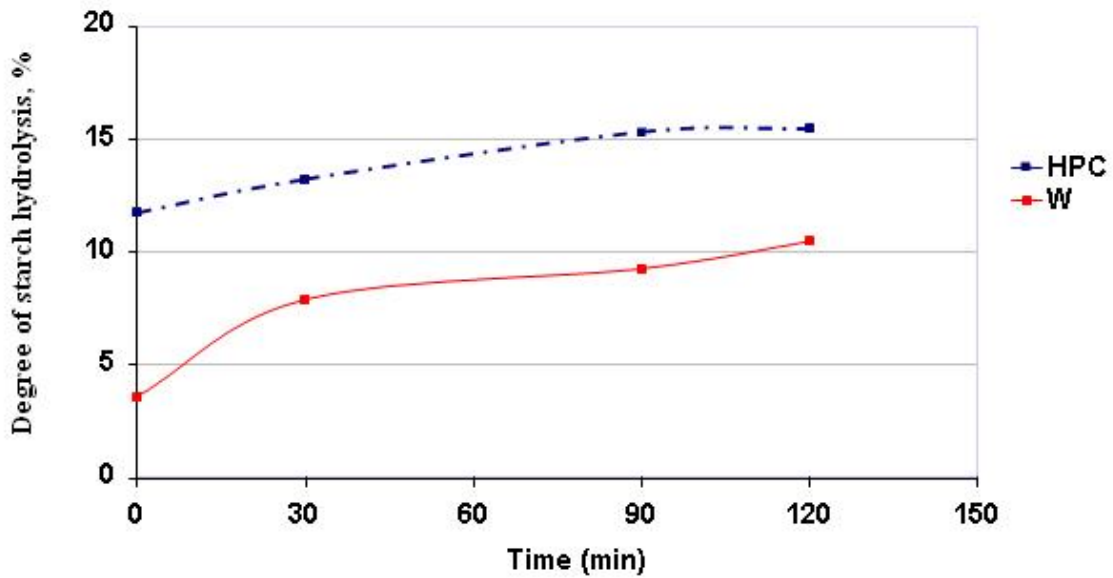
Figure 2. In vitro rate of starch hydrolysis by α-amylase of the experimental diets measured at incubation time 0, 30, 90 and 120 min.
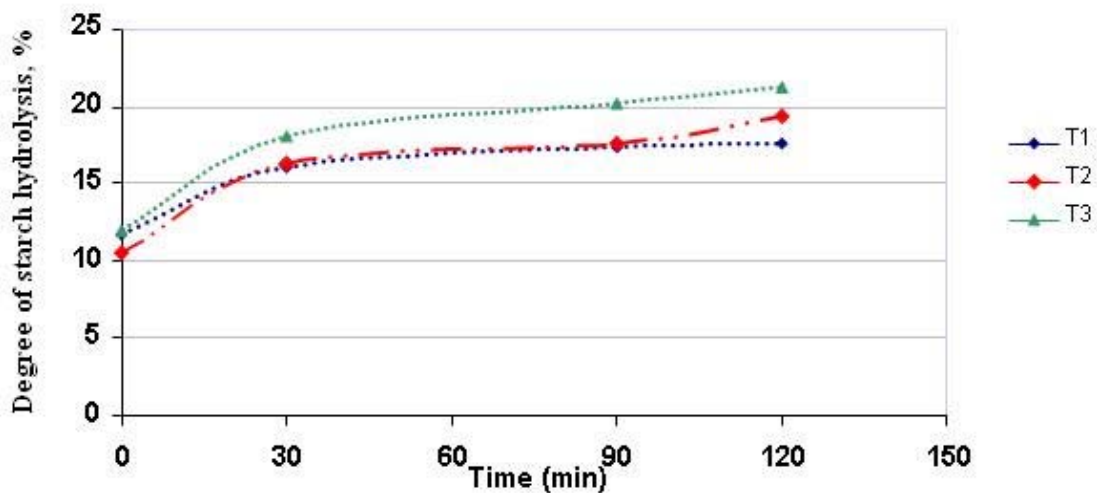
In vitro digestibility of the prestarter and starter diets by porcine pancreatin and amyloglucosidase was performed to assess the amount of essential amino acids released during digestion by the different diets (Table 6). After 6 and 24 hours of enzymatic treatment, both prestarter and starter T2 diets showed the highest level of digested proteins and released free essential amino acids. Among the prestarter diets, at 6 h the amount digested starch was higher in T3 diet.

Figure 3. Degree of starch digested by amiloglucosidase of wheat kernels and experimental diets at 60 minutes.
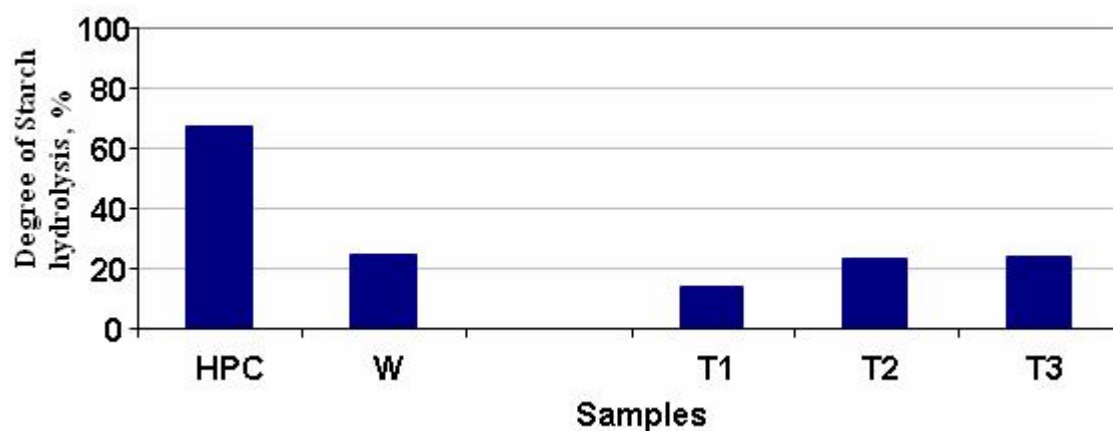
At 24 h, the T3 diets had the highest level of free amino acids and digested protein, while T2 had the highest amount of digested starch.

Table 11. Starch and protein digested and essential aminoacid released after 6 and 24 h of in vitro digestion of the experimental diets.

	Diets digestibility 6hours			Diets digestibility 24hours		
	Starch digested %	CP digested %	Free EAA %	Starch digested %	CP digested %	Free EAA %
Prestarter						
T1	31.14	54.88	4.88	53.31	72.70	6.47
T2	26.35	56.50	5.11	65.24	71.12	6.34
T3	33.06	49.60	4.66	66.39	72.72	6.67
Starter						
T1	24.15	49.35	4.43	53.31	72.57	6.61
T2	25.95	58.02	5.22	63.75	75.37	6.73
T3	37.88	57.44	5.10	57.47	76.32	6.84

Figure 4. Starch digested at 6 and 24 h of in vitro experimental diets digestion.
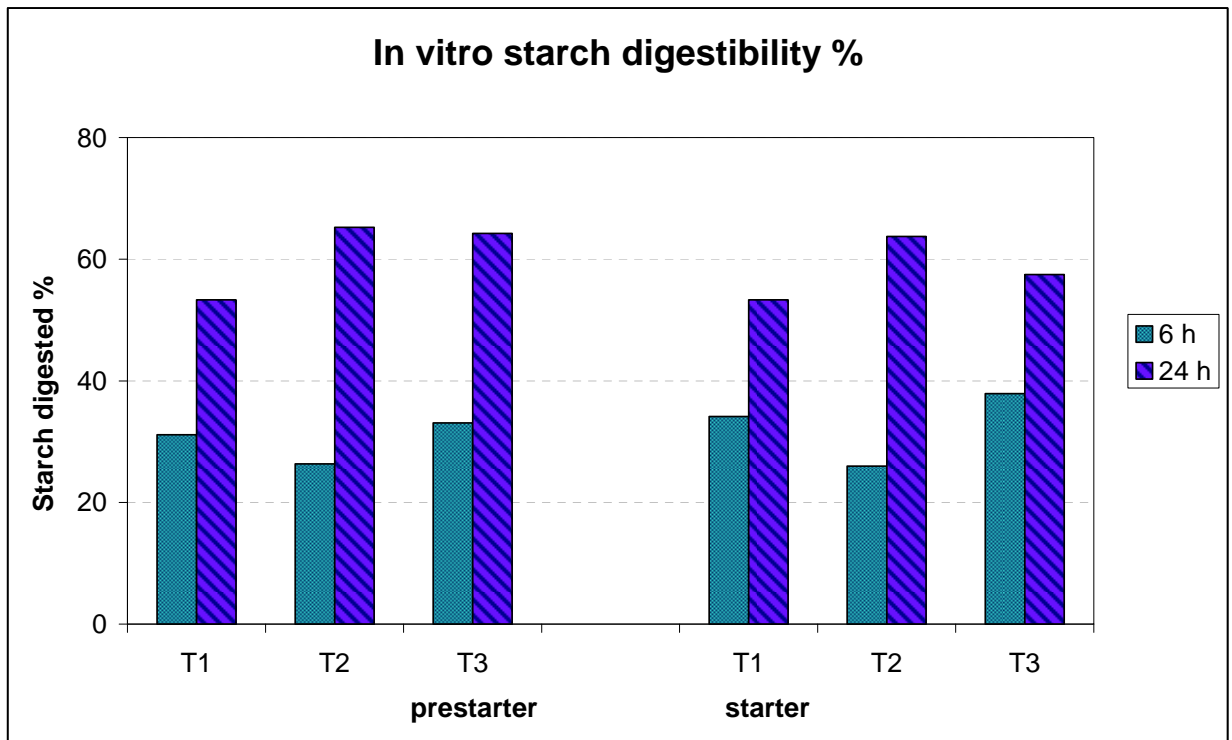
Discussion and Conclusions

Early weaning exposes piglets to simultaneous nutritional, social and environmental stresses that can lead to an impairment of health status and to a major susceptibility towards infections (Hiss and Sauerwein, 2003). Moreover, gastrointestinal diseases around weaning occur in a context of incomplete development of the digestive and immune systems physiology (Partanen and Mroz, 1999). Up to 2006, the use of antibiotics seems to have been the most effective way to control the negative effects of premature weaning on animal health and performance. Immuno-modulatory feed additives could be the alternative to AGP in livestock production by improving the animals' natural ability to fight diseases (Gallois *et al.*, 2009).

According to Lange and Lönnroth (2001), feeding hydrothermally processed cereals can induce endogenous secretion of the AF protein in both human and animals. AF-inducing diets improved intestinal fluid balance and inflammation in patients affected by inflammatory bowel diseases (Björck *et al.*, 2000). Improved intestinal fluid balance and growth performance in animals fed AF-inducing diets were reported by Göransson (1993) and Lange and Lönnroth (2001).

During this trial, none of the piglets showed signs of diarrhoea. This was not unexpected, given the experiment was conducted in an experimental facility where pathogens presence might be generally lower than on a commercial farm.

Supplementation of the wheat-barley based diet with 6% of HPC improved the average daily gain (P= 0.003) and the feed conversion rate during the whole experimental period (P= 0.053). Feed intake was higher in both T2 and T3 groups, compared to the control, during the second growing phase, even if the differences were not significant (P= 0.063). There were no significative effects of feed treatment on blood parameters. At 42 days, a higher amount in plasma level of purine metabolites, as uric acid, was found in piglets fed T3 diet (P= 0.065), likely due to a higher protein turnover in fast growing piglets. At the end of the trial, total serum bilirubin tended to be lower in the HPC-fed groups (P= 0.062), though bilirubin is correlated to heme turnover and high values are correlated to several hepatic diseases, the values were low and into the range of the reference for pigs (Kaneko, 1997).

Plasma I-FABP can be considered as an indicator of gut mucosa damage. It is found only in the mature enterocytes of the small intestine, with only trace amounts identified in the stomach and large intestine, and it can be detected in plasma and urine after cell

damage (Niewold *et al.*, 2004). Reduction of the intestinal oxygen supply in pigs, as a way to induce metabolic stress to the intestinal mucosa, was previously shown to induce intestinal cells damage and increase plasma I-FABP concentrations (Niewold *et al.*, 2004). In the current study the concentration of I-FABP in plasma were low in all groups and not affected by the dietary treatments, suggesting that, in good hygienic conditions, the dietary treatments did not affect small intestinal integrity. On average, I-FABP levels were numerically less in piglets 6 wk after weaning compared with 2 wk after weaning. However we did not detect significant differences in the plasma I-FABP level between diets, so it is not possible to clarify if the better performance observed in piglets fed the 6% HPC supplemented diet might be due to an anti-inflammatory affect of the endogenous antisecretory factor possibly induced by the HPC inclusion in the diets.

The difference in growth performance observed in this study between might be in part related to a balanced supply of energy-yielding nutrients and amino acids in the diets.

Starch in cereals has a great importance in newly weaned piglets since it constitutes the largest portion of the diet and is the major energy-yielding component (White *et al.*, 2008). The larger part of starch is digested in the upper part of the small intestine. Starch which is not digested in the small intestine will enter the large intestine and will be fermented (Cummings and Englyst, 1995). Starch digestibility is related to the source and processing of the starch (Graham *et al.*, 1989; van der Poel *et al.*, 1992). According to Wiseman (2006), the rate of starch digestion is a crucial characteristic that rules the dietary energy value of compound diets. As expected, the *in vitro* digestion assays evidenced a relevant simple sugars content and a higher rate of starch hydrolysation in the hydrothermal process wheat compared to the unprocessed cereal (Figure1). Inclusion of HPC (6%) as partial substitute of unprocessed wheat meal resulted in a higher *in vitro* starch digestion rate of T3 diet but not in T2, where the inclusion level used was 3% (Figure 2).

According to Van den Borne *et al.* (2007), pig performance is maximized when the absorption of energy-providing nutrients and amino acids, for protein synthesis, is synchronized.

After 6 hours of *in vitro* enzymatic digestion of the diets T2 (3% HPC) showed the highest amount of digested protein and free amino acids, associated to a lower diet starch digested.

As reported by Cummings and Englyst (1995), a decrease in the rate of starch digestion may reduce or delay the postprandial glucose and insulin responses. The slower rate of starch digested in T2 diet might have reduced the amount of glucose available for the intestinal tissue metabolism, leading to an increased gluconeogenesis and to a reduced amount of amino acids for protein deposition.

According to Lallès *et al.* (2004), cooked cereals are thought to improve post-weaning performance because the starch is more gelatinised and hence they assist in overcoming the (temporary) period of pancreatic amylase insufficiency that generally occurs after weaning. The first week after weaning is characterized by reduced feed intake and reduced pancreatic enzymes activity. Therefore feeding a diet with highly digestible starch guarantees a correct amount of energy, limiting the amount of feed to be fermented in caecum and colon.

In vitro assessment of the antisecretory activity of AF-16

The Ussing Chamber

The Ussing chamber method was developed in the 1950s by the Danish biologist Hans H. Ussing as a means to understand the phenomenon of active NaCl transport across epithelium.

The first model system was the frog skin because of its ability to move NaCl from the skin surface into the interstitium against more than a 100-fold concentration difference (Clarke, 2009). To distinguish the active ions transport from the passive diffusion through paracellular and intercellular pathways across the epithelium, dissected frog skin separated two halves of a chamber, each of which were perfused with identical electrolyte solutions. Therefore, paracellular ion movements driven by osmotic and electrochemical gradients were eliminated. The passive transepithelial driving force, created by the spontaneous electrical potential across the epithelium, was eliminated by clamping the potential to zero with an external current passed across the epithelium (Clarke, 2009). This current, known as the short-circuit current (Isc), is equivalent to the algebraic sum of electrogenic ion movement by active transport. Consequently, eliminating transepithelial diffusion forces, the movement of ions as measured by Isc in the Ussing chamber resulted from active transport (Clarke, 2009).

Nowadays, the Ussing chamber method is applied to every epithelium of the animal body and is extensively used for studies on cultured epithelial cells where tight junction integrity maintains apical and basolateral membrane polarity.

Ussing chamber studies of intestinal mucosa improved the understanding of transepithelial transport processes toward a molecular basis. Indeed, the Ussing chamber technology allows a direct contact between the tissue and the substances studied, provided these substances are soluble and it is time-proven method for the measurement of electrolyte, nutrient and drug transport across epithelial tissues (Clarke, 2009). As reported by Boudry (2005), the Ussing chamber is a valid method to measure the active ion transport across the intestinal mucosa and study its permeability, which are relevant parameters to assess gut health.

Moreover, this method allows to study the effects of substances whose antisecretory activity is suspected and to clarify their mechanisms of action.

The intestinal fluid secretion

Fluid secretion plays a key role in the gut physiology since it is required for hydration of the intestinal mucosa and mixing of intestinal contents. Under normal conditions, the intestine carries out the absorption of luminal fluid, electrolytes and nutrients. Several channels and cotrasporters regulate the ionic balance on both sides of the intestinal epithelium. Water and solute absorption is mainly realized by Na^+/glucose, Na^+/K^+, Cl^-/HCO_3^- exchangers (Lapointe *et al.*, 2009). At the apical side, cAMP-dependent cystic fibrosis transmembrane conductance regulator (CFTR) is fundamental to create the osmotic gradient that drives the passage of water into the lumen. Fluid secretion is driven by active Cl^- transport from the basolateral to the apical side of enterocytes: Cl^- is taken up at the basolateral membrane via NaK_2Cl cotransporter, which is driven by Na^+ and Cl^- concentration gradients produced by the Na^+K^+-ATPase and basolateral K^+ channels. Cl^- is electrochemically driven across the cell apical membrane primarily through the CFTR, as well as through $Ca2^+$-activated Cl^- channels (CaCCs) and other Cl^- channels. Both Na^+ and fluid follow Cl^- paracellularly. During gastrointestinal diseases, regulatory mechanisms of secretion are altered. Inflammation and diarrhoea, caused by either infectious or non infectious etiologies, are the result of changes in fluid and electrolytes transport in the small and/ or large intestine (Fordtran, 1967). *Escherichia coli* and *Vibrio cholera* are among the major agents causing diarrhoea in both human and animals. Bacteria enterotoxins induce secretory diarrhoea by improving active transport of anions, mainly chloride and bicarbonate, from crypt epithelial cells. Thus, a key player in the response to enterotoxin-induced intestinal fluid secretion is the apical anion channel CFTR.

Cholera toxin (CT) induced diarrhoea has been considered a prototype of enterotoxin-caused diarrhoea, because it does not cause histological changes in the intestine despite substantial rates of net fluid secretion (Field *et al.*, 1972). CT attaches the enterocytes by binding to GM1-ganglioside via the B-subunit and the binding itself triggers the toxin internalization. Into the cell, CT increases cAMP level (Moss *et al.*, 1981). CT and the increased cAMP stimulate active Cl^- secretion by activating or inserting Cl^- channels into the apical membrane of the crypt cells, and inhibit electroneutral NaCl absorption by decreasing the activity of apical membrane Na^+/H^+ and Cl^-/HCO_3^- exchange in villous cells, without interfering with the glucose stimulated Na absorption (Petri *et al.*, 2008). Moreover, CT induces diarrhoea by an indirect pathway which involves the enteric nervous system, by inducing the secretion of neurohormones as 5-HT (5-

hydroxytryptamine) and vasoactive intestinal peptide (VIP) (Cooke *et al.*, 1994; Cassuto *et al.*, 1981).

The antisecretory properties of AF has been evaluated in both rat and pig intestinal loop models challenged with several secretagogues such as bacterial enterotoxins and intestinal neurohormones.

The antisecretory activity of three AF-derived peptides (A1, A3 and A4), overlapping the antisecretory and anti-inflammatory domain of the complete protein, was evaluated in *in vivo* and *in vitro* studies (Grøndal *et al.*, 2002). In the *in vivo* experiments on pig intestinal loops, A3 showed to have the highest antisecretory activity and reduced by 60% the CT-induced fluid accumulation in the proximal small intestine. It also reduced by 60% LT-induced hypersecretion in the proximal and distal intestinal loops, while no effect were seen on 5-HT challenged loops.

The antisecretory properties of A3 were also evaluated by electrophysiological experiments performed on pig intestinal mucosa, stripped of outer muscle layers, in an Ussing chamber trial. The peptide (10 nmol/l) was added bilaterally 30 minutes before the challenge with secretagogues, including substance P, VIP, 5-HT and theophylline which were added to the serosal side of the tissues. A3 had no effect on the secretagogues-induced increase of short circuit current (Isc). Grøndal and colleagues (2002) suggested that the antisecretory effect of AF-derived peptides involves the neuronal structures of the intestinal mucosa. It has been suggested that AF might attenuate the secretory reflexes in the enteric nervous system (Kim *et al.*, 2005).

However, the pathway for the antisecretory effect of AF is not yet clear. Flotillin-1, an integral protein localized in the lipid rafts, was recently identified as an interacting protein for antisecretory factor (Johansson *et al.*, 2008). Both CT and AF receptors seem to be localized in the lipid rafts, specialized cell membrane area with a high concentration of receptors and ion channels. Johansson *et al.* (2008) suggested that cytoplasmatic AF can modulate the signal pathway of CT by interacting with flotillin-1. This in turn might affect the localization of signal proteins in the lipid rafts. On the other hand, exogenous addition of AF or AF peptides reproduced the effects of endogenous AF in many systems, indicating the presence of interacting structures exposed on the external part of the plasma membrane.

The antisecretory effect of AF-16 was evaluated using an *in vitro* model of CT-induced hypersecretion measured electrophysiologically in IPEC-2J cells. All the experiments

were performed at Katholieke Universiteit of Leuven, Belgium, under the guidance of prof. T.A. Niewold.

Materials and Methods

Cell culture

Intestinal porcine epithelial cell line (IPEC-J2, derived from newborn unsuckling piglet jejunum) were cultured in Dulbecco Modified Eagle Medium (DMEM)/ Ham's F-12 (1:1) medium supplemented with 5% fetal bovine serum, 1,6 % sodium bicarbonate, 1,5 % HEPES, 0.5% sodium pyruvate and 1% antibiotic-antimycotic and incubated at 37°C, in 5% CO_2 atmosphere. Medium was refreshed every 3 days and the cultures were split weekly.

IPEC-J2 cells were seeded ($6x10^5$ cells/ml) on permeable Transwell-col inserts (Collagen-Coated membranes; 0.4 μm pore size, 1.12 cm^2, 12 mm diameter, Corning Inc., Corning, NY) which were placed in 12-well plates. Once seeded on a permeable filter, the cells polarized and attached themselves to each other by forming tight junctions thus developing an epithelial structure. The culture medium was changed every 3 days at the luminal side (500 μl) and serosal side (1500 μl). To ensure that the monolayers exhibited the properties of a tight biological barrier, confluence was measured by checking trans epithelial electrical resistance (TEER) using an epithelial voltohmmeter (EVOM). Confluence, on average, was reached after 9-10 days of culture.

Figure 1. Transswell Plate (Corning Inc., Corning, NY).

Reagents

AF-16 peptide (VCHSK TRSNP ENNVG L, m.w. 1754.95), synthesized by ordinary solid phase technique, was generously provided by Prof. Stefan Lange of the Göteborg University (SW).

The peptide was carefully dissolved to a concentration of 1 mg/ml in MilliQ water (57×10^{-5} M), divided into aliquots of 50 μl and stored at -20°C until use. The molar AF-16 concentration used to perform the electrophysiological assays was 5.7×10^{-4}M.

The Ussing chamber experiments were performed using the HEPES-buffered Krebs-Ringer's solution (K-R) containing (in mM): 115 NaCl, 1.2 $MgCl.6H_2O$, 5.9 KCl, 2.5 $CaCl_2.2H_2O$, 26.1 HEPES buffer and 10.0 D-glucose. All chemicals were purchased from standard sources and were generally of the highest purity available. The HEPES-buffered Krebs-Ringer solution was prepared fresh for each experiment and osmolality was measured by an osmometer (Vapor Pressure Osmometer 5500 WESCOR) and adjusted to 320 mosmol/kgH_2O with saccharose, pH 7.4 at 37°C.

Purified cholera toxin (Sigma) was first diluted in MilliQ water and after in Ringer-Krebs solution used to do the tests. Diphenylamine 2-carboxylic acid (DPC, Sigma) diluted to a stock solution and used at 2mM in all Ussing chamber experiments.

Apparatus

The electrophysiological measurements were performed in modified Ussing chambers apparatus designed by Prof. Dr. Willy Van Driessche (KU Leuven, Belgium).

The chamber was shaped to host a transwell insert $(A = 1.12\text{cm}^2)$ and was provided with holes for the electrodes. The monolayer divided the chamber in two compartments representing the apical side and the basolateral sides of the membrane.

Both compartments of the Ussing chamber were continuously perfused by the bathing solutions, aerated with 95% O2/5% CO2 by a gas lift system. The solutions were pumped in and out the chamber from beakers via polyethylene tubes. In order to ensure a

Figure 2. Ussing chamber used to perform the tests.

proper seal, the edges were coated, with a layer of silicone grease. During the experiments, the chamber and the solution were placed inside a close scaffolding, kept at 37°C. The short-circuit current (Isc) was recorded continuously using salt bridges (KCl 3M) and Ag/AgCl electrodes, and a multichannel computer- controlled voltage-clamp unit. The silver chloride electrodes which measured the voltage were applied so that the tips were close to the monolayer. The tips of electrodes which measured the current were at around 15 mm from the epithelium.

Electrophysiological measurement of net electrogenic transport

The transwell insert containing an IPEC-2J cells monolayer was mounted into the Ussing chamber. The exposed surface area of the monolayer was 1.12 cm^2. After that, the electrodes were inserted in the proper position and the chamber was placed inside the scaffold, heated at 37°C. To help maintain adhesion of the monolayer to the insert's membrane, the apical and the basolateral compartment were carefully filled with the Krebs-Ringer solution by the means of polyethylene tubes and syringes. Attention was paid to avoid air bubbles close to the electrodes tips. Once the chamber was filled with the bathing solution, the pump for the inflow and outflow of the solutions was activated. Then, the Ag/AgCl electrodes were connected to automatic voltage clamp apparatus, realized by Prof Van Driessche. The voltage was clamped to zero and the short-circuit current (Isc) was continuously recorded as a measure of net active ion transport across the monolayer. Chamber fluid resistance was automatically subtracted. The transepithelial short-circuit current was continuously and simultaneously recorded (ImpDsp1.4; KU Leuven) and analyzed with Origin 7.0 software (Origin Lab, Northampton, MA). An initial series of experiments were performed to characterize the effect of different dosage of cholera toxin (0.02, 0.1 and 1 μg/ml) on Isc and evaluate the proper concentrations and duration of the experiment. After ~ 20 min of equilibration period to achieve a steady-state of Isc, CT was added at the bathing solution at the apical side; the concentration tested were To evaluate the in

Figure 3. Ussing chamber apparatus used to perform the in vitro trials at the Department of Biosystems, Katholieke Universiteit of Leuven, Belgium

vitro antisecretory property of AF-16 (5.7×10^{-4} M), it was added before or after CT (1 μg/ml) at the apical or basolateral side. DPC (2 mM) was added at the apical side, according the experiment schedule.

Data Analysis and Statistics

The secretory responses were calculated as the difference between the basal Isc and the maximal effects (Δ Isc). Values for Isc were normalized per cm^2 exposed area. The number of experiments represented independent measurement on separate monolayers. Comparisons between groups of data were made by Student t-test (MiniTab Release 14.1). Treatment effects were considered significant at $P < 0.05$.

Results

Basal values of Isc were collected before the addiction of the toxin and AF- 16. In basal conditions, IPEC-2J cell monolayers maintained an Isc of 5.68 ± 0.03 μA/cm^2 (mean ± SE, n= 15).

Effects of CT on Isc. Different cholera toxin concentrations (0.02 to 1 μg/ml) were screened to evaluate the dose response, (Table 1). Once added to the apical bathing solution, CT induced a concentration-dependent increase of basal Isc. After a period of lag phase, that lasted about 30 minutes for all toxin concentrations used, Isc increased slowly, the highest value and the plateau were reached around 90 minutes after the beginning of the toxin treatment, (Figure 4). CT-induced secretory response was dose dependent and reached a value of Δ Isc= 5.60 μA/cm^2 (n=6) at the highest concentration (1 μg/ml).

Table 1. Secretory responses as changes of Isc (μA/cm^2) evoked by different CT concentrations.

Isc μA/cm^2	CT concentration (1 μg/ml)		
	0.02	**0.01**	**1**
Max	8.911	9.643	10.304
Min	5.089	5.340	4.696
Δ Isc	3.821[a]	3.802[a]	5.607[b]
Time Max (min)	91	96	90

[a, b] P<0.001

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Figure 4. Effects of CT (1 μg/ml) added to the apical side of the chamber on Isc in IPEC-2J cells.
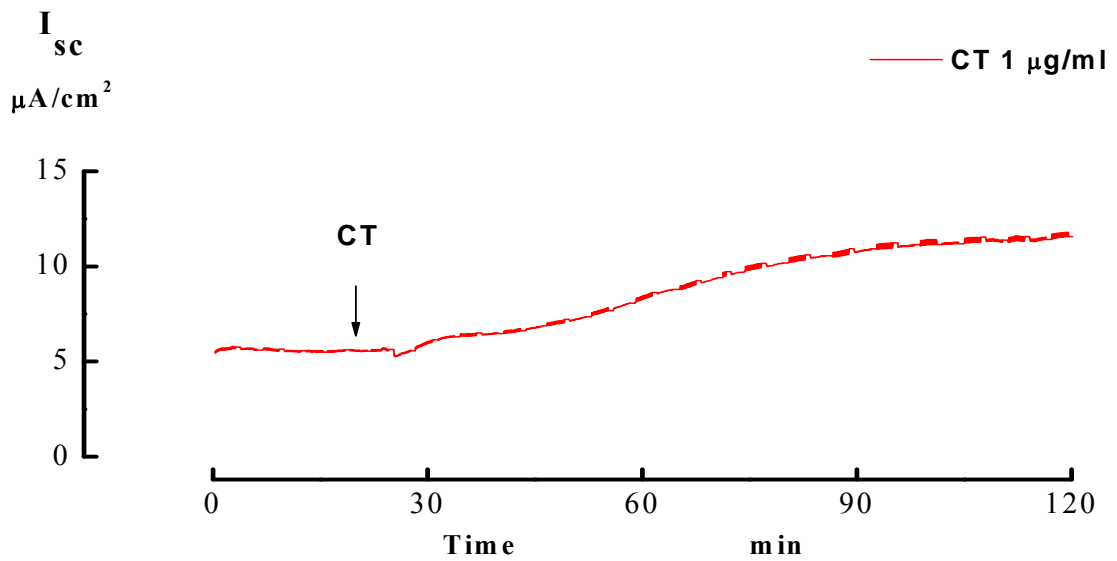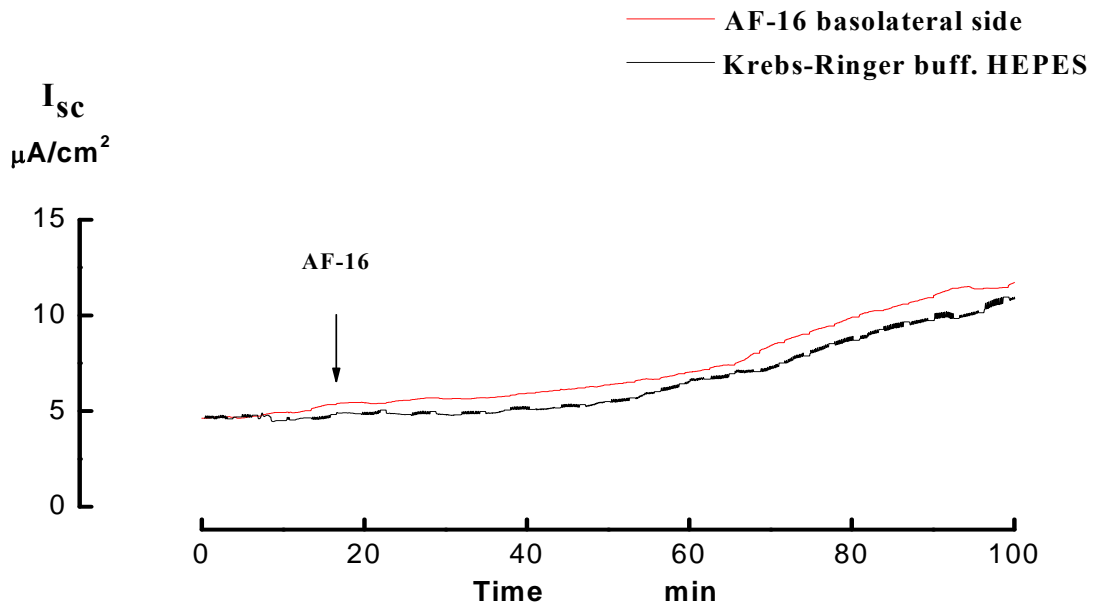

Figure 5. Effects of AF-16 (5.7x10-4 M) added to the basolateral side of the chamber**.**

Effects of AF-16 on Isc. The AF-16 concentration ($5.7x10^{-4}$ M) used to perform the tests was considerably higher than that used by Grøndal (2002). However, the peptide (A3) used by Grøndal and colleagues had a different aminoacids sequence, though it

overlapped the active region of the protein (AF-16) and, used at nanomolar concentration, it was unsuccessful on reducing different secretagogues-induced Isc increase. Observations of the *in vitro* tests done to assess the AF-16 anti-inflammatory properties induced us to use a higher concentration. Preliminary test were made to evaluate the effect of AF-16 on the basal Isc. As shown by Figure 5, AF-16 applied to basolateral bathing solution led to a slow increase of Isc. Diphenylamine 2-carboxylic acid is a non specific Cl^- channels blocker and a voltage-dependent CFTR/Cl^- blocker, which is thought to be the primary pathway for Cl^- and hence intestinal fluid secretion in c-AMP mediated diarrhoeas (Thiagarajah and Verkman, 2003). Applied after the AF-16 treatment DPC (2mM) reduced Isc by 30%, (Figure 6).

Figure 6. Effect of apical administration of DPC (2mM) on Isc of IPEC cells which were treated apically with AF-16 (5.7×10^{-4} M).
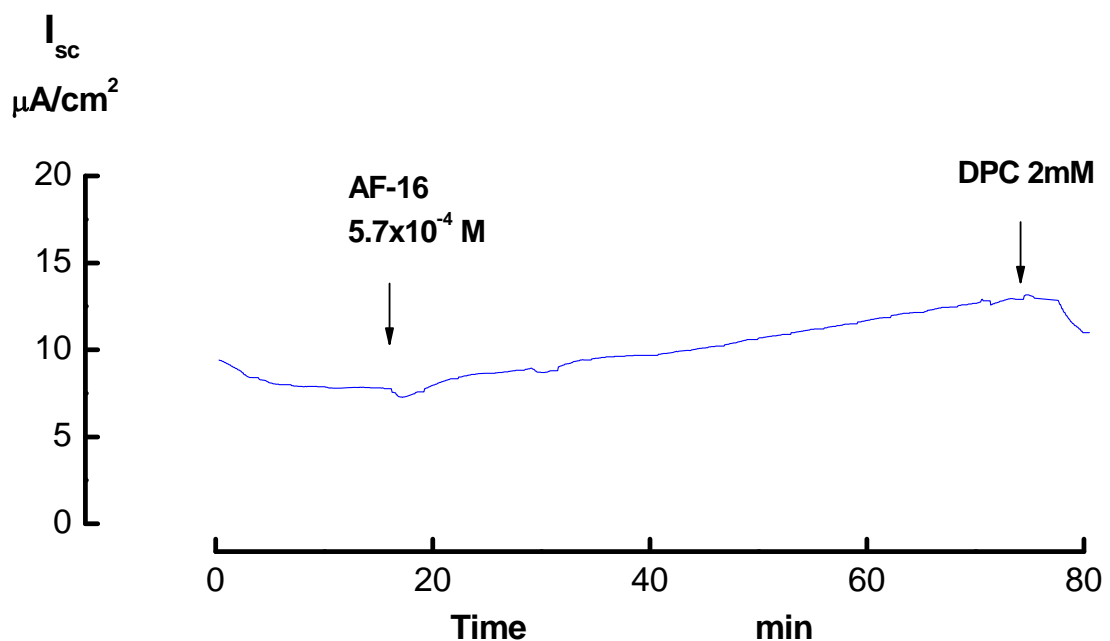
Effects of AF-16 on CT-stimulated Isc. The capability of AF-16 (5.7×10^{-4}M) on CT-induced (1 µg/ml) increase of Isc was studied. Depicted in *Figure*7 are the results from the experiments were CT and AF were administrated simultaneously. AF administration at both sides before and during the toxin treatment caused a slowly mounting increase in Isc (ΔIsc=2.03 and ΔIsc=1.79 µA/cm^2, respectively). When AF-16 was replaced by the bathing solution at the basolateral side, Isc increased faster +ΔIsc= 5.99 µA/cm^2, (+ 41% *vs* + 9%) see Table 2. The current increase was drastically blocked by DPC (2mM)

applied at the apical side: Isc lowered immediately by 30% (ΔIsc= - 4.69 $\mu A/cm^2$). When AF-16 was administrated after CT treatment, the same trend on Isc increase was noted since AF-16 reduced the rate by which Isc increased, but it did not cause a reduction of Isc (Figure 8).

Figure 7. Effect of AF-16 (5.7×10^{-4} M) added to both sides before CT challenge (1 $\mu g/ml$) and only at the basolateral side after the toxin challenge. DPC (2mM) was added apically at the end of the test to evaluate its effect on CT-induced anion secretion.
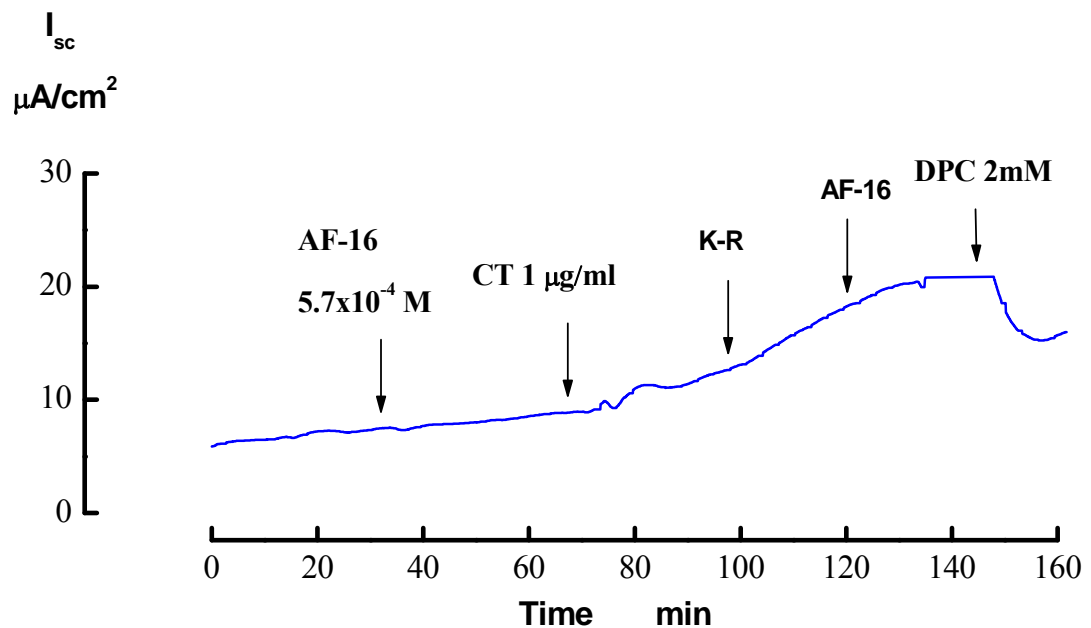
Table 2. Average Isc values and Δ Isc referred to Figure 7: AF-16 (5.7×10^{-4} M) was initially added to both sides. During and after CT challenge (1 $\mu g/ml$) the peptide was added only at the basolateral side. P value of Δ Isc reported under the table.

Isc $\mu A/cm2$	Basal	AF	CT +AF	CT_1	CT+AF	CT_2	DPC
Average Isc	6.83	8.10	9.85	13.62	19.25	20.38	15.68
SD	0.50	0.48	1.03	2.26	0.89	3.29	0.11
Δ Isc	1.46	1.39	2.03	5.99	1.79	4.65	-4.69

Basal *vs* **AF**   P< 0.001;   **AF** *vs* **CT+AF**   P< 0.01;
CT+AF *vs* **CT$_1$**   P< 0.001;   **CT$_1$** *vs* **CT+AF** P< 0.001;
CT+AF *vs* **CT$_2$**   P< 0.001;   **CT$_2$** *vs* **DPC**   P< 0.01;

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Figure 8. Effect of AF-16 (5.7×10^{-4} M) added to both basolateral and apical bathing solution and DPC (2mM) on CT-induced (1 μg/ml) Isc.
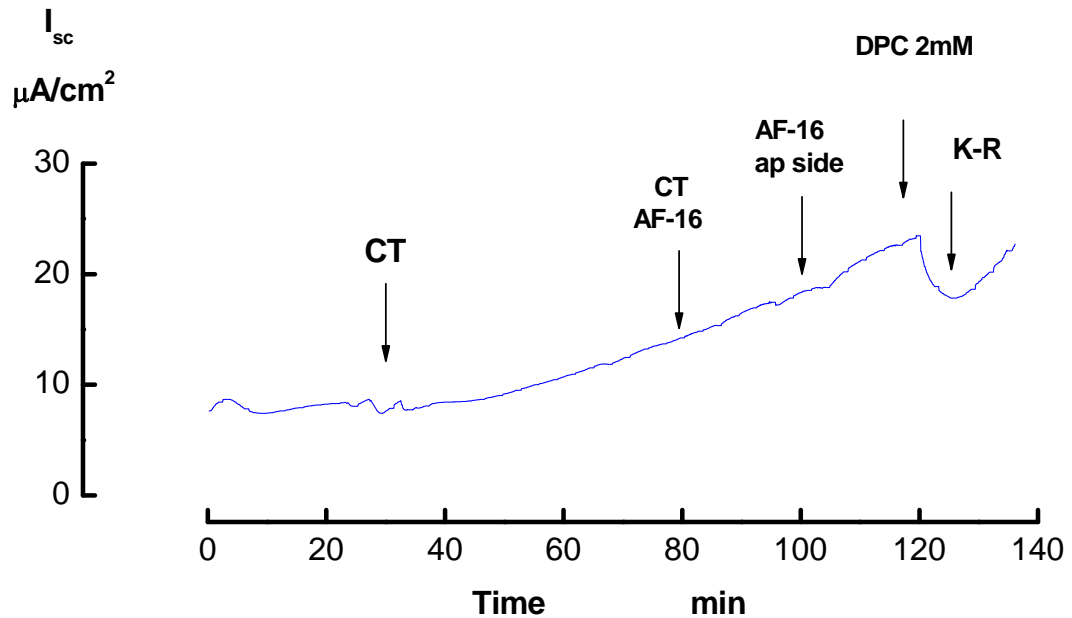

Table 3. Average Isc values and Δ Isc referred to Figure 8: AF-16 (5.7×10^{-4} M) was added to both sides during CT challenge (1 μg/ml) and only at the apical side after the toxin treatment. P value of Δ Isc reported under the table.

Isc μA/cm2	Basal	CT	CT +AF	AF	DPC
Average Isc	7.170	9.768	15.296	18.599	17.480
SD	0.39	2.00	0.90	1.32	1.56
Δ Isc	2.938	6.723	3.152	4.018	5.045

Basal *vs* **CT**   P< 0.001;   **CT** *vs* **CT+AF** P< 0.001;
CT+AF *vs* **AF** P< 0.001;   **AF** *vs* **DPC**   P< 0.01;

Discussion

Bacterial infections can cause severe fluid accumulation that may be associated to life threatening dehydratation. The bacterial enterotoxins stimulate secretion from crypt epithelial cells with luminally directed active transport of anions, mainly chloride and bicarbonate, which is the driving force for fluid secretion. However, when secretion increases beyond the ability of the colon to reabsorb water and electrolytes, it results in diarrhoea that may lead to severe dehydration if not treated in a timely manner.

AF-16 contains the active region of the human antisecretory protein. As previously discussed, the antisecretory factor has a strong antisecretory activity *in vivo*. Immuno-histochemical analysis and experimental evidences suggest the involvement of the nervous system in the AF-mediated antisecretory action. Electrophysiological studies on Deiter's cell membranes showed that AF-derived peptides, which were actives as antisecretory molecules in a rat *in vivo* model, inhibited GABA and chloride permeation (Rapallino *et al.*, 2003). As reported before in the text, bicuculline blocks the effect of AF on Deiters' cells, suggesting that AF acts via the GABA$_A$ receptor (Lange *et al.*, 1987).

According to Rapallino *et al.* (2003), AF and its derivatives counteracted intestinal hypersecretion by blocking the permeation of anions, with a possible inhibitory effect on the generation of action potentials in enteric nerve cells, which control the intestinal water and ion transport system.

Indeed, the intestinal secretion is controlled by local nervous reflexes and likely involves GABAergic neurons. GABA, acting on GABA$_A$ receptors, has a depolarizing and excitatory in the enteric nervous system. Kim *et al.* (2005) hypothesized that AF may act at the level of the enteric nervous system as a modulator of GABAergic transmissions in the gut, counteracting hypersecretion by decreasing the activity in secretory reflexes. The GABA$_A$ receptor agonists are known to act as secretagogues, whereas the GABA$_A$ receptor blockers exert antisecretory effects (MacNaughton *et al.*, 1996). According to Kim and co-workers (2005) AF does not appear to be a general GABA antagonist or agonist.

In the *in vitro* experiments here presented, AF-16 did not inhibit the toxin-induced hypersecretion in cultured IPEC cells, thus supporting the hypothesis of a neuronal-mediated activity of AF. Indeed the results demonstrated that secretory responses, induced by application of CT into the mucosal bathing media were reduced by AF, but

not suppressed. As reported by Li *et al.* (2003), cholera toxin and AF receptors are co-localized in the lipid raft of cell membrane. It is possible speculate that the reduced increase rate of CT-induced Isc observed during simultaneous administration of CT and AF-16, might be due to a kind of steric "disturbing effect" of AF-16 binding to its receptor flotillin-1, as suggested by Johansson *et al.* (2008). Although the effects of AF-16 on CT-stimulated Isc observed were significative (P= 0.001), it is difficult to find an explanation to our observation, since it is well known that the Ussing chamber technique performed with polarized cell monolayer has some limitation. More trials are required to define the direct effect of AF exogenous in the intestinal lumen, i.e. as consequences of AF-rich products administration, on enterocytes. *In vitro* studies with intestinal mucosa provided with outer muscles layers are necessary to elucidate which neuronal structures of the enteric nervous system are involved in the antisecretory activity of this multi-functional protein.

In vitro assessment of anti-inflammatory activity of AF-16

The immune system has been conventionally divided into innate immunity and adaptive immunity. The innate immune system non-specifically recognises foreign antigens and constitutes the first line of defence of the organism. It consists of cells such as polymorphonuclear leucocytes, mononuclear phagocytes, dendritic cells, mast cells and platelets. Moreover, humoral inflammatory mediators such as complement components are involved in the nonspecific response.

The adaptive cellular immune system consists of B and T lymphocytes, characterised by the presence of different receptors and can thus specifically recognise antigens.

Macrophages play a role in both the innate and the adaptive immune system. In the acquired immune system, macrophages act as antigen presenting cells (APCs) while in the innate immune system, they act as phagocytic cells and release different biologically active molecules such as oxygen and nitrogen radicals (Kròl *et al.*, 1995). Therefore, macrophages provide a link between the innate and adaptive immune response. During inflammatory reactions, macrophages have 3 major tasks: antigen presentation, phagocytosis and immunomodualtion through production of a variety of cytokines and "growth factors" (Fujiwara and Kobayashi, 2005).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are known to play a double role in biological systems, since they can be both harmful and beneficial to the organism (Valko *et al.* 2006).

NO is a critical mediator of inflammation and it is released by different cell types such as activated macrophage (Hibbs *et al.*, 1988; Azadmehr *et al.*, 2009; MacMicking *et al.*, 1997). Nitric oxide (NO) is a short-life free radical synthesized from arginine that has a regulator/mediator role in several biological processes, including host defence, neurotransmission, blood vessel dilatation and platelet aggregation (Moncada *et al.*, 1991; Nathan, 1992).

Under physiological conditions, NO mediates anti-inflammatory reactions in order to prevent autoimmunity, although it may exert regulatory or cytotoxic effects depending on the concentration acting on the target cell (Stuehr and Nathan, 1989). Excessive NO production has been related with chronic inflammatory diseases, septic shock and autoimmune diseases (MacMicking *et al.*, 1995; Guzik *et al.*, 2003).

Reactive oxygen species (ROS), such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are commonly produced during inflammatory processes and play an important role in host defence against infections. Phagocytosis and the capability to initiate a respiratory burst by producing ROS, are the main cytotoxic properties of phagocytes (Miller and Britigan, 1997). At moderate concentrations, ROS play an important role in redox homeostasis as regulatory mediators in the signaling pathways and in maintaining physiological cell processes and functions (Dröge, 2002). However, when cellular production of ROS overwhelms its antioxidant capacity a state of oxidative stress is reached, leading to serious cellular injuries and contributing to the pathogenesis of several diseases. As previously discussed, the endogenous AF protein counteracted inflammation and abnormal fluid transport in relation to intestinal diarrheal diseases (Björck *et al.*, 2000; Lange and Lönnroth, 2001), and Ménière's disease (Hanner *et al.*, 2004). The immunohistological distribution of AF suggests that this protein may play a role in the immune system as a neuromodulator and a potent anti-inflammatory agent. In rat jejunal loops, AF inhibited the inflammatory response caused by toxin A from *Clostridium difficile* (Björck *et al.*, 2000). AF has been reported to counteract inflammatory reactions in experimental autoimmune encephalomyelitis (EAE). Davidson and Hickey (2004a, 2004b) hypothesized that the increased AF expression observed in rats during the course of EAE could be a means of counteracting the pro-inflammatory environment and of limiting the tissue damage. We tested the AF-16 peptide anti-inflammatory activity in cultured macrophages challenged with inflammatory stimuli. The lipopolysaccharide (LPS)-challenged RAW 264.7 murine macrophages and phorbol myristate acetate (PMA)-challenged pig alveolar macrophages were used as model system.

All the *in vitro* experiments were performed at the Katholieke Universiteit of Leuven under the guidance of Prof. T.A. Niewold.

Materials and methods

Test substance

The AF-16 peptide (VCHSK TRSNP ENNVG L, m.w. 1754.95)**,** synthesized by ordinary solid phase technique, was generously provided by Prof. Lange of the Goteborg University (SW).

The peptide was carefully dissolved to a concentration of 1mg/ml in MilliQ water, divided into aliquots of 50 μl and stored at -20°C until use.

The molar AF-16 concentrations used to perform the NO assay were (M):1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, 1×10^{-8}, 1×10^{-9}.

ROS test was performed using the following concentrations (M): 1×10^{-5}, 1×10^{-7}, 1×10^{-9}.

Nitric oxide assay

In RAW 264.7 cells, LPS induces inducible nitric oxide synthase (iNOS), and then NO production. Therefore, this macrophage cell line provides an excellent model for the evaluation of AF-16 as potential inhibitor of the pathway leading to the induction of iNOS. The reactive free radical NO synthesized by iNOS is a major macrophage-derived inflammatory mediator and has been reported to be involved in the development of inflammatory diseases. NO released from cells can be detected and quantified photometrically, as its stable product nitrite, by a simple colorimetric reaction (Griess reaction).

Cell Culture

Murine macrophage RAW 264.7 cell line, obtained from the American Type Culture Collection, was cultured in antibiotic free Dulbecco Modified Eagle Medium (DMEM +4mM $^{L-}$ glutamine, 4.5 g/l glucose, GIBCO) with 10% heat-inactivated fetal bovine serum and 2% of $NaCO_3$, at 37 °C under 5% CO_2.

Before performing the tests, cell viability was assayed by trypan blue exclusion test, based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, whereas dead cells do not. Briefly, 10 μl of cell suspension were dissolved in 90 μl of trypan blue solution and mixed. A drop of the solution was placed on a Burker chamber and cells were observed and counted under a binocular microscope.

Reagents

The AF-16 solutions with known peptide concentration used to perform the test were always prepared fresh in DMEM medium supplemented with 10% of FBS. Lipopolysaccharide (LPS) stock solution (5 mg/ml in milliQ water) was diluted with medium to a final concentration of 50 ngLPS/ml. The $NaNO_2$ stock solution was obtained by dissolving 690 mg of $NaNO_2$ in 10 ml of RAW cell medium, to obtain a

1M concentration. The stock solution was serially diluted with medium to obtain the known molar concentration of $NaNO_2$ for the calibration curve: 100-50-25-12, 5-6.25-3.13-1.56 and 0μlM. Oxytetracycline (OTC; Sigma Chemical Co., St. Louis, MO, USA) was used as positive control in the plate (700μg of OTC in 10 ml of medium). GRIESS-reagent used was a ready stock reagent (Sigma Chemical Co., St. Louis, MO, USA).

Experimental protocol

The grown RAW cells were removed from the culture flask, transferred to falcon tubes (50 ml) and centrifuged for 10' at 1500 rpm. The pellet was resolved in fresh medium: cells were counted and diluted to a final concentration of $1x10^6$ cells/ml. After that, 100 μl of cell suspension ($1x10^6$ cells/ml) were seeded in a 96-well microtiter plate and incubated for 24 hours at 37°C in 5%CO_2 humified atmosphere. At the end of incubation, the medium was removed and, following the experimental schedule, 50 μl of fresh medium or medium with various AF-16 concentrations were added to the wells. The plate was then incubated for 4h at 37°C in 5%CO_2 atmosphere. OTC was used as positive control in the last two rows of the plate. The incubation time for OTC was 10 minutes. In order to stimulate the inflammatory response by cultured macrophages, 50 μl of fresh medium with LPS (10 ng/ml) were added to the wells, following the experimental schedule (Figure 1). The plate was incubated for 24h at 37°C in 5%CO_2 atmosphere. After challenge with LPS, aliquots of cell culture supernatant (100 μl) were transferred to a new plate and mixed with 100 μl of Griess reagent. The plate was then incubated at room temperature for 10 min. Sodium nitrite was used to generate a standard curve and the nitrite concentration was determined by measuring the optical density at 540 nm with a Wallac Victor2 1420 Multilabel Counter. Assays were performed in quadruplicate.

Figure 1. Plate scheme used for NO assay.

		AF		AF		AF		AF			
	St. control	med	LPS	med	LPS	med	LPS	med	LPS	med+OTC	OTC+LPS
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Reactive Oxygen Species Assay

Reactive oxygen species (ROS) are known to play important roles in cellular systems, they are fundamental in the clearance of invading microorganisms by phagocytes of the innate immune cells.

Stimulation with phorbol myristate acetate (PMA) induces ROS release by alveolar macrophages.

The fluorescent probe dihydrorhodamine 123 (DHR) has been shown to respond to a number of species including superoxide, hydrogen peroxide and peroxynitrite, which oxidize DHR123 to yield the fluorescent product rhodamine 123 (Henderson *et al.*, 2009). The fluorescence observed is therefore directly proportional to intracellular concentrations of ROS.

Cell culture

Alveolar macrophages (AMs) were isolated from the lungs of piglets as described by Ure at al. (2002). Briefly, lungs were aseptically removed from euthanized pigs. A funnel was placed in the trachea and 200 ml of PBS were poured into the lungs. The

lungs were gently massaged, the lavage fluid was collected into sterile falcon tubes (50 ml), immediately put on ice. The procedure was repeated two more times and the tubes were centrifuged at 1500 rpm for 10 minutes at 4°C. The supernatant was removed and the pellet was resolved in PBS and centrifuged at the same conditions to eliminate the erythrocytes. Macrophage cells were resolved in RPMI 1640 medium (Sigma Aldrich, St. Louis, MO) with 10% FBS (GIBCO) and 1% of antibiotic/antimycotic (10 mg streptomycine and 25 μg amphotericine per ml, Sigma Aldrich, St. Louis, MO). The cell viability was evaluated with the trypan blue dye exclusion test (>95%). The purity and morphology of AMs was determined by May-Grünwald-Giemsa staining of air-dried cytospin smears. The cells were counted, divided into aliquots and stored in liquid nitrogen until use.

Reagents

Phorbol 12-myristate 13-acetate (PMA) (Sigma, St. Louis, MO) was dissolved in dimethyl-sulfoxyde (DMSO) to obtain a stock solution (10^{-3} M) and further diluted in Hanks balanced salt solution (HBSS) before use. Dihydrorhodamine 123 (DHR, Molecular Probes Europe, Leiden, The Netherlands) stock solution (2.5 mg / ml in DMSO) was diluted in HBSS before use.

Experimental protocol

Tests were performed using a cell suspension with a final concentration of 1×10^6 cells/ml.

Briefly, 50 μl of cell suspension were seeded in a microtiter black plate. The plate was incubated for 2 hours at 37°C in 5%CO_2 atmosphere, to allow cells adhesion to the wells bottom.

After incubation the supernatant was removed with a multichannel pipette and 50 μl of medium with or not variuos AF-16 concentrations were added to the wells following the experiment scheme (Figure 2). The plate was incubated for two hours at the same temperature and atmosphere conditions. After that, wells were rinsed with 100 μl heated HBSS. The rinsing step was repeated 3 times.

Following the experiment schedule, 50 μl HBSS or 50 μl medium containing PMA were added to the wells. The plate was wrap with an aluminium foil and incubated for 15' at 37°C on a plate shaker. After the first step, 50 μl HBSS or 50 μl DHR were added to the

wells (Figure 2) and the wrapt plate was incubated at the same conditions for 25 minutes.

Production of intracellular ROS was measured by the fluorescence dye dihydrorhodamine-123. Fluorescence was measured using a Victor 1420 Multilabel Counter, with excitation at 488 nm and emission at 530 nm.

Figure 2. Plate scheme used for ROS assay.

step 1		HBSS		HBSS		PMA		HBSS		HBSS		PMA
step 2		HBSS		DHR		DHR		HBSS		DHR		DHR
	1	2	3	4	5	6	7	8	9	10	11	12
AF-16												
AF-16												
AF-16												
AF-16												
AF-16												
AF-16												
C												
C												

Results and Discussion

As previously reported, the antisecretory factor is expressed by cells of the immune system, primarily macrophages, and appears to regulate the immune response. Furthermore, the expression levels and distribution of AF are markedly altered during an immunological response. According to Davidson and Hickey (2004b), *in vitro* administration of TLD-1A8A antibody that specifically recognizes AF, resulted in increased proliferation of memory/effector T cells. In a rat model of experimentally induced autoimmune encephalomyelitis, administration of anti-AF antibody enhanced the severity of the symptoms and increased the proinflammatory cytokines production (Davidson and Hickey, 2004a).

The anti-inflammatory activity of the antisecretory factor has been reported by Johansson *et al.* (1997) who found that AF, at picomolar concentration, abolished

cytoxic and inflammatory reactions in intestinal mucosa of rats challenged with Toxin A from *C. difficile*.

AF-rich egg yolk provided to patients affected by chronic inflammatory bowel disease, as a supplement to conventional pharmacological therapy, was found to improve inflammation, as demonstrated by rectal biopsies (Eriksson *et al.*, 2003). In this work the *in vitro* anti-inflammatory activity of AF-16, at different concentrations, on stimulated macrophages was investigated. Macrophages play a critical role in the initiation, maintenance, and resolution of inflammation. Upon activation, macrophages release a set of primary inflammatory mediators involved in both beneficial and detrimental outcomes in inflammation. LPS, as an outer membrane component of bacteria, triggers the secretion of nitric oxide, which is extremely reactive and has a wide range of physiological activities involved in the immune response (Ignarro, 2002). The large amount of NO produced in response to bacterial lipopolysaccharide plays an important role in endotoxemia and inflammatory conditions (Bellot *et al.*, 1996). In this experiment, the basal NO production of the RAW 264.7 culture, in absence of drugs, was 0.76 ± 0.10 μM (n=8). In agreement with the data of others (Shen *et al.*, 2002; Wadsworth *et al.*, 2001), we found a huge NO release by LPS-stimulated cells as opposed to untreated control. It was found that AF-16 treatment reduced nitric oxide production by LPS-stimulated macrophages in a dose depend fashion, with major effects observed at the highest dosages (10^{-4} and 10^{-5} M, P< 0.05), see Table 1. AF-16 showed a mild anti-inflammatory effect only at the highest dosage. Indeed, nitric oxide production by LPS-stimulated macrophages was significatively reduced only by AF-16 at 10^{-4} M, (28.01 *vs* 32.17 μM, P< 0.05). As shown by Figure 3, NO production by activated macrophages was also reduced by AF-16 used at lower concentration, though the differences with the control were not significant.

The mild anti-inflammatory activity of AF-16 was not confirmed by the ROS assay, neither at the highest dosage, (Figure 4). In fact, pre-treatment with various AF-16 concentrations for 2 h did not significantly reduced the PMA-induced ROS generation in pig alveolar macrophages (P< 0.05). In macrophages, like in other cell types, ROS influence signalling pathways in diverse ways and participate in the signalling cascade triggered by inflammatory mediators, including cytokines and chemoattractants. The contradictory results of the tests might be related to errors in the experimental design. The alveolar macrophages used to do the experiments were from different piglets, so this may be a source of error. The reduction of NO release after AF-16 treatment could

be due to the blockage of LPS activity and consequently the iNOS inhibition. Unfortunately this enzyme was not determined in our experiment.

Figure 3. Evaluation of AF-16 effects of NO production by LPS stimulated RAW 264.7 macrophages. AF-16 reduced LPS-induced release of NO at the higher doses. Values are mean ± SEM of 6 experiments performed in quadruplicate. * $P < 0.05$ compared with untreated control.
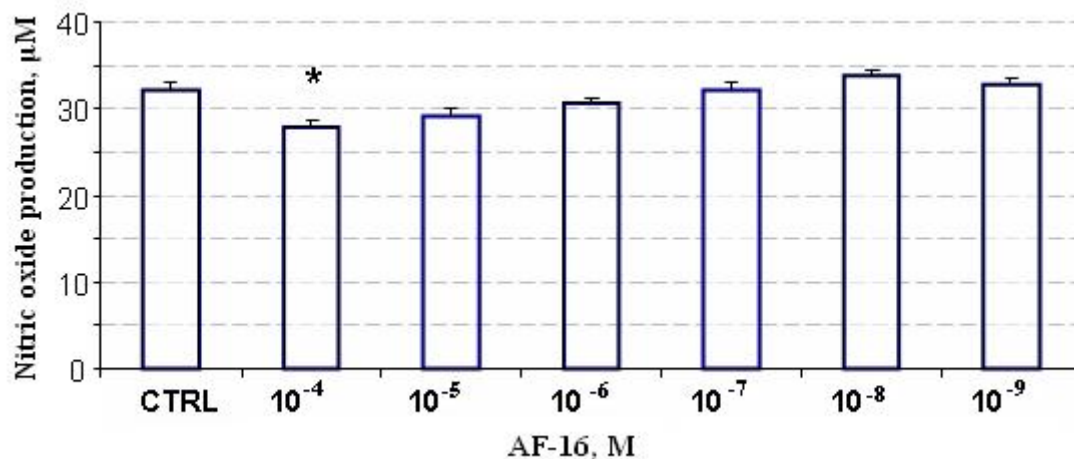

Moreover, the presence in AF-16 of cysteine, which contains a SH group, and proline, which is a well known free-radicals scavenger, might have acted as a scavenger for unpaired electrons, thus reducing free radicals generation (Sunman *et al.*, 1993; Nunes *et al.*, 1995). Since NO is a vasodilatative molecule, a reduction in its synthesis fit well with the *in vivo* reduction of gut permeability in cholera-challenged rats after AF administration (Lange *et al.*, 1998). NO synthesized normally diverts O_2 from ROS pool to produce peroxynitrite and reduce ROS concentration (Chabot *et al.*, 1998). However the lack of evidence about an effect of AF on ROS production could not be explained in this way because pigs alveolar macrophages do not produce NO (Zelnickova *et al.*, 2008).

Table 1. NO production (μM) by RAW 264.7 macrophages grown with different amounts of antisecretory factor (AF).

		AF concentration (μM)								
CTRL	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	SEM	Linear	Quadratic	Cubic
32.17[bc]	28.01[a]	29.16[ab]	30.63[ab]	32.15[bc]	33.81[b]	32.86[bc]	1.64	0.0167	0.4288	0.4166

[a,b,c] ($P<0.05$)

Figure 4. Production of intracellular ROS in pig alveolar macrophages, stimulated by PMA, after 2 h of incubation with different concentration of AF-16. Values are mean ± SEM of 6 experiments performed in duplicate. There were no significative differences between treatment and between treatment and control. P < 0.05 compared with untreated control.
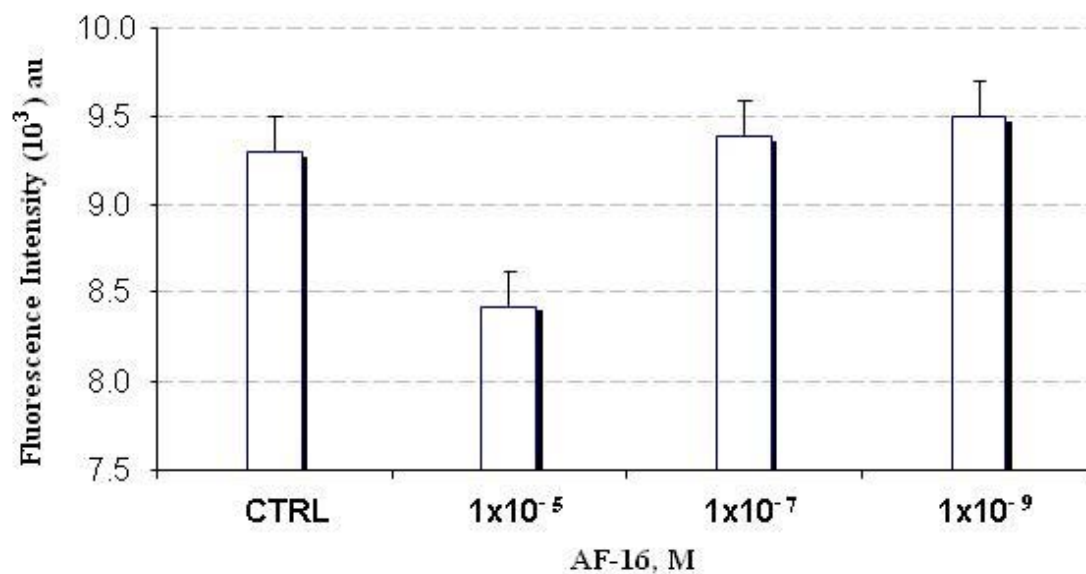
General Discussion and Conclusions

In recent years, nutrient supply has ceased to be the main goal of nutrition, both for humans and animals. Foods and feeds are now considered as possible tools to improve health and wellness. Well-known foods like grains, tomatoes, or milk are studied for health promoting activity of nutrients such as β-glucans, lignans, lycopene or conjugated linoleic acid.

In pig intensive husbandry nutritional, social and environmental stresses are common, particularly at weaning. For more than 50 years, AGP provided a protection against the negative effects associated to intensive breeding practices on animals' health, and permitted better growth performance. After the AGPs complete ban in European Union, the detrimental effects observed on piglets health and growth performances prompted the research for new feed additives. New nutritional and management strategies, aimed to reducing the economic loss caused by increased production length and higher veterinary expenses, have also gained interest.

So far, none of the substances proposed is as satisfactory as the in-feed antimicrobials.

According to Piè *et al.* (2004), weaning in piglets is associated with a transient inflammatory response that may contribute to both anatomical and functional intestinal disorders in piglets.

Therefore, immunomodulating compounds are gaining interest, since the correct development of both innate and acquired immune system of the piglets is strategic in determining the outcomes of a large exposure to antigens and stress conditions, especially in intensive farming (Gallois *et al.*, 2009). AF-inducing diets such as the hydrothermally processed cereals appear to be suitable candidates as replacers of antibiotic growth promoters. Lönnroth *et al.* (1988) found a correlation between AF concentration in milk and incidence of diarrhoea in the offspring.

In vitro and clinical studies evidenced its potent capability to counteract intestinal fluid imbalance of various aetiologies, meanwhile recent reports focused on its immunomodulatory and anti-inflammatory properties.

We tested the effect of two different HPC level of inclusion in a wheat-barley based diet fed to weaned piglets growth performance, reared in an experimental farm. The results from the field trial confirmed the efficacy of the HPC as growth promoters in piglet nutrition: compared to the 3% supplemented diet and the control group, 6% of HPC supplementation improved average daily gain and feed conversion rate. The growth

improvement observed might be explained by two theories. First, according to Göransson (1997) the hydrothermally processed cereals present in the diet might have induced higher AF values in piglets plasma. Therefore, given the physiological inflammatory state of the intestinal mucosa, AF might have exerted an anti-inflammatory activity due to the down-regulation of the immune response. The energies saved might be conveyed to growth. However, the trial was conducted in an experimental facility, with optimal sanitary conditions and both blood haptoglobin and I-FABP were low for all the piglets.

The differences in growth performance between HPC-fed piglets and the control group might due to a balanced supply of energy-yielding nutrients and amino acids. The *in vitro* digestion assays evidenced relevant simple sugars content and a higher rate of starch degradability in the HPC diets compared to the control. Starch in cereals has a great importance in newly weaned piglets since it constitutes the largest portion of the diet and is the major energy-yielding component, so the rate of starch digestion is a crucial characteristic that rules the dietary energy value of compound diets (Wiseman, 2006; White *et al.*, 2008). The first week after weaning is characterized by reduced feed intake and reduced pancreatic enzymes activity, therefore feeding a diet with highly digestible starch guarantees a correct amount of energy, limiting the amount of starch available for microbial fermentation in caecum and colon.

In vitro test were made to improve our knowledge about the antisecretory and anti-inflammatory properties of the AF protein and its mechanism of action by using AF-16, a16-meric peptide defined as the active region of the protein.

The Ussing chamber provides a valuable, time-proven method for the measurement of electrolyte, nutrient, and drug transport across epithelial tissues (Clarke, 2009). This is a useful tool to study the effects of alternatives to in-feed antibiotics applied to the animals *in vivo*.

The Ussing chamber experiments performed on polarized IPEC-J2 cells confirmed the neuronal involvement in the antisecretory activity of AF. In fact, AF-16 did not inhibit CT-induced Isc increase as did DPC, a voltage-dependent CFTR/Cl⁻ blocker. A slower rate of Isc increase was observed during the simultaneous administration with AF-16 and CT.

A recent study by Johansson *et al.* (2008) hypothesized that AF binding to the integral membrane protein flotillin-1, localized in the lipid rafts, causes an alteration of ion channels and receptors localization. It is possible to speculate that the effects observed

on CT-induced Isc might be due to a kind of "disturbing effect" on the toxin binding to GM1, which is also localized in the lipid rafts. However, it is difficult to find a correct explanation to our observation, since the Ussing chamber technique performed on polarized cell monolayer has some limitation. As reported by Clarke (2009), polarized cell monolayers produce lower responses in transport function compared with epithelial cells layer of the native intestine because of the reduced number of available epithelial cells within the same surface area. Also the microscopic architecture of the intestine contributes to the alterations in drug responses by the epithelium.

Furthermore, cholera toxin requires a long time to exert its effect on Isc, while the monolayer cells have not a long viability. More *in vitro* experiments are required to define which are the direct effects of exogenous AF, due to AF-rich products intake, or those related to endogenous AF, i.e. induced by HPC, on the enterocytes. *In vitro* studies on the intestinal mucosa, provided with outer muscles layers, are necessary to elucidate which neuronal structures of the enteric nervous system are involved in the antisecretory activity of this multi-functional protein.

As mentioned above, the intestinal mucosa has been defined an organ in state of constant and controlled inflammation, processes that require a high energy expenditure (McDonald and Monteleone, 2005).

Hyckey and Davidson (2004a, 2004b) found that AF is expressed in immune system cells and that it is able to down regulate the immune response in a rat model of encephalomyelitis.

 To evaluate the anti-inflammatory activity of AF, as a possible immunomodulating feed additive, two kind of in vitro assays were made.

High dosages of AF-16 were found to reduce the LPS-stimulated NO production in RAW264.7 cell, thus supporting the hypothesis of an anti-inflammatory action. The effects observed could be due to the blockage of LPS activity and consequently the iNOS inhibition. Moreover, the presence in the peptide sequence of cysteine, which contains a SH group, and proline, which is a well known free-radicals scavenger, may act as a scavenger for unpaired electrons, thus reducing free radicals generation. On the contrary, no significant results were obtained on PMA-stimulated ROS generation in pig alveolar macrophages.

The literature review showed how the AF-inducing diets and the AF-therapy is an interesting approach to improve welfare in humans affected by several disease

Furthermore, the inclusion of HPC in the diet of lactating women could be exploited in order to reduce intestinal disorders in suckling children, particularly in developing countries, where breastfeeding is highly recommended and the access to anti-diarrhoeal drugs and/or rehydrating solutions are poor. As suggested by Zaman *et al.* (2007), these active cereals can be used as prophylaxis in much exposed populations during the diarrhoea season.

The recent study by Säljö *et al.* (2009b) showed the effect of diet supplementation with HPC on the prevention of harmful effects of exposure to low levels of blast overpressure in rats, reducing the extent of intracranial pressure increase and cognitive function impairment. So far, there are few studies on the effects of blast related brain injuries on brain function in the long term period, but their prevention by dietary tools is an interesting perspective.

Moreover cereals are part of daily food intake in many countries, so it could be easy to improve the consumption of active cereals particularly by people exposed to the risk of blast related brain injuries, such as soldiers during training session or conflicts (Säljö *et al.*, 2009b). Cereals might also be helpful for people subjected to the risk of intracranial pressure increase, i.e. patients affected by brain pathologies like tumors in conditions of sudden pressure changes, as during long flies, or exposed to overpressure as divers. AF-rich egg yolk powder gives the opportunity of a direct and fast AF administration to young children and to people with impaired food consumption. It is remarkable that the hydrothermally processed cereals, being simple cereals kernels, may have a positive image towards the modern European consumers, who are increasingly sensitive to ethical considerations and whose opinion has a great influence on legislation (Florkowsky *et al.*, 1998). Compared to other possible alternatives to antimicrobial growth promoters, these processed cereals do not contain any active compound that can be lost in the upper gut, since their action is to stimulate the animal capability to produce it. In addition, they do not pose any environmental harm such as the use of molecules like ZnO, that even with proved growth promoting and anti-diarrhoeic properties, accumulates in the soil (Jondreville *et al.*, 2003). In our field trial, we showed that the AF-inducing diet improved the feed conversion rate. It is well known that a more efficient use of nutrients present in the diets leads to decreased nutrition costs and reduced nitrogen and phosphate excretion in manure and their accumulation in the soil.

Aknowledgements

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