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Nutrition-based health in modern animal production

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CHAPTER 1

Foreword

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1.1 Animal health in modern animal production: current challenges

Animal health in modern animal production is a major challenge on a global basis as it impacts upon both human health and economics, being a primary factor in determining safety, quality and health benefits of food of animal origin. Traditionally, the control of animal diseases relied upon the use of in-feed antibiotics, which have been used in animal production for more than 50 years as feed additives. Inclusion of antibiotic growth promoters (AGP) in animal diets has been documented to improve growth performance and feed efficiency, to reduce morbidity and mortality (by approximately 50% in pigs), as a consequence of suppression of pathogenic bacteria in the digestive tract, increased feed utilization and stimulation of metabolic processes. However, the use of AGP has raised increasing concerns about antibiotic residue problems in animal products and about the development of antibiotic resistant bacterial strains, which may impair the effectiveness of certain antibiotics in the treatment of human diseases. The possible association between in-feed antimicrobials and the spreading antibiotic resistance to bacterial infections in humans caused the ban of the use of antibiotics as growth promoters within the EU (EC Regulation 1831/2003).

Reduced performances, increased incidence of disease and mortality and increased therapeutic use of antibiotics in swine production has been documented in some European countries as a consequence of the discontinuation of using AGP (Kil and Stein 2010). These issues accentuated the need for alternative strategies to prevent diseases, focusing attention on nutrition as a practical solution for health maintenance and disease avoidance of animals raised for food. Animal nutrition, for long time based on the need to avoid deficiency symptoms and to support the economically important production criterion, in recent years has driven the research of new strategies for disease prevention, and a better understanding of the interactions between nutrition and health introduced the concept of nutrition-based health for animal production (Adams, 2006). Various nutritional approach have thus been tested in the past decades in order to manage critical periods in the lifespan of the animal, such as the weaning period of the piglet or the transition from late pregnancy to lactation in dairy cattle, when the animal is more exposed to diseases.

The main theme of this research concerns the nutritional management of some health problems frequently occurring during the weaning of piglets, focusing in particular on the use of feed additives and immunogenic products derived from engineered plants.

With regard to the management of critical periods of the productive cycle of ruminants, I have also examined the involvement of oxidative stress in certain disorders of dairy cattle and the role of micronutrients, in particular of vitamin E and selenium, in maintaining pro-oxidant/antioxidant balance, thus contributing to the safeguard of animal health and quality of food of animal origin.
The results of this literature research are the subject of a published review paper reported in the Appendix.

1.1.1 Critical periods in pig farming: the weaning transition

Italian pig farming, with a consistency of 9,321 thousand heads (53% fattening pigs from 50 to over 110 kg, 39% pigs up to 50 kg, 8% breeding pigs) represents 6.1% of the total pig production in the EU-27 (data 2010, Source: Eurostat).
About 85% of pig production is concentrated in the Italian Pianura Padana, in regions traditionally dedicated to the production of heavy pigs (over 110 kg), for the production of hams and cured meats. In the last decades, Italian pig farming was involved in deep structural and organizational changes, in order to comply with the changing needs and demands from the domestic and European markets. In this regard, over the years intensive production systems where animals are placed in separated "sites", for reproduction, post-weaning and growth-fattening phase respectively, have become increasingly common in order to develop an appropriate biosecurity system to improve the health of animals and their production performances.
Among the different phases of production, the weaning transition is a particularly complex period, during which the piglets are confronted by multiple stressors. Weaning involves complex social changes, including abrupt separation from the sow, a new housing system, separation from littermates and mixing with other litters in a new environment. Diet composition also changes at weaning: the liquid highly digestible milk from the sow is replaced by a less digestible and more complex dry feed. Early weaning (21 days or before) in intensive production systems is more likely to exacerbate the level of general stress, contributing to increased mortality, decreased growth performances and abnormal behaviour of piglets (Kil and Stein, 2010).
The immediate effect of weaning is a dramatic reduction in feed intake and a consequent 'growth check' (Pluske *et al.*, 1997), which continues to represent a major source of production losses in commercial piggeries.
Weaning also causes alterations in intestinal architecture and function, predisposing to diarrhoea and intestinal infections, particularly from enterotoxigenic *E. coli* and *Salmonella*. Intestinal alterations often seen in newly-weaned piglets include reduction in villous height and increased crypt depth, reduced activities of intestinal digestive enzymes, disturbed intestinal absorption, secretion and permeability (Boudry *et al.*, 2004; Lalles *et al.*, 2004).

Low feed intake and stress related to weaning seem to play a major role in intestinal tissue damage and in the small intestine epithelial barrier function impairment (Pluske *et al.*, 1997, Spreeuwenberg *et al.*, 2001, Lalles *et al.* 2007).

1.2 The management of post-weaning disorders

A correct and timely functional development of the gastrointestinal tract (GIT) play a key functional role in the growth of the young piglet and in particular for growing digestive, absorptive and immune functions (Domeneghini *et al.*, 2006). Recent insights into GIT physiology, microbiology and immunology and considerable advances in the understanding of intestinal nutrient utilization provided a basis for the nutritional management of GIT health around weaning. Formulation of young pig diets assumed the specific task of optimizing growth, function and health of the gut and a large amount of research has been conducted in recent years to evaluate the impact of several feed ingredients and feed additives on various aspect of gut health and development. Extensive recent reviews have summarized all research conducted on this area (Lalles *et al.*, 2007; de Lange *et al.*, 2010).

The management of post-weaning disorders without the use of AGP should consider management strategies minimizing stress at weaning (i.e. weaning age, hygiene conditions, ventilation and heating), as well as feeding strategies stimulating feed intake and improving gut structure and function, such as the use of high-quality feed ingredients, application of enzymes, reduced content of protein fermented in the gut, minimal buffering capacity, minimal content of anti-nutritional factors and supply of gut health promoting feed additives (de Lange et al, 2010; Kil and Stein 2010). As proposed by Lalles *et al.* (2010), gut health promoting feed additives can be classified by the purpose of their use into: (1) enhancing the pig's immune response (e.g. immunoglobulin; ω-3 fatty acids), (2) reducing pathogen load in the pig's gut (e.g. organic and inorganic acids, zinc, plant extracts, some types of prebiotics and anti-microbial peptides), (3) stimulating establishment of beneficial gut microbes (probiotics and some types of prebiotics), and (4) stimulating digestive function (e.g. butyric acid, gluconic acid, lactic acid, glutamine, threonine, cysteine, and nucleotides).

All these additives have the potential to improve growth performances and to minimize disease susceptibility around weaning, and most of them show promising results, although not yet comparable with those of AGPs.

However, more research will be needed to assess metabolic pathways and optimal dosage of these compounds..

To date, not a single strategy has been proved to be fully effective and a combination of different approaches seems to be the most effective alternative to the use of AGPs.

1.2.1 Milk biologically active components as potential health-enhancing nutraceuticals for the weaning phase

The benefits of milk go beyond its ideal balance of nutrients for digestive and metabolic requirements of the newborn. Over the years, scientific research in humans has shown a "functional" role of milk, due to the presence of several bioactive components, with beneficial properties not only for the development of the newborn, but also for the health and the prevention of diseases in adults (for reviews, see Severin and Wenshui 2005, Bauman *et al.* 2006). The results of these studies, conducted mostly *in vitro* or in animal models, including pig in particular, have opened up interesting prospects for the use of these substances as supplements or food products, for a nutritional prophylaxis alternative to AGPs in pigs.

Among the native components of milk, specific proteins and peptides, nucleotides, fatty acids, vitamins, enzymes and oligosaccharides play "extranutritional" functions, such as modulation of intestinal microflora, gut-trophic effects, protection from pathogens or immunomodulatory activities. These substances may also be called "nutraceuticals", for their protective effects and benefits on piglet health, both in the newborn and in the later stages of its growth. A better characterization and understanding of the biological functions of these substances may represent an interesting basis for the development of new prophylactic and therapeutic agents to be used in the early stages of weaning, in order to sustain growth and performance and to reduce the incidence of health problems, thus ensuring continuity in the critical transition from milk to feed.

During the first year of my PhD I explored the "functional" role of milk and the protective effects and benefits of milk biologically active components on piglet health, in the perspective of their possible use as novel nutraceuticals in the early stages of weaning. The results of this literature research are the subject of a review paper reported in the Appendix.

1.2.2 In vivo evaluation of nutritional additives for prevention of E. coli diarrhoea

Post-weaning diarrhoea (PWD) is one of the most frequent causes of heavy economic losses in pig herds. Outbreaks affect a high proportion of pigs, are often recurrent and demand expensive control measures (Fairbrother *et al.*, 2005). PWD can be caused by a number of causative agents. The major bacteria involved are enterotoxigenic *Escherichia coli* (ETEC) strains, most commonly serogroups O8, O139, O141, O145 and O149 (Fahy *et al.*, 1987). Despite ETEC have been identified as the primary infectious agents, other factors are necessary for diarrhoea to occur (Pluske *et al.*, 2002). All stress factors involved with weaning may influence the establishment of infection; the loss of the passive protection provided by milk, the rise in stomach pH, the slower gut transit time

and the morphological (Hampson, 1986) and functional alterations of the small intestine (Kidder and Manners, 1980; Hampson and Kidder, 1986) can all contribute to creating a suitable environment for the proliferation of *E. coli* in the small intestine. Social stresses from mixing, fighting and moving into a new pen can increase transit time and depress immune response through the release of cortisol. Predisposing factors to post-weaning diarrhoea include rearing conditions, in particular environmental temperatures, hygiene and dietary composition. The presence of other pathogens in the environment increases the risk and severity of the disease (Pluske *et al.*, 2002). Cold stress, due to inappropriate temperatures and to the inability of piglets to adequate thermoregulation, is thought to be an important predisposing factor to PWC by altering intestinal motility. It has also been reported that low feed intake in the first week after weaning (Madec *et al.*, 1998) and high amounts of crude protein in the diet of newly weaned piglets (Prohάska and Baron, 1980; Macfarlane and Macfarlane, 1995; Heo *et al.* 2009; Opapeju *et al.*, 2009) are risk factors for PWC.

Alternatives to antibiotic growth promoters in prevention of E. coli diarrhoea

Before 2006, AGP were widely used, added to feed for piglets from birth to weaning, with the aim of improving the composition of intestinal microflora in piglets and of reducing enteric infections (Sorensen *et al.*, 2009). Due to the fact that the use of antibiotics as growth promoters has been banned in the EU, intensive research has focused on the development of alternative strategies for prevention of diarrhoea in weaned piglets (for reviews, see Hodgson and Barton 2009; Vondruskova *et al.*, 2010). At present, various materials - such as probiotics, prebiotics, organic acids, enzymes, zinc, plant extracts and others - have been tested or are under investigation (Corino *et al.*, 2008; Hodgson and Burton, 2009; Zhang *et al.*, 2010). Among them, zinc oxide (ZnO) at pharmacological concentrations (2,000 to 3,000 ppm) has been reported to reduce diarrhoea during weaning (Poulsen, 1995) and it has been included in piglet weaning diets for many years to improve growth rates and as a preventative measure against PWD, although its mode of action is not entirely clear yet. However, there is concern about the high rate of excretion of this element into the environment and, in this respect, there is growing interest in the potential role of organic minerals more biologically active and bioavailable. If the mode of action of Zn is based on a systemic effect, an organic form of Zn with greater bioavailability would allow to reduce the concentration in feed and the subsequent release in the environment, while maintaining benefits to the animals. The efficacy of other Zn sources as growth promoters and diarrhoea preventative in weaned pigs has been investigated (Castillo *et al.* 2008), although results are yet not comparable with those related to pharmacological levels of inorganic Zn from ZnO (Hollis *et al.* 2005).

Experimental models of porcine post-weaning colibacillosis

Using an appropriate disease model represents an effective approach to evaluate substitute products for antibiotics in the prevention of diarrhoea *in vivo*. Numerous researchers (e.g. Sarmiento *et al.*, 1988; McDonald *et al.*, 1999; Madec *et al.*, 2000; Van Dijk *et al.*, 2002; Owusu-Asiedu *et al.*, 2002, 2003a,b; Montagne *et al.*, 2004; Jensen *et al.*, 2006; Bhandari *et al.*, 2008) have adopted a challenge model of PWD, using specific pathogenic ETEC strains, to examine whether a particular feed additive or dietary strategy is effective in controlling PWD.

A major advantage of using an *in vivo* model is that the impact of a particular product or diet can be assessed in the context of an infectious pathogenic agent acting within the ecosystem of the gastrointestinal tract. However, a criticism often directed at disease challenge models to induce diarrhoea after weaning is that incidence and severity of the diarrhoea observed are often less than what experienced in commercial herds (de Lange *et al.*, 2010). In many cases, clinical signs cannot be provoked by an oral challenge alone and a stressor, such as cold stress, is introduced into the model (Sarmiento *et al.*, 1988; McDonald *et al.*, 1999).

For this reason, the first study (chapter 3) was addressed to set up experimental conditions to experimentally reproduce *E. coli* diarrhoea as observed in the field through a controlled *E. coli* challenge. Factors predisposing piglets to PWD were brought into the experimental challenge model, including stress factors (i.e. related to weaning, transport and group formation), iperproteic diet (28% of crude protein) and 30 mL 10% bicarbonate solution orally administered with purpose to neutralize gastric acid and to increase gastric survival rate of the challenger strain. One day after arrival piglets were orally inoculated with $3.7*10^8$ CFU of *E. coli* O149 strain. Daily health status, faecal score and faecal colour were individually recorded for 20 days after the challenge and Polymerase chain reaction, serotyping and biochemical identification were established for the evaluation of *E. coli* strains from faecal samples.

1.2.3 Plant-based veterinary vaccines, a potential novel strategy for the control of Oedema Disease

Given the devastating impact that serious disease outbreaks can have on farmers, society and economy, the European animal health strategy is based on the principle that "prevention is better than cure" (European Commission, 2007). In this respect, the administration of vaccines has been used very effectively over the decades to control and even to eradicate a number of important animal infectious disease. An efficacious vaccine affords protection, removing the need for antibiotic treatments and guarding against the economically harmful consequences of disease, e.g. reduced weight gain and productivity.

In recent times, the development of vaccines has rapidly advanced thanks to a significant progress made in the use of recombinant gene technologies. Plants have been recognized as an expression system for the production of edible vaccines thanks to the possibility of introducing antigenic proteins into their genoma. Stable transformation allows transgene integration into the plant genetic material and, consequently, the transfer of the acquired character to next generations.
Transgenic plants would also permit large scale, low-cost production of selected genes and have the potential for crossing transgenic lines to obtain multiple proteins expressed in the same plant. Moreover, they are able to confer heat stability to the heterologous protein and, if edible plants are used, the antigen can be attractively delivered through oral administration in feed, thus avoiding antigen purification and needle administration.
Delivery of a vaccine to a mucosal surface induces a mucosal immune response, providing a first line of defence against infection. Since several orally-delivered vaccine candidates induced mucosal immune response in the gastrointestinal tract, the most promising targets for plant-based oral vaccines may be gastrointestinal pathogens. However, even pathogens invading a body via other mucosal surfaces are good targets, because the linked nature of the mucosal immune system allows the delivery of an antigen to any mucosal surface to potentially induce immunity to others.
Their relatively low cost of production and their delivery method make plant-based vaccines very competitive in the field of veterinary vaccines, and in recent years there has been an increasing number of studies investigating candidate vaccines antigens for animal health.

Currently, no vaccine protecting piglets against OD is available and treatment relies upon the use of antibiotics, which, however, are usually used too late for piglets with visible clinical signs, as toxin has been already produced in the gut, with systemic spread and consequent lesions. Moreover, due to the increase of antibiotic-resistant enteric bacteria, especially *E. coli* strains, treatment with antimicrobials has come under increased scrutiny (Aarestrup *et al.* 2008). Due to the pathogenesis of OD, oral vaccination could represent an interesting strategy to control the disease.
The work presented in this thesis is part of a wider project, still in progress at our Department, in which genetically modified tobacco plants have been constructed for the expression of vaccine antigens against OD.
Two proteins actively involved in different parts of the pathogenic process of OD were chosen as antigens for oral immunization of weaned piglets: the F18 fimbriae adhesive, the primary pathogenic factor responsible for the adhesion of *E. coli* strains to enterocyte receptors, and the B subunit of the toxin VT2e,

responsible for the receptor binding capacity and the consequent systemic damage.
Tobacco seeds were transformed via *agroinfection* (*Agrobacterium tumefaciens* EHA 105) using pBIpGLOB bynary vector, as previously described (Rossi *et al.*, 2002). pBIpGLOB carried Kanamycin resistance gene and a soybean basic 7S globulin promoter which is able to direct the transgene expression in seeds (Reggi *et al.* 2005). The second generation (R_1) of transgenic tobacco plants was evaluated for the presence of the gene codifying for VT2e-B and F18 proteins by PCR, using internal primers of gene sequence, as previously described (Rossi *et al.* 2003).
The seed transcription ability of the two transgenic genes was evaluated by Northern Blot, using RNA probes labelled with digoxigenin, specific for mRNA F18 and mRNA VT2eB. The seed expression ability of VT2eB protein was evaluated by Western Blot, using a polyclonal serum obtained through parenteral immunization of New Zealand rabbits with VT2e-B (from pEt system). The seed expression ability of F18 was evaluated by agglutination on slides, using 50 µl of total soluble protein and 100 µl of F18+ polyclonal serum.
Oral administration of whole transgenic tobacco seeds as edible vaccine for newly-weaned piglets raises doubts about an effective exposure of the antigen to the sites of activation of the mucosal immune system. The use of milled transgenic tobacco seeds would appear more appropriate for animals with immature digestive capacity, however there are no data available concerning the gastric degradability of antigenic proteins expressed in both whole and milled tobacco seeds in weaned piglets. For this reason, in a preliminary study (chapter 4), we assessed the gastric degradation of the VT2e-B antigen expressed in transgenic tobacco seeds, evaluating the residual amount of transgenic proteins after *in vitro* digestion of both milled and whole seeds.
In the subsequent study (chapter 5), transgenic tobacco seeds expressing antigenic proteins F18 and VT2e-B were used as edible vaccine in weaned piglets and evaluated for the induction of an intestinal immune response and a protective effect against O138 *E. coli* infection.

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CHAPTER 2

Objectives

2. Objectives

The main theme of this research concerns the management of some health problems that frequently occur during the weaning of piglets, focusing in particular on the use of feed additives and immunogenic products derived from engineered plants.
Three different trials were designed to study novel strategies for the control of two important diseases typically occurring during the weaning phase of the piglet, specifically *Escherichia coli* diarrhoea and Oedema Disease (OD).
The first experimental work was addressed to the study of appropriate experimental conditions to evaluate i*n vivo* nutritional additives for prevention of *E. coli* diarrhoea.
The two subsequent experimental works are part of a wider project, still in progress at our Department, in which genetically modified tobacco plants have been constructed for the expression of vaccine antigens against verotoxin-producing *E. coli* strains responsible for OD.
Below, the specific objectives of each study are described.

2.1 Experimental induction of *Escherichia coli* diarrhoea in weaned piglets

After the ban of antibiotics as growth promoters in the EU, intensive research has focused on the development of alternative strategies for prevention of *E. coli* diarrhoea in weaned piglets. Using an appropriate disease model represents an effective approach to evaluate substitute products for antibiotics in the prevention of diarrhoea *in vivo*. However, a criticism often directed at disease challenge models to induce diarrhoea after weaning is that incidence and severity of the diarrhoea observed are often less than what experienced in commercial herds.
The aim of the present work was to set up appropriate experimental conditions to reproduce the disease in research units, in order to evaluate the effectiveness of substitute products for antibiotics *in vivo*.

2.2 Evaluation of gastric degradability of antigenic protein expressed in tobacco seeds

The second experimental work represents a preliminary step for the subsequent study, in which tobacco seeds expressing antigenic proteins against Oedema Disease were used for oral immunization of recently weaned piglets.

An essential prerequisite for the effectiveness of oral vaccines is their survival to digestive processes. Since there are no data available concerning the gastric degradability of antigenic proteins expressed in tobacco seeds, the aim of the present study was to evaluate the effect of swine gastric fluid, derived from weaned piglets, on VT2e-B antigenic protein expressed in whole and milled tobacco seeds, in the perspective of their use for immunization trials on piglets.

2.3 Oral administration and evaluation in piglets of tobacco seeds expressing antigenic proteins against Oedema Disease

Demonstrating the induction of immune response is a key step in developing new vaccines and protective efficacy, particularly in the target species, is of course the principal goal during development of a vaccine candidate.
The aim of this work was to evaluate whether oral immunization of weaned piglets with transgenic tobacco seeds expressing antigenic proteins against Oedema Disease could induce an increase of mucosal antibodies and a protective effect against a subsequent O138 *E. coli* infection.

CHAPTER 3

Experimental induction of *Escherichia coli* diarrhoea in weaned piglets

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3. Experimental induction of *Escherichia coli* diarrhoea in weaned piglets

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3.1 Abstract

Escherichia coli diarrhoea is a multifactorial disease which usually occurs during the postweaning period and is responsible for important economic losses in pig production. The aim of study is the induction of diarrhoea in weaned piglets after *E. coli* challenge, in order to set up experimental conditions to evaluate innovative compounds to control or to treat it. Thirty-five healthy piglets, weaned at 33±2 days, from a selected farm, were divided into three experimental groups: control group (CG), infected group 1 (IG1) and infected group 2 (IG2). One day after arrival piglets of IG1 and IG2 were orally inoculated with $3.7*10^8$ CFU of *E. coli* O149 strain. Piglets received a diet containing 28% of crude proteins for three days around the challenge. Daily health status, faecal score and faecal colour were individually recorded for 20 days after the challenge in IG1 and CG and for 2 days after the challenge in IG2. Polymerase chain reaction, serotyping and biochemical identification were established for the evaluation of *E. coli* strains from faecal samples. Diarrhoea was observed in 96.67% (58.6% severe; 41.4% mild) of all infected piglets and occurred on average 1.3 days after the challenge. The CG group presented one piglet with a transient mild diarrhoea. The *E. coli* challenge significantly affected the consistency and colour of faeces ($P<0.001$). The 70% of piglets with severe diarrhoea shed *Escherichia coli* O149 in their faeces.
The O149 challenger strain was detected in 17 out of 30 (56.7%) infected piglets two days after experimental infection, and 15 out of 17 isolated O149 *E. coli* strains (88%) were also hemolytic.
Zootechnical parameters did not show significant differences. The experimental conditions described in this study allowed to effectively induce diarrhoea in weaned piglets.

Keywords: Piglet, diarrhoea, *Escherichia coli*, challenge, weaning period

3.2 Introduction

In the weaning period diarrhoea is one of the most important causes of economic losses in pig livestock and during outbreaks of the disease the morbidity may be over 50% among weaned piglets (Hong *et al.*, 2006). Several bacterial and viral agents are responsible for post-weaning diarrhoea (PWD) (Thomsson *et al.*, 2008; Vondruskova *et al.*, 2010). PWD is a multifactorial disease and in its pathogenesis plays a significant role the proliferation of pathogenic strains of *Escherichia coli* throughout the intestinal tract of piglets after weaning. This condition is defined as Post-Weaning Colibacillosis (PWC) (Callesen *et al.*, 2007). PWD occurs most frequently 1 to 3 weeks post-weaning but it was also observed to affect pigs later, together with stressful events (Fairbrother *et al.*, 2005). The act of weaning is a precipitating factor for PWC, regardless of the age at weaning (Pluske 2002), as piglets are exposed to several stress factors that may influence the establishment of the infection. The loss of the passive protection provided by milk, the rise in stomach pH, the slowed gut transit and the morphological and physiological changes in the small intestinal tract, which occur at weaning, allow bacterial adhesion and colonization. Stresses from mixing and moving into a new pen also cause increased transit time and depressed immune response through the release of cortisol. Predisposing factors to post-weaning diarrhoea include rearing conditions, in particular environmental temperatures, hygiene and dietary composition (Madec *et al.*, 2000; Laine *et al.*, 2008). Veterinary antibiotics were used to reduce enteric infections and the occurrence of pathogens able to adhere to the intestinal mucosa (Barton, 2000). The increase of antibiotic resistance and the related negative consequences for human health, animal health and the environment caused in Europe a ban on the use of antibiotics as growth promoters in animal nutrition according to EC Regulation 1831/2003 (Sarmah *et al.*, 2006; Smith *et al.*, 2010), thus making necessary to develop sustainable alternative strategies or tools to control diseases (Camerlink *et al.*, 2009). Nutrition is obviously a critical determinant in the functional development and growth of the gastrointestinal tract and the weaning phase represents a critical period (Domeneghini *et al.*, 2006), so various natural materials such as probiotics, prebiotics, organic acids, zinc and plant extracts have been tested (Corino *et al.*, 2008; Hodgson and Burton, 2009; Zhang *et al.*, 2010). The effects of dietary factors or functional feed additives on spontaneous weaning diarrhoea are difficult to study because of the variable incidence of PWD and PWC. Moreover, because of the multifactorial aetiology of PWC, it has been difficult to develop a reliable and repeatable experimental model of post-weaning diarrhoea. For these reasons, the aim of this study was to set up appropriate experimental conditions to simulate the outbreak of PWD through a

controlled *E. coli* challenge, in order to assess the effectiveness of alternative molecules to control or to treat PWC in pigs.

3.3 Materials and methods

3.3.1 Selection of the farm

A conventional herd free from diseases according to the A-list of the International Office of Epizootic, and from Aujeszky's disease, atrophic rhinitis, transmissible gastroenteritis, porcine reproductive and respiratory syndrome and salmonellosis, without history of PWD and Oedema Disease (OD) was chosen for piglets supplying.

A total of 50 piglet randomly selected were weaned at 33±2 days and maintained in the original herd in groups of 10 animals per box. For 15 days all animals were examined for clinical status and two faecal samples were collected from rectum for microbiological evaluations, respectively on day 3 and 7 after weaning, in order to assess the presence of *E.coli* strains soon after the loss of the passive protection provided by milk. Therefore, due to the capacity of the facilities, 35 out of 50 healthy weaned piglets (Large White x Landrace), female and castrate (1:1), homogenous for weight, with two bacteriological analysis of the faeces negative for hemolytic *E. coli* and for *E. coli* O149, were selected and transferred in the University facilities.

3.3.2 Animals and experimental design

The piglets were allocated in pens, each pen containing 2-3 piglets, under the same environmental conditions. The environmental temperature of the experimental facility was regulated at 28 °C. The relative humidity has been always maintained at 60%.

Piglets were randomized into a group of 30 piglets challenged with an enterotoxigenic strain of *Escherichia coli* O149 (infected group, IG) and a group of 5 piglets not challenged, serving as controls (control group, CG).

The IG group was randomly divided into one group of 10 piglets (IG1) and one of 20 piglets (IG2). CG, IG1, IG2 were allocated in the same room, in order to standardize environmental conditions, with the control group separated by the infected group by two empty pens, so that physical contact between piglets of the two groups was excluded. In the experimental site, two separate manure collection pits ran under the holding pens: one manure pit collecting manure from control and IG1 and one manure pit collecting manure from the IG2. Separate boots for each group were used by the staff having access to the pens and the not-infected animals were always handled before the *E. coli* inoculated animals, to avoid any cross contamination.

The IG1 group was monitored for 20 days after the challenge to evaluate the time to complete recovery from diarrhoea by daily clinical examination. The piglets of IG2 were evaluated for two days after the challenge in order to assess diarrhoea onset; then, to avoid unnecessary pain, they were treated with antibiotic molecules.
During the first three days of the trial, from the day before the challenge until the second day after the challenge, the same antimicrobial-free diet, containing 28% of crude protein on dry matter, was administered to all experimental groups (Table 1). Before the administration, the chemical analysis of the diet was performed to confirm the high level of protein previously calculated by specific software (Plurimix, Fabermatica, Cr, Italy) and to measure the principal components: crude protein (CP), according to the official method of Analysis of Association of Analytical Communities, procedure 2001.11 (AOAC, 2005); dry matter (dm), according to procedure 930.15 (AOAC, 2005); fat (EE) according to DM 21/12/1998; crude fiber (CF), according to procedure Ba 6a-05 of the official method of the American Oil Chemists Society (AOCS, 1998); ash, according to the procedure 942.05 (AOAC, 2005).
From the third day after the challenge onwards the piglets were fed *ad libitum* with a weaning feed without antibiotics; the composition of both the experimental diets is reported in Table 1. Fresh water was available throughout the experiment.

3.3.3 Experimental infection

The day of challenge, one day after the arrival, was considered day 1 and the subsequent timetable was based on this criterion.
Both IG1 and IG2 group were challenged with an enterotoxigenic strain of *Escherichia coli* O149, with toxins LT and VT2e, provided by the Lombardy and Emilia Romagna Experimental Zootechnic Institute (IZSLER). The strain was isolated in field from a piglet died for severe diarrhoea caused by *E. coli* O149. The challenger strain was grown on liquid LB medium for 24 hours at 37 °C in a sterile bottle with shaking.
The inoculum was given to the piglets via oral route in a single dose of 5 mL of bacterial medium with $3.7*10^8$ colony forming units (CFU) of the challenger strain. At the same time, piglets of the CG group were orally inoculated with 5 mL of sterile physiological saline to equilibrate the level of stress associated with the oral challenge.
About 10-15 min before bacterial inoculation piglets orally received 30 mL of a 10% bicarbonate solution (SIGMA, Italy), a procedure used in attempt to neutralize gastric acid and increase the survival rate of the challenger strain in the stomach (Madec *et al.*, 2000, Jensen *et al.*, 2006).

Ingredients (%)	High protein diet[a]	Standard diet[b]
Soybean meal	29,00	
Corn	12,78	18
Barley	11,36	16
Corn extruded	7,81	11
High protein soybean meal	7,10	10
Wheat bran	6,39	9
Full fat soybean extruded	5,68	8
Wheat middlings	5,33	7,5
Wheat	4,26	6
Barley flakes	2,84	4
Milk whey	1,78	2,5
Harring meal	1,07	1,5
Soya oil	0,71	1
Calcium formiate	0,57	0,8
Molasse	0,43	0,6
Coconut oil	0,43	0,6
Acidifier	0,36	0,5
Calcium sulphate	0,36	0,5
Peas fiber	0,36	0,5
Dicalcium phosphate	0,36	
L-Lys HCl	0,36	0,5
L-Thr	0,28	
Vitamin and trace mineral premix	0,18	
Trp	0,14	
DL-Met	0,11	
Calculated chemical composition, % DM		
CP, %	28,08	19,87
Fat, %	4,8	6,28
Crude fiber, %	5,43	4,87
Ash %	6,49	6,20
NE, Mc/Kg	2,45	2,59
Lys, %	1,85	1,39
Met, %	0,54	0,47
Met +Cys, %	1,02	0,83
Thr, %	1,39	1,15
Trp, %	0,52	0,45

Table 1 – Composition of experimental diets and calculated chemical analysis
[a] used for all experimental groups during the first 3 days of the trial
[b] used for all experimental groups from the third day after challenge onwards.

3.3.4 Clinical and zootechnical examinations, faecal score

Every day and during all the experimental period piglets of the CG and IG1 were individually given a clinical examination; piglets of IG2 were clinically monitored until day 3.

Clinical examination included observation of faecal consistency and colour, behavioural disturbances and cyanosis.

Individual faecal samples were collected from rectum daily. A scale of four levels was used to score faecal consistency: 0 = normal (faeces firm and well formed), 1= soft consistency (faeces soft and formed), 2= mild diarrhoea (fluid faeces, usually yellowish), 3= severe diarrhoea (faeces watery and projectile). A faecal consistency score ≤1 (0, 1) was considered normal, whereas a faecal score >1 (2, 3) was defined as a clinical sign of diarrhoea. Faecal colour was evaluated using a three points scale: 1= yellow, 2= green, 3= brown. A faecal colour ≥2 (green-brown) was considered normal, while a faecal colour <2 (yellow) was considered pathological. Disturbed behaviour was defined as slow reactions, an unsteady and slow gait whilst walking and an inattentive response when encouraged to move. Cyanosis was defined as a blue discolouration of the ears or limps related to the production of toxins. Pigs of CG and IG1 group were individually weighed on days 0, 6, 8, 10, 14, 17 and the feed intake (FI) was measured daily, weighing the residual feed. *Post mortem* examination was performed on dead subjects.

3.3.5 Microbiological analysis of faecal samples

On day 3 *Escherichia coli* strains were respectively collected from individual faecal samples coming from pigs. Each strain was cultured on MacConkey agar (OXOID), Blood Agar incubated at 37°C for 24 hours. Every compatible colony was isolated on Trypticase Soy Agar (TSA) (OXOID) and incubated at 37°C for 24 hours. The biochemical identification was carried out using the API-20E method (Bio-MERIEUX). All *E. coli* strains were considered hemolytic and non hemolytic and tested for the characterization test. Serotyping was carried out using monospecific antisera towards 40 different somatic O antigens (O1, O2, O4, O6, O8, O9, O10, O11, O15, O18, O20, O21, O22, O26, O45, O49, O64, O68, O73, O75, O78, O83, O85, O86, O88, O92, O101, O103, O109, O111, O115, O128, O132, O138, O139, O141, O147, O149, O153, O157) in U bottom polystyrene microtitre plates incubated for 24 hours at 37°C in a moist box (Blanco and Blanco, 1993; Blanco *et al.*, 1996; Farina *et al.*, 1996). Biochemical identification and serotyping were carried out on *E. coli* strains isolated from all faecal samples (IG and CG). The genetic characterization was performed on isolated pathogenic *E. coli* strains to demonstrate VT using polymerase chain reaction (PCR) screening (Lin *et al.*, 1993), followed by identification of VT1, VT2 (Russmann *et al.*, 1995) and VTe (Franke *et al.* 1995) by PCR (Karch *et al.*, 1993).

3.3.6 Statistical analysis

The individual piglet was considered to be the experimental unit for evaluation of faecal samples, body weight and average daily gain (ADG). Daily feed intake could only be determined per pen, so the statistical analysis could not be

performed on an individual basis. Faecal Score, Faecal Colour, Feed Intake and Body Weight variables were statistically compared among the two groups by analysis of variance (ANOVA) in a continuous design approach. Treatment effects were analyzed by a multivariate repeated measures ANOVA using PROC GLM of the SAS System (Version 9.2; SAS Institute, Inc., Cary, NC). A separate analysis of variance for the two groups was performed using the PROC GLM on ADG calculated as difference between final and initial body weights divided by number of days of feeding. Simple correlation between the least square daily means of Faecal Score and Faecal Colour was also investigated by means of the PROC CORR of SAS, in order to confirm the coherence between the two scales of measurement.

3.4 Results

3.4.1 Diarrhoea

All 50 piglets that underwent clinical examination and microbiological analysis of faecal samples in the herd of origin, presented two consecutive samples negative for hemolytic *E. coli* and for *E. coli* O149. Two subjects were excluded for respiratory problems and, among those remaining, 35 healthy piglets were randomly selected to be included in the study. The chemical analysis of the experimental diet administered during the first three days (CP 27.93% dm, EE 5.14% dm, CF 2.8% dm) confirmed the high level of protein previously calculated by Plurimix software. On day 0 no subjects presented signs of enteric disease, in fact the average faecal score of piglets in the experimental groups was 0. Twenty-four hours after the challenge (day 2) diarrhoea occurred in 76.67% of the IG piglets, (43.5% severe; 56.5% mild) and the CG group presented one piglet with a transient mild diarrhoea; on day 3 the highest percentage of diarrhoea was observed in the infected piglets (96.67%; 58.6% severe; 41.4% mild). At the same time no signs of diarrhoea were observed in the CG group. Diarrhoea occurred on average 1.3 days after the challenge (minimum 24 hours, maximum 48 hours after the challenge). To evaluate the trend of diarrhoea in pigs of the IG1 group, frequency, individual faecal score and average faecal score were considered in relation to time. By exploratory analysis of the raw faecal evaluation data over the entire experimental period, days 1-17, major differences relating to healthy/pathological faeces were found between the IG1 and CG groups during the first week after the challenge. The frequency of diarrhoea, calculated as the ratio between subjects with diarrhoea and 10 (number of piglets of IG1), was at the highest level (0.9) on day 3, when the highest faecal score was also observed, similarly to the IG group. It decreased gradually up to score 0 on day 11. During days 2-8 the Least Square (LS) means of the faecal score for IG1 were pathological (> 1), while during days 8-17 they returned to normal (= 1).

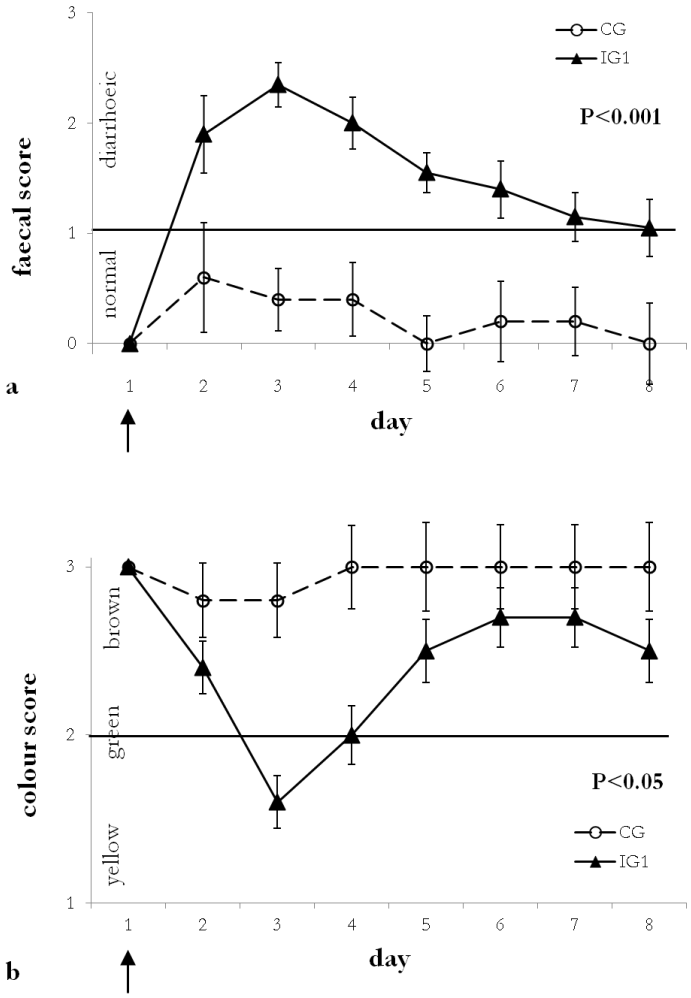

Figure 1 – a) Least square means of faecal scores from days 1-8 recorded in individual piglets challenged with *E. coli* (IG1, triangles in solid line) and non-challenged piglets (CG, circles in broken line). b) Least square means of colour scores from days 1-8 recorded in individual piglets challenged with *E. coli* (IG1, triangles in solid line) and non-challenged piglets (CG, circles in broken line).
The day of the challenge is indicated by an arrow.
Faecal scores were based on the following scale: 0 = normal (faeces firm and well formed), 1 = soft consistency (faeces soft and formed), 2 = mild diarrhoea (fluid faeces, usually yellowish), 3 = severe diarrhoea (faeces watery and projectile). A faecal consistency score ≤ 1 (0, 1) was considered normal, whereas a faecal score >1 (2, 3) was defined as a clinical sign of diarrhoea. Faecal colour was evaluated using a 3 points scale: 1 = yellow, 2 = green, 3 = brown. A faecal colour ≥ 2 (green-brown) was considered normal, while a faecal colour < 2 (yellow) was considered pathological.
The CG and IG1 groups showed significant differences in faecal score (P<0.001) and colour (P<0.05). SE of each point is indicated.

Moreover, diarrhoea in IG1 lasted 3.6 days on average, with a range of 1-8 days. For this reason, although it was possible to detect significant differences between the groups (CG *vs* IG1) throughout the entire experimental period (P<0.001), the shown statistical analysis refers to data collected during days 1-8. The trend of the LS means of the faecal score for IG1 was higher than for the CG

(P<0.001), as represented in Figure 1a. The LS means of the colour score for IG1 were lower (P<0.05) than CG during days 1-8 (Figure 1b). The IG1 group showed a significant negative correlation (r=-0.89; P<0.001) between faecal colour and faecal score. From the day of challenge, when the colour of faeces was brown in all piglets, an increase in the percentage of yellow faeces was observed, corresponding to score 1. On day 3, when the diarrhoea incidence was at its highest, the LS means±standard error (SE) of faecal colour were 1.6±0.16 in IG1, *vs* 2.8±0.22 in CG. On day 4, when the severity of the diarrhoea was decreasing, the average colour became normal again in the IG1 with a score of 2±0.18 (LS means±SE). Green faeces was often observed in the transition periods from healthy to pathological status or vice versa. The observed behavioural disturbances were mainly signs of depression correlated with a severe diarrhoea status. The behavioural disturbances were detected immediately after the challenge (day 2), when 5/30 of challenged piglets (IG1 and IG2) showed signs of depression; after that, the percentage of behavioural disturbances in the IG1 group decreased. One piglet in this group presented a disturbed behaviour, together with other clinical signs (cyanosis of ears and snout and ear necrosis) for a longer period.

3.4.2 Mortality

A mortality rate of 6% (1/15) was observed on animals monitored for 20 days after the challenge (CG and IG1); in particular, no mortality occurred in the CG, while one pig in the IG1 group died. *Post mortem* examination confirmed systemic symptoms related to *E. coli* infection responsible for OD (severe diarrhoea together with cyanosis of ears and snout, ear necrosis and neurological signs). Piglets of the IG2 group did not present mortality.

3.4.3 Faecal samples analyses

Microbiological evaluation of IG faeces showed (Figure 2) the presence of *E. coli* strains in all 30 subjects, composed by 16 (53%) hemolytic *E. coli* strains and 14 (47%) not hemolytic *E. coli* strains. The O149 challenger strain was detected in 17 out of 30 (56.7%) piglets two days after experimental infection. Fifteen out of 17 isolated O149 *E. coli* strains (88%) were also hemolytic. One faecal sample showed *E. coli* O157 without VT2e gene. Verotoxin genes were found in 13 out of 22 (59%) analyzed samples and in 13 out of 17 (76%) O149 *E. coli* strains isolated from piglets. Moreover, 14 of the 20 piglets with severe diarrhoea showed the presence of *E. coli* O149 in faecal samples. In the CG there was no shedding of both hemolytic strains and *E. coli* O149.

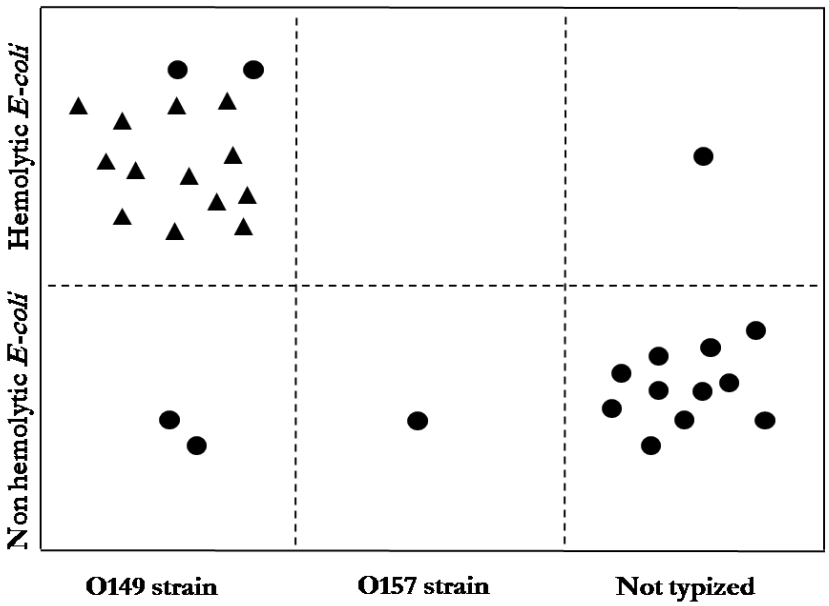

Figure 2 – Serotyping and genetic characterization of *E. coli* strains isolated from faecal samples of piglets two days after challenge with *E. coli* O149.
▲ : PCR–positive strain for the detection of VT2e genes;
● : PCR–negative strain for the detection of VT2e genes.

3.4.4 Zootechnical parameters

Although the average body weight (ABW) of the CG was higher than the ABW of the IG1, significant differences were not detected (Figure 3). So individual body weights were not influenced by the challenge.

In the 7 days following challenge, the piglet who was seriously ill has certainly influenced zootechnical parameters (ABW, ADG, FI) of the IG1 group, showing a reduced ADG during days 1-6 (182±262 g/d, weight ± standard deviation) when compared with ADG of CG (247± 103 g/d, weight ±standard deviation). Considering the ADGs on a longer post-inoculation period (days 0-17), the difference between the CG and the IG1 group tended to decrease as the piglets rapidly recovered from diarrhoea. In fact CG registered a ADG of 359±62 g/d (weight±SD) and IG1 360±78 g/d (weight ±SD).

Similarly, the daily FI decreased after experimental infection; in particular, on day 4, the average FI of the IG1 was 360±166 g/d *vs* 496±21 g/d (weight±SD) for the CG. However, considering the entire experimental period, it was not affected by the challenge. In fact the average daily FI calculated on days 0-17, although slightly higher in CG than in IG1 (691±157 g/d *vs* 654±181 g/d), showed no significant differences.

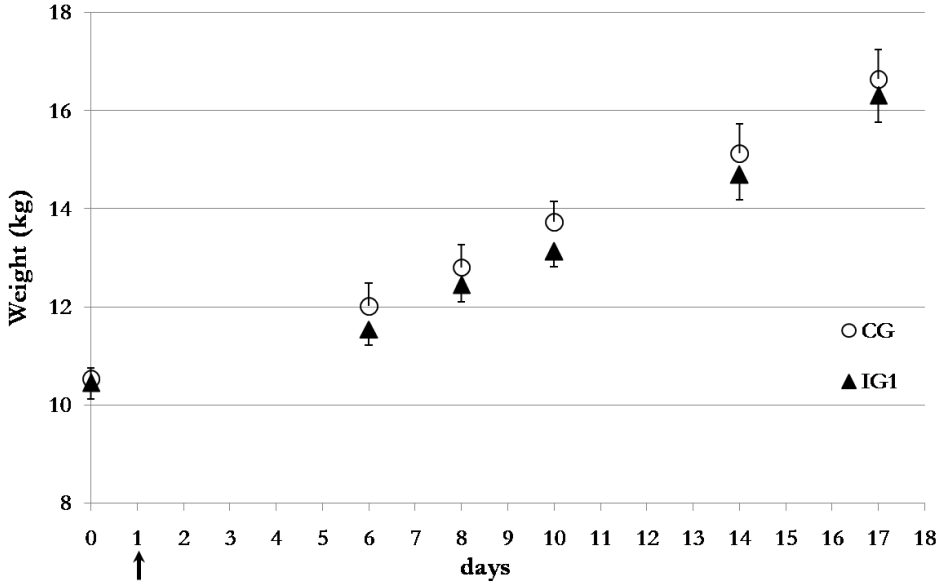

Figure 3 – Least square means of the weight of piglets challenged with *E. coli* (IG1, triangles) and non-challenged piglets (CG, circles) during the experimental period.
SE of each point is indicated.
Black arrow represents the *E. coli* challenge (day 1).

3.5 Discussion

Post-weaning digestive disorders are multifactorial and experimental infection with *E. coli* alone is not sufficient to reproduce the syndrome as observed in the field (Cox *et al.*, 1991). In this study, factors predisposing the piglets to PWD were introduced into the experimental challenge model, including stress factors (i.e. related to weaning, transport and group formation), a iperproteic diet and 30 mL 10% bicarbonate solution orally administered with purpose to neutralize gastric acid and to increase gastric survival rate of the challenger strain. The diet administered during the first three days, containing a high level of crude protein, was intended to be a "high-risk" diet, since several pathogens preferentially ferment proteins and high amounts of crude protein in the diet of newly weaned piglets have been identified as one of the predisposing factors of PWC (Prohàska and Baron, 1980; Macfarlane and Macfarlane, 1995; Heo *et al.* 2009; Opapeju *et al.*, 2009). Moreover, the high level of CP was due to the presence in the feed of soybean meal, an ingredient that seems to favour the occurrence of PWD (Fairbrother *et al.* 2005). Nevertheless the stress factors alone were not sufficient to influence significant changes in the consistency and colour of faeces, as evidenced by the findings in the CG. According to the multifactorial aspects of this disease, the tested experimental conditions with *E. coli* challenge affected faecal characteristics ($P<0.001$) and faecal colour. This study showed that the a single dose of 10^8 CFU was able to reproduce diarrhoea in 96.67% of the challenged piglets. Moreover, diarrhoea started 1-2 days after the challenge

and lasted an average of 3.6 days, which is in agreement with data reported by Sarmiento *et al.* (1988). The significant correlation between faecal colour and faecal score supported the validity of the scale of colour score applied in this study. The microbiological analysis confirmed the effect of the challenger strain on faecal samples. The detection of the inoculated strain from the faeces corresponded to the presence of diarrhoea; however, on the contrary, *E. coli* was not detected in all diarrhoeic piglets. In fact, as reported by Van Dijk *et al.* (2002), the onset of diarrhoea did not invariably coincide with the start of shedding of a particular enterotoxigenic *E. coli* strain. The challenger strain, hemolytic and verocytotoxic in most of faecal isolates showed high virulence, related to the onset of severe diarrhoea. Piglets included in the IG1 group, monitored for 20 days after the challenge, showed a spontaneous recovery after eight days, as evidenced by increased FI and ADG. The mortality observed in the IG1 was probably associated with the proliferation of *E. coli* and the release of shiga-like toxins related to OD. In fact, clinical findings and *post mortem* examination showed important nervous signs and typical mucosal oedema. The rate of mortality (10%) in the challenged group was comparable with the mortality normally occurring in the field (Nabuurs *et al.*, 1993). The measurement of zootechnical parameters evidenced a depressed FI which persisted for about five days after the challenge and a subsequently reduced ADG, as encountered in the field.

3.6 Conclusions and possible applications

The evaluation *in vivo* of substitute products for antibiotics for prevention of *E. coli* diarrhoea presents the major advantage that the impact of a particular product can be assessed in the context of an infectious pathogenic agent acting within the ecosystem of the gastrointestinal tract. However, a critical point when using specific pathogenic ETEC strains to induce diarrhoea after weaning is that the incidence and the severity of the diarrhoea observed under experimental conditions is often less than that experienced in commercial herds.
In the present study, the effect of environmental risk factors and etiologic agent factors has been combined in order to experimentally reproduce *E. coli* diarrhoea under experimental conditions.
A single oral dose of 10^8 CFU of challenger strain, hemolytic and verocytotoxic, combined with a diet containing a high level of crude protein, stress factors related to weaning, transport and group formation and the oral administration of 30 mL 10% bicarbonate solution allowed to effectively induce a high percentage of diarrhoea in weaned piglets.
Such an approach may be used in future to evaluate *in vivo* substitute products for antibiotics for the prevention of *E. coli* diarrhoea.

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CHAPTER 4

Evaluation of gastric degradability of antigenic protein expressed in tobacco seeds

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4. Evaluation of gastric degradability of antigenic protein expressed in tobacco seeds

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4.1 Abstract

Plants have been recognized as an expression system for the production of edible vaccine because of the possibility of introducing antigenic proteins into their genoma. In livestock, transformed plants for the expression of immunogenic proteins could be administered, orally, in feed to induce mucosal immune response in the gastrointestinal tract. Moreover, edible vaccines for veterinary use could reduce the costs of traditional vaccines associated with the production, the cold storage, and parenteral administration. However, the most important problem related to the oral delivery route is the potential for antigen degradation in the gastrointestinal tract. For these reasons, the aim of this study was the evaluation of the effect of swine gastric fluid on VT2e-B antigenic protein, derived from a *E. coli* strain responsible for Oedema Disease, expressed in tobacco seeds by *agroinfection*. Samples of transgenic tobacco seeds, both milled and whole, were incubated with porcine gastric fluid at 38° C in Dubnoff Shaker for 1, 2 and 3 hours. After gastric fluid removal, by centrifugation and washing with PBS, samples were homogenized in the presence of protein extraction buffer. A Western blot was performed on representative samples of extracted proteins, quantified by the Bradford method, using rabbit polyclonal serum. The Vt2e-B specific signal was observed in all samples derived from transgenic tobacco seeds. Nevertheless, from 0 h to 3 h, a progressive reduction of intensity of signal was observed. No significant differences were detected on the reduction of signal intensity between samples derived from whole and milled tobacco seeds.

4.2 Introduction

Oral delivery of antigens has been shown to be capable of inducing mucosal immune responses in the gastrointestinal tract and immune responses are commonly observed in serum and at secondary mucosal surfaces. Mucosal immunization offer the potential to mount an immune response to a pathogen at the site of invasion, so providing a first line of defense against infection. Moreover oral vaccines for veterinary use are appealing as an immunization strategy, due to the simplicity and the low costs of their administration. However, vaccines administered by oral route are subject to antigen degradation in the gastrointestinal tract and especially subunit vaccines, where protein antigens are vulnerable to proteolytic digestion.

The production of antigens in plant material has the advantage of a natural encapsulation in the tissues of the expression host. This encapsulation offers the potential for antigen to be protected against rapid and complete degradation and to be gradually released as host tissues are digested. It has been shown that the protein avidin expressed in transgenic corn products fed to mice can be detected in the faeces, whereas a corresponding dose of purified avidin is completely degraded (Bailey, 2000).

The present work is part of a wider project in which genetically modified tobacco plants have been constructed for the expression of vaccine antigens against Oedema Disease (OD).

Tobacco seeds were transformed via *agroinfection* (*Agrobacterium tumefaciens* EHA 105) using pBIpGLOB bynary vector (Rossi *et al.*, 2002), for the expression of two proteins actively involved in the pathogenic process of the disease: the F18 fimbriae and the B subunit of toxin VT2e, responsible for receptor binding capacity and consequent systemic vascular damage. It has been shown that purified VT2e, injected intravenously in pigs, reproduces all the clinical signs and pathological lesions of OD (Macleod *et al.* 1991) and is therefore a vaccine candidate for the stimulation of protective immunity in pigs. VT2e toxins are bipartite 70-kDa proteins composed of a single enzymatically active A subunit and five B subunits. The A subunit inhibits protein synthesis at the ribosiomial level, resulting in citotoxicity and the clinical syndrome, while the B subunits are responsible for binding to specific receptors on cell surface. In this study, the B subunit of the toxin VT2e was selected as potential immunogen against OD and expressed in tobacco seeds.

Nevertheless, oral administration of whole transgenic tobacco seeds as edible vaccine for newly-weaned piglets raise the problem of an effective exposure of the antigen to the sites of activation of the mucosal immune system. The use of milled transgenic tobacco seeds would appear more appropriate for animals with immature digestive capacity, however there is no data available concerning the

gastric degradability of antigenic proteins expressed in both whole and milled tobacco seeds. For this reason, the aim of the present study was to evaluate the effect of swine gastric fluid on VT2e-B antigenic protein expressed in whole and milled tobacco seeds, in the perspective of their use for immunization trials on piglets.

4.3 Materials and methods

4.3.1 Analysis of the chemical composition of tobacco seeds

The chemical analysis of tobacco seeds was performed to measure the principal components: dry matter (dm, AOAC method 930.15); crude protein (CP, AOAC method 2001.11); ether extract (EE, DM 21/12/1998); crude fibre, (CF, AOCS method Ba 6a-05); neutral detergent fibre, (NDF, AOAC method 2002.04) and ash (AOAC method 942.05).

4.3.2 Digestion of tobacco seeds

Samples of gastric fluid from 5 slaughtered weaned piglets were collected in sterile tubes and maintained at -20°C. At the time of the trial, samples were defrosted at 4° C and used to create a pool of gastric fluid composed by equal volumes of each sample.

Ten samples of 250 mg of transgenic whole seeds and 10 samples of 250 mg of transgenic seeds previously ground in a mortar were incubated in a Dubnoff Shaker at 38°C with 1,2 ml of porcine gastric fluid.

Samples of both whole and milled seeds with different incubation times of 1h, 2h and 3h were centrifuged and washed several times with PBS in order to remove the gastric fluid.

The seeds were ground in a mortar and each sample was incubated for 1 h at -20°C with 500 µl of extraction buffer (50mM pH8 Tris, 5mM EDTA, 200mM NaCl, 0.1% Tween 20). For each sample, total soluble proteins were obtained after centrifugation for 15 minutes at 14,000 rpm and collection of the supernatant and were quantified by the Bradford method. The Vt2e-B subunit protein in tobacco seeds was determined by a Western blot analysis using a specific polyclonal rabbit serum as described below.

4.3.3 Production of primary antibodies specific for VT2e-B

Primary antibodies were obtained from two New Zeland rabbits 15 days after the third immunizations with VT2e-B protein produced in *E. coli* BL21 by pET-system.

For the initial immunization, rabbits were immunized by subcutaneous (on the back of the neck) and intramuscular (on thigh muscle of the rear legs) injection

with the protein (100 μg per rabbit) emulsified in 1 ml of PBS and in Freund's complete adjuvant. Subsequent booster shots were given every 2 weeks with immunogens (100 μg per rabbit) in Freund's incomplete adjuvant. Fifteen days after the third immunization, blood samples were collected from the jugular vein. The blood was incubated for 1 h at 37 ° C and then centrifuged at 6000 rpm for 40 minutes. The supernatant serum was taken to determine antibody titer by Western Blot. In order to determine the antibody titre that was needed to run Western Blot analysis, Dot Blot was performed using different concentrations of proteins produced by pET-system (50, 100 and 200 μg) and different dilutions of the serum.

4.3.4 Detection of VT2e-B by Western blot analysis

Proteins were separated by electrophoresis on SDS-polyacrylamide gels, loading 15 μg of total protein per well, and transferred to nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech) in a transfer buffer containing 20% methanol.

Membranes were blocked for 60 min with PBS-T (PBS plus 0.1% Tween-20) containing 5% of skim milk powder. Blots were then incubated (25 °C, 1 h) with primary antibodies specific to Vt2e-B protein (diluted 1:5000) and washed three times (one time for 15 min and two times for 5 min each) with PBS-T. After rinsing the membrane was incubated (25 °C, 1 h) with anti-rabbit secondary antibodies diluted 1:5000. The blots were washed three times (one time for 15 min and two times for 5 min each) with PBS-T. Detection was performed using ECL Western Blotting Detection Reagents (Amersham Pharmacia).

4.4 Results and discussion

4.4.1 Analytical composition of tobacco seeds

The chemical analysis of principal components of tobacco seeds is reported in Table 1.

Chemical composition		
	% DM	*% as fed*
CP, %	23,71	22,79
Fat, %	35,35	33,97
NDF, %	43,2	41,51
Ash %	2,81	2,7

Table 1 – Analytical composition of tobacco seeds.

4.4.2 Digestion of tobacco seeds

The Vt2e-B specific signal was observed in all samples derived from transgenic whole and milled tobacco seeds (Figure 1, 2).

No significant differences were observed in relation to the times of treatment of the samples derived from transgenic whole seeds (1h, 2h and 3h, Figure 1).

These results confirm the potential of plant material, particularly seeds, to protect selected antigens against digestion in the stomach, as previously shown by other studies (Bailey, 2000).

From 0 h to 3 h, a progressive reduction of the intensity of the signal was observed in samples derived from transgenic milled tobacco seeds (Figure 2). This reduction was more evident between 2 and 3 hours of incubation with gastric fluid, indicating a partial antigen degradation after 2 hours. However, even after 3 hours of incubation with gastric fluid it was possible to detect the specific VT2e-B signal in the samples derived from milled transgenic tobacco seeds.

4.5 Conclusions

One of the most important issues related to the oral delivery of plant-based vaccines is the potential for antigen degradation in the gastrointestinal tract. Since there is no data available concerning the gastric degradability of antigenic proteins expressed in tobacco seeds, the present study investigated the effect of gastric fluid derived from weaned piglets on VT2e-B antigenic protein expressed in whole and milled tobacco seeds.

After incubation with porcine gastric fluid, a progressive reduction of the intensity of the signal for VT2e-B was observed from 0 h to 3 h, more evident in milled transgenic tobacco seeds than in whole seeds. Even after 3 hours of incubation with gastric fluid it was possible to detect the VT2e-B signal in both whole and milled seeds. Our results confirmed those of previous studies (Bailey, 2000; Daniell, 2006), showing that natural encapsulation of antigenic proteins within plant cells offers protection against digestion in the stomach.

The ability of the specific polyclonal rabbit serum to recognize the antigen even after 3 hours of incubation with porcine gastric fluid suggests that the residual amount of transgenic proteins after digestion of both milled and whole seeds appears sufficient for their use in immunization trials on piglets. This study represents a preliminary step for a subsequent study, in which it was decided to use milled tobacco seeds expressing antigenic proteins against OD for the oral immunization of recently weaned piglets.

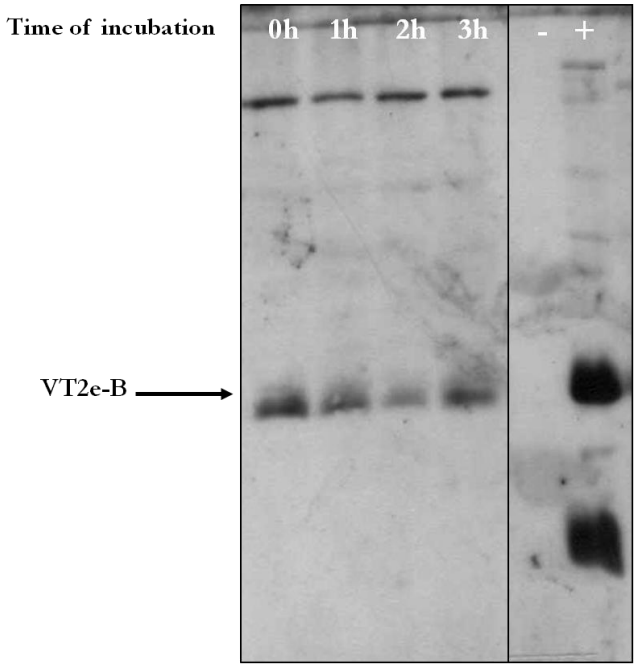

Figure 1 –Vt2e-B specific signal observed in samples derived from transgenic whole tobacco seeds. Positive control was represented by VT2e-B protein expressed in pET system.

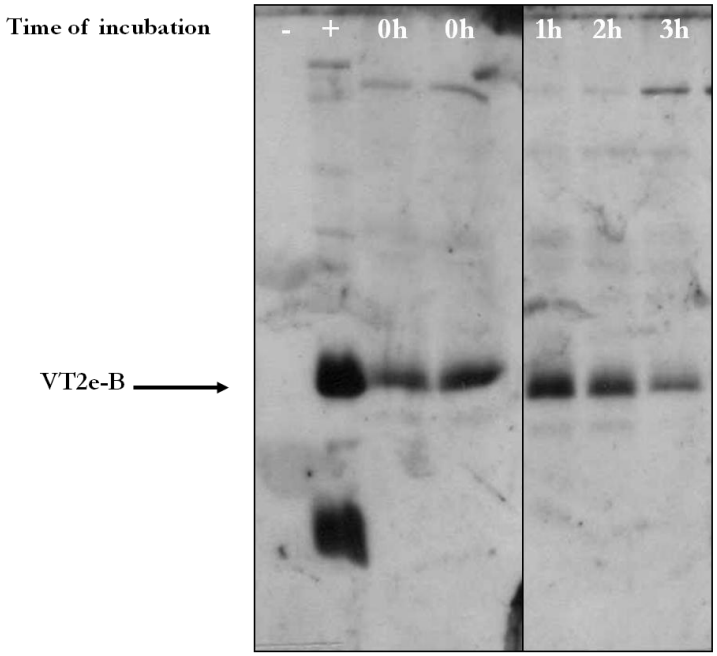

Figure 2 –Vt2e-B specific signal observed in samples derived from transgenic milled tobacco seeds. Positive control was represented by VT2e-B protein expressed in pET system.

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CHAPTER 5

Oral administration and evaluation in piglets of tobacco seeds expressing antigenic proteins against Oedema Disease

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5. Oral administration and evaluation in piglets of tobacco seeds expressing antigenic proteins against Oedema Disease

5.1 Abstract

Vaccination is an important and cost-effective way to control animal and human infectious diseases. The use of transgenic plants as delivery system for vaccine proteins is attractive for its simplicity and increases likelihood for local immune response at sites of infection. In particular in livestock, vaccination allows to reduce antibiotic treatments, as suggested by EC Regulation 1831/2003.
The aim of this study was to evaluate the use of transgenic tobacco seeds as edible vaccine in piglets. Tobacco seeds were previously transformed *via agroinfection* for the expression of antigenic proteins of verotoxin-producing *E. coli* (VTEC) strains responsible for Pig Oedema Disease: the F18 fimbriae adhesive and the VT2e-B subunit. Forty-three piglets, weaned at 20±2 days, were randomly divided into 4 experimental groups. Three immunized groups (T1, T2, T3) orally received a bolus of tobacco seeds (TS) mixed with chocolate on days 0, 1, 2, 14 of the trial. In particular, the T1 group received 10 grams of TS-F18+ and 10 grams of TS-VT2e-B+, the T2 group received 10 grams of TS-VT2e-B+ and the T3 group received 25 grams of TS-VT2e-B+. Control Group (CG) received 20 grams of wild type TS. The amount of transgenic protein was estimated about 0.6 mg/gram of whole TS. In this immunization phase faecal and blood samples were collected weekly to evaluate IgA and IgG amounts by ELISA assays. On day 22, the piglets were orally challenged with $1*10^{10}$ CFU of O138 *Escherichia coli* strain. Faecal score, body temperature and clinical signs related to OD (eyelids, epiphora, neurological and respiratory symptoms, vitality) were determined, daily for 15 days after challenge, for each piglet through specific point scales. Zootechnical performances and haematocrit percentages (HT) were evaluated during the experimental period. T1, receiving both the antigens, showed a higher level of IgA in faeces than other groups on day 21 (22,000±13,000 ng/ml *vs* control group: 7,200±3,000 ng/ml). No differences were observed among groups in relation to the total IgG titre in faeces and total IgA and IgG levels in serum. For each clinical sign, the average total score (the sum of the average daily score from day 1 to day 9 post-challenge) was significantly higher in the control group compared to orally immunized groups ($P<0.05$) and the latter showed a faster recovery than in CG. In the same period (day 1 to 9 post-challenge), T1 showed a significantly higher consistency of faeces compared to T1 and T2 ($P<0.05$). No differences were observed in body

temperature and HT. After challenge (day 21 to 25), the average daily feed intake ($P<0.05$) and the average daily gain were higher in T1 and T2 than CG. For all measured parameters, no differences were observed between T2 and T3, suggesting that no dose-response effect was shown for the Vt2e-B antigen in our experimental conditions. In conclusion, oral administration of recombinant tobacco seeds expressing antigenic proteins against VTEC strains can induce an increase in mucosal antibodies and a protective effect against challenger strain in piglets.

5.2 Introduction

Oedema disease (OD) is a systemic disease that usually occurs in piglets shortly after weaning (mainly within the first 2 weeks post-weaning) and causes significant economic losses due to high morbidity, mortality and reduction of growth rates. Clinical signs of the disease include palpebral oedema, neurological symptoms (i.e. ataxia, paralysis), recumbency, sudden death. Diarrheoa and rise of temperature may occur, but these are not typical symptoms (Imberechts *et al.* 1992, Bertschinger and Gyles 1994).
Such disease is attributed to a heat-labile toxin from the Shiga toxin family (also called verotoxin or Shiga-like toxin), produced by verotoxigenic *Escherichia coli* (VTEC) strains belonging to serogroups O138, O139, and O141 (Gannon *et al.* 1988, Bertschinger and Gyles 1994) and called Shiga toxin 2e (Stx2e or VT2e). VT2e toxins are bipartite 70-kDa proteins composed of a single enzymatically active A subunit and five B subunits. The A subunit has N-glycosidase activity that removes a specific adenine base from the 28S rRNA of the 60S ribosomal subunits, inhibiting protein synthesis at the ribosomal level and resulting in citotoxicity and the clinical syndrome. The B subunits of Stx2e bind to globotetraosylceramide present on the surface of gut cells and are responsible for binding the toxin to the cell surface.
The aetiology of the disease is complex, since changes on food composition and consistency, loss of passive protection from the sow and genetic susceptibility of the pigs are involved in the pathogenesis (Imberechts *et al.* 1992).
The VTEC strains colonize the ileum via host-specific F18 fimbriae that allow the bacteria to adhere to F18 receptors on small intestinal enterocytes.
The F18 fimbrial adhesin recognizes and binds to specific glycosphingolipids, having blood group ABH determinants on the type 1 core present on enterocytes of weaned piglets (Coddens *et al.*, 2009). Colonization is followed by secretion of verotoxins responsible for the pathogenic effect.
Currently, no vaccine that protects piglets against OD is available and treatment relies upon the use of antimicrobials that, however, are frequently used too late for piglets with visible clinical signs, when the toxin has already been produced

in the gut, has systemically spread and caused consequent lesions. Moreover, due to the excessive use of antibiotics in intensive and large-scale swine production and due to the consequent increase of antibiotic-resistant enteric bacteria, especially *E. coli* strains, treatment with antimicrobials has come under increased scrutiny (Aarestrup *et al.* 2008).
Due to the pathogenesis of OD, oral vaccination could represent an interesting strategy to control the disease. In fact oral delivery of antigens has shown to be capable to induce mucosal immune responses. The mucosal immune system plays a crucial role in the primary defence against pathogens that invade the gastrointestinal tract, by preventing adhesion of the pathogens and/or neutralizing toxins (for review, see Streatfield 2006).
It has been shown that purified VT2e injected intravenously in pigs reproduces all the clinical signs and pathological lesions of OD (Macleod *et al.* 1991) and is therefore an important virulence factor. The F18 fimbria has been also indicated as an important virulence factor related to OD (Ansulini *et al.*, 1994; Gannon and Gyles, 1989; Rippinger *et al.*, 1995), and pig intestinal colonization with live (F18-positive) *Escherichia coli* strains resulted in a significantly increased level of anti-fimbriae antibodies, especially IgA, in serum and intestinal wash fluids (Sarrazin and Bertshinger, 1997). In this regard there is a great deal of interest in developing vaccination strategies that lead to mucosal immunity, with production of IgA instead of IgG.
In this study, two proteins actively involved in the pathogenesis of the disease were selected as potential immunogens against OD: the F18 fimbria, responsible for the adherence of the bacteria on small intestinal enterocytes, and the B subunits of VT2e, responsible for binding the toxin to specific receptors on cell surface. In a previous phase, tobacco seeds of *Nicotiana tabacum* specie were transformed via *agroinfection* for the expression of the antigenic proteins. Tobacco presents many advantages as a laboratory model plant, including high transformation efficiency and easy cell culture protocols. Furthermore, unlike the leaves, seeds do not contain significant levels of nicotine and, once included in diets for weaned piglets, they showed a good palatability (Rossi *et al.* 2007).
The aim of this study was to evaluate whether oral immunization of weaned piglets with transgenic tobacco seeds expressing antigenic proteins against OD could induce an increase of mucosal antibodies and a protective effect against a subsequent O138 *Escherichia coli* infection.

5.3 Materials and methods

In the present study, a total amount of 500 grams of transgenic tobacco seeds transformed for the expression of F18, and 1,900 grams of transgenic tobacco seeds transformed for the expression of VT2eB were used as edible vaccines. In

particular, seeds of the two lines of transgenic tobacco were collected after flowering and were evaluated with the methods described below.

5.3.1 Analysis of the chemical composition of tobacco seeds

The chemical analysis of tobacco seeds was performed to measure the principal components: dry matter (dm, AOAC method 930.15); crude protein (CP, AOAC method 2001.11); ether extract (EE, DM 21/12/1998); crude fibre, (CF, AOCS method Ba 6a-05); neutral detergent fibre, (NDF, AOAC method 2002.04) and ash (AOAC method 942.05).

5.3.2 Transgenic tobacco seeds evaluation

Tobacco seeds were transformed via *agroinfection* (*Agrobacterium tumefaciens* EHA 105) using pBIpGLOB bynary vector, as previously described (Rossi *et al.*, 2002). pBIpGLOB carried Kanamycin resistance gene and a soybean basic 7S globulin promoter which is able to direct the transgene expression in seeds (Reggi *et al.* 2005).

The second generation (R_1) of transgenic tobacco plants was evaluated for the presence of the gene codifying for VT2e-B and F18 proteins by PCR, using internal primers of gene sequence, as previously described (Rossi *et al.* 2003a).

The seed transcription ability of the two transgenic genes was evaluated by Northern Blot, using RNA probes labelled with digoxigenin, specific for mRNA F18 and mRNA VT2eB.

Total proteins were obtained from all mature transformed tobacco seeds (positive for Northern blot) by homogenization with liquid N2 in a mortar and protein extraction with the solubilisation buffer (50 mM Tris, pH 8, 5 mM EDTA, 200 mM NaCl, 0.1% Tween 20). Protein content was estimated by a Bradford assay (BioRad, Hercules, USA) using bovine serum albumin as the standard. The presence of the F18 protein was evaluated by agglutination on slides with F18+ polyclonal serum (Biovac, Beaucouze Cedex, France), according to the modified method previously described by Chen *et al.* (2004). Total proteins extracted from untransformed tobacco seeds were used as negative control. A sample of 50 ml of total proteins was put onto a slide with 50 ml of F18 antibody solution and evaluated for the presence/absence of a reaction by optical microscopy (×100).

The expression of VT2e-B in the total protein sample was evaluated by Western blotting with specific polyclonal antibodies obtained from New Zealand rabbits. Samples (80 mg total protein) were loaded in a 10% polyacrylamide gel together with the Precision Standards (BioRad, Hercules, USA). The proteins were transferred to an Immobilon-PSQ membrane (Millipore, Billerica, USA) with the Trans-Blot SD apparatus (BioRad, Hercules, USA); the filters were incubated overnight with rabbit polyclonal anti-VT2e-B serum (1:5000). After incubation

for 1 hour with HRP-conjugated secondary antibody (1:10,000), chemiluminescence was developed using the SuperSignal West Pico Trial Kit (Pierce, Rockford, USA).
The amount of transgenic protein contained in 1 gram of whole seeds was estimated by the comparison of results with a positive control (VT2e-B produced by pET-system).
Transgenic and wild type tobacco seeds used in the present study were ground with a suitable mill to obtain a uniform milling, paying attention to avoid the overheating of the seeds and the subsequent proteins denaturation.

5.3.3 Pigs

Forty-three piglets (Landrace x Large White) were selected from a conventional herd free from diseases according to the A-list of the International Office of Epizootic, and from Aujeszky's disease, atrophic rhinitis, transmissible gastroenteritis, porcine reproductive and respiratory syndrome and salmonellosis, without history of PWD and OD and with bacteriological analysis of the faeces negative for hemolytic *E. coli*.
Piglets were early weaned at 20±2 days to ensure the animals' sensitivity upon experimental infection and were transported to the experimental facilities specific for pigs at the Experimental Animal Research and Application Centre in Lodi.
Animals were allocated in pens, each pen containing 2-3 piglets, under the same environmental conditions (environmental temperature regulated at 28 °C and relative humidity maintained at 60%), with water and feed *ad libitum*. The composition of the experimental diet is reported in Table 1. The pigs were orally treated with colistine (150,000 units/kg body weight/day) up to 5 days post-weaning to prevent *E. coli* infections.
Experimental and management procedures were approved by the Ethics Committee of the Faculty of Veterinary Medicine (Favourable opinion about the project "Evaluation of immunogenity of tobacco seeds expressing antigenic protein to OD in weaned piglets", February 17th, 2011).
Piglets were randomly divided into 4 experimental groups:
Treatment 1 group (T1): composed of 12 piglets receiving 20 grams of milled transgenic tobacco seeds (10 grams containing 6 mg VT2e-B and 10 grams containing 6 mg F18)
Treatment 2 group (T2): composed of 9 piglets receiving 10 grams of milled transgenic tobacco seeds containing 6 mg VT2e-B.
Treatment 3 group (T3): composed of 10 piglets receiving 25 grams of milled transgenic tobacco seeds containing 15 mg VT2e-B

Ingredients (%) of the experimental basal diet.	
Wheat flakes	23,20
Barley	21,00
Maize flakes	12,00
Wheat	7,60
Soy protein concentrate	7,50
Harring meal	5,00
Milk whey	5,00
High protein soybean meal	5,00
Soya oil	3,45
Dextrose	2,50
Lactose	2,50
Citric acid	1,00
Dicalcium phosphate	0,73
Calcium carbonate	0,55
L-Lys HCl	0,50
Butyric acid monoglyceride (Monobutyrin)	0,45
Vitamin and trace mineral premix	0,45
Acidifier	0,42
L-Thr	0,40
DL-Met	0,25
Inulin powder	0,20
Aroma substances	0,15
Trp	0,10
Sweetener	0,05
Calculated chemical composition, % DM	
CP, %	20,96
Fat, %	5,96
Crude fiber, %	2,74
Ash %	5,55
NE, Mc/Kg	2,73
Lys, %	1,63
Met +Cys, %	0,99
Thr, %	1,22
Trp, %	0,34
Ca, %	0,81
P, %	0,65
Na, %	0,13

Table 1 – Composition of the experimental diet and calculated chemical analysis

Control group (CG): composed of 12 piglets receiving 20 grams of wild type milled tobacco seeds.

The dose of antigen used for oral immunization was determined after a thorough literature search, considering the doses used by other researchers (Verdonck *et al.* 2007, Lamphear *et al.* 2002, Streatfield *et al.* 2001).

5.3.4 Oral immunization

Five days after weaning, three groups of piglets (T1, T2, T3) were orally immunized on 3 subsequent days (0, 1 and 2 days post primary immunization, dppi) and again on 14 dppi (booster immunization). On the same days, the control group received wild type milled tobacco seeds. Tobacco seeds were individually administered mixed with chocolate and water in palatable boluses.
Three hours before immunization up to 3 hours after it, animals were deprived of food. About 10-15 min before each immunization piglets orally received 60 mL of a 10% bicarbonate solution to neutralize the gastric acid pH and to protect the antigen against possible denaturation (Snoeck *et al.*, 2004).

5.3.5 Challenge infection

About one week after booster immunization (22 dppi), the animals were orally challenged with O138 *Escherichia coli* strain, isolated from one weaned piglet that had died because of OD (provided by Prof. Sala, Department of Animal Pathology, Hygiene and Veterinary Public Health, Università di Milano).
Sixty minutes before challenge, piglets were sedated with azaperon (StresnilTM, Janssen Cilag SpA, 2 ml/head), thereafter 30 mL of a 10% bicarbonate solution (SIGMA, Italy) was orally administered in the attempt to neutralize gastric acid and to increase the survival rate of challenger strain in the stomach (Madec *et al.*, 2000, Jensen *et al.*, 2006). After 10-15 min, the *inoculum* was given via oral route in a single dose of 5 mL of bacterial medium with $1*10^{10}$ colony forming units (CFU) of challenger strain, using 16G catheter. Animals were fasted 3 hours before and 3 hours after challenge.
For each group, two animals were not challenged and housed in pens separated by means of two empty pens from the infected group, so that physical contact between challenged and not challenged piglets was excluded.
From the day of challenge until the second day after challenge, the same antimicrobial-free diet, containing 27% of crude protein on dry matter, was administered to all experimental groups. Before administration, chemical analysis of the diet was performed to confirm its high protein level and to measure its principal components: crude protein (CP), according to the official method of Analysis of Association of Analytical Communities, procedure 2001.11 (AOAC, 2005); dry matter (dm), according to procedure 930.15 (AOAC, 2005); fat (EE) according to DM 21/12/1998; crude fibre (CF), according to procedure Ba 6a-05 of the official method of the American Oil Chemists Society (AOCS, 1998); ash, according to procedure 942.05 (AOAC, 2005).

5.3.6 Clinical and zootechnical examinations, sample collection

Blood was collected from the jugular vein of each animal on 0, 7, 14, 21, 25, 29 and 36 dppi. Samples were taken in double to determine hematocrit value and serum antibodies, as described below.

Faecal samples were taken from rectum weekly on 0, 7, 14, 21 dppi to determine total IgA levels in the immunization phase, as described below. In addition, from the day after challenge (23 dppi) until 7 days post-challenge (29 dppi), faeces were sampled daily for determining the excretion of challenger strain.

From the day of challenge until day 14 post-challenge (36 dppi), rectal temperature was daily recorded and the clinical signs of the disease - in particular: palpebral oedema, epiphora, vitality, respiratory and neurological problems - were daily checked and scored using the specific point scales reported below:

Respiratory score: 0=normal; 1=slightly quick; 2= quick.

Oedemal score in palpebra: 0=normal; 1=mild; 2=severe.

Epiphora score: 0=normal; 1=mild; 2=severe.

Vitality score: 0=good; 1=loose; 2=bad.

In the same period, faecal consistency was daily evaluated through a scale of four levels: 0=normal faeces; 1=soft consistency 2=mild diarrhoea; 3=severe diarrhoea.

All piglets were individually weighed weekly during the entire experimental period and twice in the week following challenge (25 and 28 dppi); the feed intake (FI) was daily measured weighing the residual feed at the pen level (experimental unit for FI evaluation).

On day 10 post-challenge (32 dppi), 12 piglets (one challenged and one unchallenged piglet for each group plus 4 challenged controls) were euthanized for *post mortem* examination and for histological studies.

Animal care and euthanasia at the end of the trial were conducted in accordance with the European Union guidelines (86/609/EEC) approved by the Italian Ministry of Health.

5.3.7 Detection of serum and faecal antibodies

Total IgA and IgG concentrations in serum and faecal samples were evaluated through Pig ELISA quantitation set.

Faecal antibody extraction. Briefly, 0.5 g of stool was diluted in 1 ml of sterile water and thoroughly mixed for 1 hour. Two ml of PBS-Tween 20 were added to 1 g of the homogenate and centrifuged at 1,600 × g for 15 min at 4°C. The supernatant was collected and further centrifuged at 7,200 × g for 10 min at 4°C. The supernatant was then removed and stored at −20°C until use.

ELISA. "Pig ELISA IgA quantitation set" was performed to detect and quantify IgA and "Pig ELISA IgG quantitation set" was performed to detect and quantify IgG in serum and faecal samples.
Stool samples were diluted 1:100 for quantification of IgA and used without further processing for the quantification of IgG.
Serum samples were diluted 1:5,000 for titration of IgA and 1:20,000, using serial dilutions in PBS and Sample Diluent, for titration of IgG ("ELISA quantitation set", Bethyl Laboratories, Texas).
Microtitration 96-well plates were coated for 1 h at ambient temperature (20-25°) with 100µl of a solution containing 1 µl of Affinity purified Pig IgA (or IgG) diluted in 100 µl of coating buffer. After aspiration of the antibody solution and after washing the plate five times, the wells were blocked with 200 µl of Blocking Solution for 30 minutes and then washed five times with 300 µl of Wash Solution. To each well 100 µl of standard or sample were added and incubated at room temperature for 1 hour. After washing the plate five times, 100 µl of diluted HRP detection antibody were added to each well and incubated at room temperature for 1 hour. After washing the plate five times, 100 µl of TMB Substrate Solution were added to each well. The plate was developed in the dark at room temperature for 15 minutes, then reaction was stopped by adding 100 µl of Stop Solution to each well. Absorbance was measured on a plate reader at 450 nm. For each plate, a standard curve was constructed to calculate the IgA or IgG concentration of each sample using "Curva Expert 1.3" software. The concentrations determined were expressed as nanograms of IgA or IgG per 1 ml.

5.3.8 Microbiological analysis of faecal samples

Microbiological analysis of faecal samples was performed by the Department of Animal Pathology, Hygiene and Veterinary Public Health, Università di Milano, Scientific Responsible Prof. Sala. For each sample 1 gram of faeces was homogenized with 1 ml of saline and incubated overnight at 37 ° C on sheep blood agar plates 5% (Blood Agar Base No. 2 - Oxoid), to examine the presence of hemolytic colonies. Up to 5 hemolytic colonies were selected from each plate and growth on MacConkey agar (MacConkey Agar - Oxoid), Triple Sugar Iron (Triple Sugar Iron Agar - Oxoid), citrate (Simmons Citrate Agar - Oxoid) and peptone water (Oxoid Buffered Peptone Water). Colonies positive for glucose oxidation–fermentation, fermentation of lactose (MacConkey agar), indole production (peptone water + reagent Kovacs - Kovac's reagent for indoles-Fluka) and sodium citrate-negative were then subjected to confirmatory biochemical test strips with the API system (API 20 NE-Biomerieux).

5.3.9 Histological analyses

Histological analyses were performed by the Anatomy Laboratory of the Department of Veterinary Sciences and Technology for Food Safety, Università di Milano, Scientific Responsible Prof. Domeneghini. Twelve samples of the ileum (one for each euthanized animal) were collected immediately after the sacrifice of each animal and fixed in a buffered formalin solution. Each sample was dehydrated in alcohols and embedded in paraffin. Microtome sections (3 μm) of the 12 ileum samples were prepared and stained with hematoxylin/eosin. Pictures of image analyses were taken by a digital camera attached to a light microscope (Olympus BX51).

5.3.10 Statistical analysis

Data were analysed using the GLM procedure (SAS System, Version 9.2; SAS Institute, Inc., Cary, NC).

For the performance data, ADG, ADFI and G:F were averaged over a pre-challenge period (0-21 dppi) and 3 post-challenge periods (21-25, 25-28 and 21-28 dppi), in order to evaluate the effect of *E. coli* infection on the zootechnical performances of the experimental groups.

In the experiment, pen means were treated as the experimental unit for daily feed intake measurement. All other measurements were assessed using the individual pig as experimental unit.

The values are represented by means ± sem.

5.4 Results and discussion

5.4.1 Analytical composition of tobacco seeds

The chemical analysis of principal components of the tobacco seeds is reported in Table 2. No differences in the analytical composition were observed between transgenic seeds and wild-type seeds.

Chemical composition		
	% DM	*% as fed*
CP, %	23,71	22,79
Fat, %	35,35	33,97
NDF, %	43,2	41,51
Ash %	2,81	2,7

Table 2 – Analytical composition of tobacco seeds.

5.4.2 Tobacco seeds evaluation

All the selected plants showed the specific band corresponding to VT2e-B gene by electophoresis in agarose gel (Figure 1).

The analyses of transcription of specific genes performed by Northern Blotting showed the signal corresponding to VT2e-B mRNA in all selected plants (Figure 2).

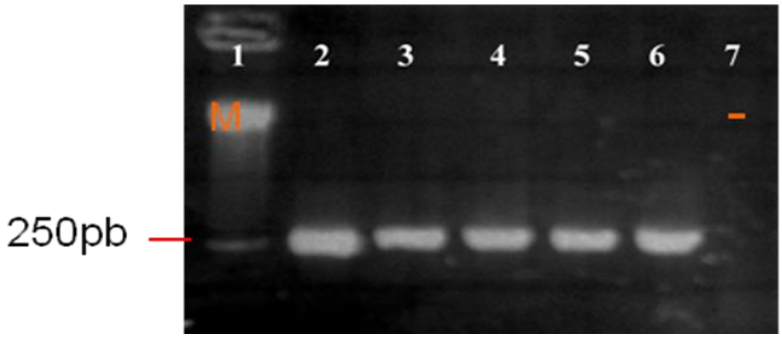

Figure 1 – All selected plants showed the specific band corresponding to VT2e-B gene, corresponding to a fragment of 250pb.

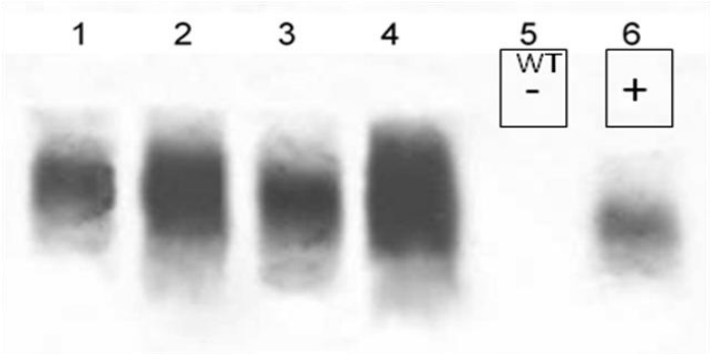

Figure 2 – The analyses of transcription of specific genes performed by Northern Blotting showed the signal corresponding to VT2e-B mRNA in all selected plants.

The presence of Vt2e-B subunit protein in tobacco seeds was observed by Western blot analysis using a specific rabbit serum (Figure 3). All samples positive to the VT2e-B mRNA signal were also positive to the Vt2e-B subunit protein.

Previous studies (Reggi *et al.* 2005) demonstrated that the expression level of specific proteins in tobacco seeds under the control of GLOB promoter ranges from 0.3 to 0.5% of total proteins. Considering a minimum value (0.3%), it is possible to estimate a content of 0.6 mg of transgenic protein in 1 gram of whole seed with 200 mg of CP, in agreement with results previously reported. These findings allowed to determine the amount of transgenic tobacco seeds to be used for oral immunization *in vivo*. The residual amount of transgenic proteins after *in*

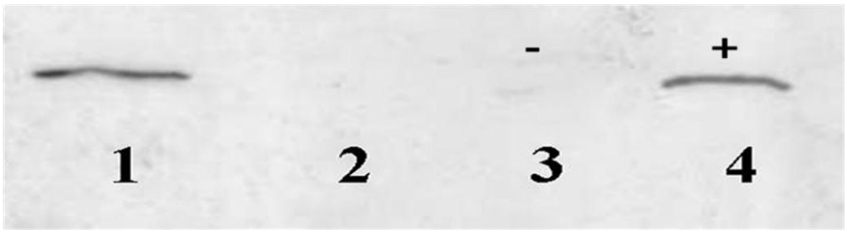

Figure 3 – Vt2e-B subunit protein in tobacco seeds was determined by Western blot analysis using a specific rabbit serum. Negative control: total soluble proteins extracted from wild type tobacco seeds. Positive control: VT2e-B produced by pET-system

vitro digestion of milled seeds was previously evaluated (chapter 5) and appeared sufficient for their use in immunization trials on piglets.

5.4.3 Diet composition

Chemical analysis of the experimental diet administered from the day of challenge until the second day after challenge confirmed the high level of protein previously calculated by Plurimix software (CP 27.46% dm, EE 5.38% dm, CF 6.73% dm, ashes 5.54% dm). The diet containing a high level of crude protein was intended to be a "high-risk" diet, since several pathogens preferentially ferment proteins and clinical OD was found to result after experimental infection, just when the protein content of the diet was increased (Bosworth *et al.* 1996).

5.4.4 Detection of fcecal antibodies after oral immunization

Secretory immunoglobulin A (sIgA) is a protective molecule of the mucosal immune system (Snoeck *et al.* 2006). It mediates the primary immunological defense line in the mucosal immune system. In the case of pigs, maternal sIgA is supplied to piglets by maternal colostrum and milk. Faecal IgA detected before 10 days of life is likely to be mostly derived from maternal fluids ingested during the first 3 days and IgA detected after 22 days to be produced by the piglet (Thompson *et al.*, 2008). In piglets, the key elements of the induction system in the mucosa develop over the first 2 weeks after birth and an almost adult organization of Peyer's patches is typically present by 12 days (Bailey *et al.*, 2001). The final stage of pig immune system maturation is thought to start around 28 days of age, when the CD8+ T cells infiltrate the intestinal tissue, and the mucosal immune system has a largely adult architecture by 6 weeks of age (Bailey *et al.*, 2001). At this age, IgA-secreting plasma cells are also present in the intestine (Bianchi *et al.*, 1999).

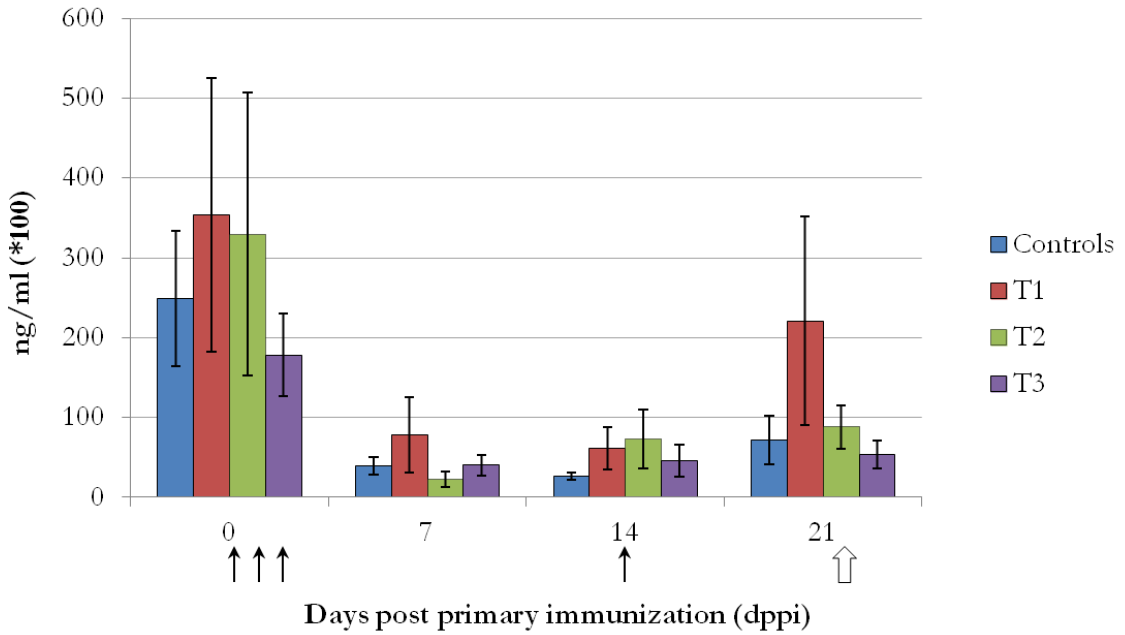

Figure 4 – Mean faecal IgA levels of piglets orally immunized with transgenic tobacco seeds containing about 6 mg of F18 and 6 mg of VT2e-B (T1), with transgenic tobacco seeds containing about 6 mg of VT2e-B (T2), with transgenic tobacco seeds containing about 15 mg of VT2e-B (T3) and with wild type tobacco seeds (Controls) in the pre-challenge period. SE of each point is indicated. Black arrows represent immunization and white arrow the VTEC challenge.

In our experiment (Figure 4), from the moment of primary oral immunization (0 dppi) to 7 dppi a decline of total IgA levels was observed, consistently with the rapid decline of luminal level of sIgA observed a few days after weaning (Ushida *et al.* 2008).

Interestingly, one week following booster immunization, the day before challenge (21 dppi), mean IgA faecal levels were increased and were clearly higher in the T1 group, compared to the others (22,000±13,000 ng/ml *vs* control group: 7,200±3,000 ng/ml). The lack of significance of this finding is probably due to the large intra-individual variation in faecal immunoglobulin levels observed in our experiment, higher than that observed in preliminary studies performed on a mouse model (Rossi *et al.*, 2003b). This figure was obviously influenced by the variability linked to colostrum intake and by the fact that the piglets included in this trial were not genetically selected and especially standardized for clinical trials. In this study, the choice of the animals was made according to the specific purpose to evaluate the efficacy of transgenic tobacco seeds under conditions as more similar as possible to those of intensive pig farming. However, the increased levels of faecal IgA observed in T1 group may suggest a higher local immune response activity in the group immunized with both antigens. The use of the multicomponent vaccine, based on two important

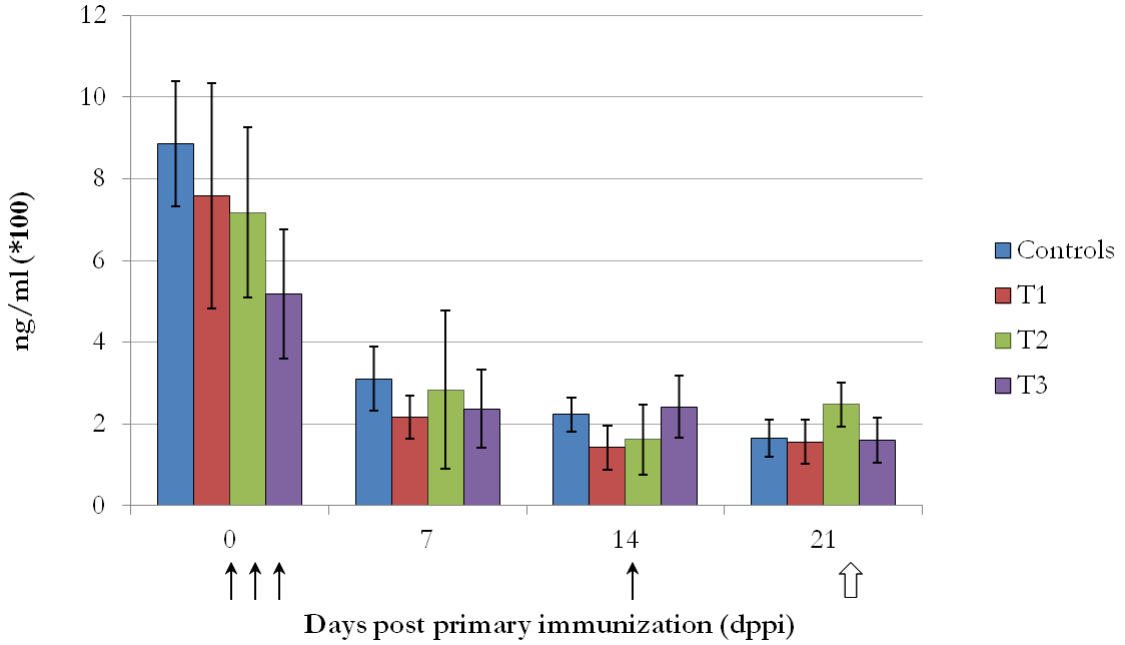

Figure 5 – Mean faecal IgG levels of piglets orally immunized with transgenic tobacco seeds (T1, T2, T3) and with wild type tobacco seeds (Controls) in the pre-challenge period. SE of each point is indicated. Black arrows represent immunization and white arrow the VTEC challenge.

virulence factors of VTEC strains, appeared to be more effective for the activation of the local immune response.

With regard to IgG levels in the faeces, statistically significant differences among experimental groups were not detected (Figure 5). Considering the general trend over the time, with the exception of day 0, when a high title was recorded due to maternal transfer of immunity, in the rest of the pre-challenge period faecal IgG levels kept almost constant in all groups. The IgG was present in very low quantities in the faeces compared with IgA, in agreement with data reported by other authors (Franz and Corthier 1981).

5.4.5 Detection of serum antibodies after oral immunization

Serum immunoglobulins (IgA and IgG) were measured to have an overview of the immune system in the period corresponding to the oral administration of antigens. For each group, blood levels of IgG were high at day 0, due to maternal antibody transmission (Figure 6). Thereafter, a decrease (from day 0 to day 14) and a subsequent rise (from day 14 to day 21) in serum IgG levels over the time was found, consistent with a framework of maturation of the immune system typical of this age (Frenyo *et al.* 1981). In fact, the reduction in serum antibody levels in piglets after weaning was reported by other authors and can be attributed to weaning stress (Deng *et al.* 2007). Serum IgA levels showed an upward trend over time (Figure 7). As expected, total IgA and IgG serum levels

monitored in the pre-challenge period showed no significant differences among experimental groups, suggesting that the immunization protocol adopted in this study did not affect serum total immunoglobulin levels.

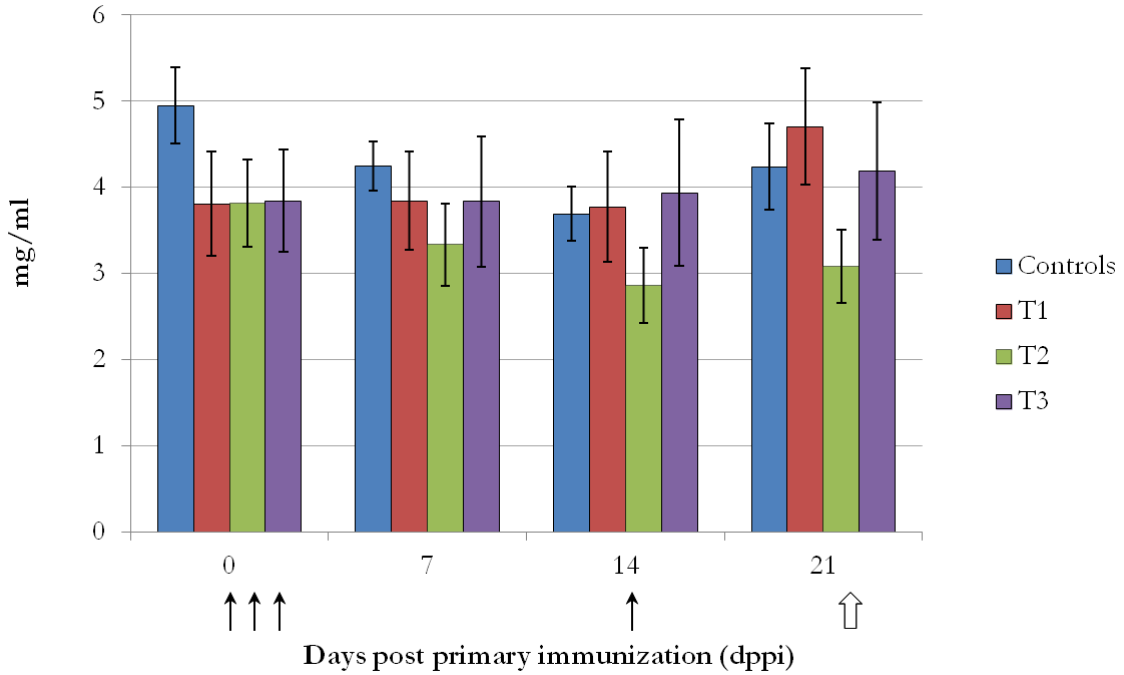

Figure 6 – Mean serum IgG levels of piglets orally immunized with transgenic tobacco seeds (T1, T2, T3) and with wild type tobacco seeds (Controls) in the pre-challenge period. SE of each point is indicated. Black arrows represent immunization and white arrow the VTEC challenge.

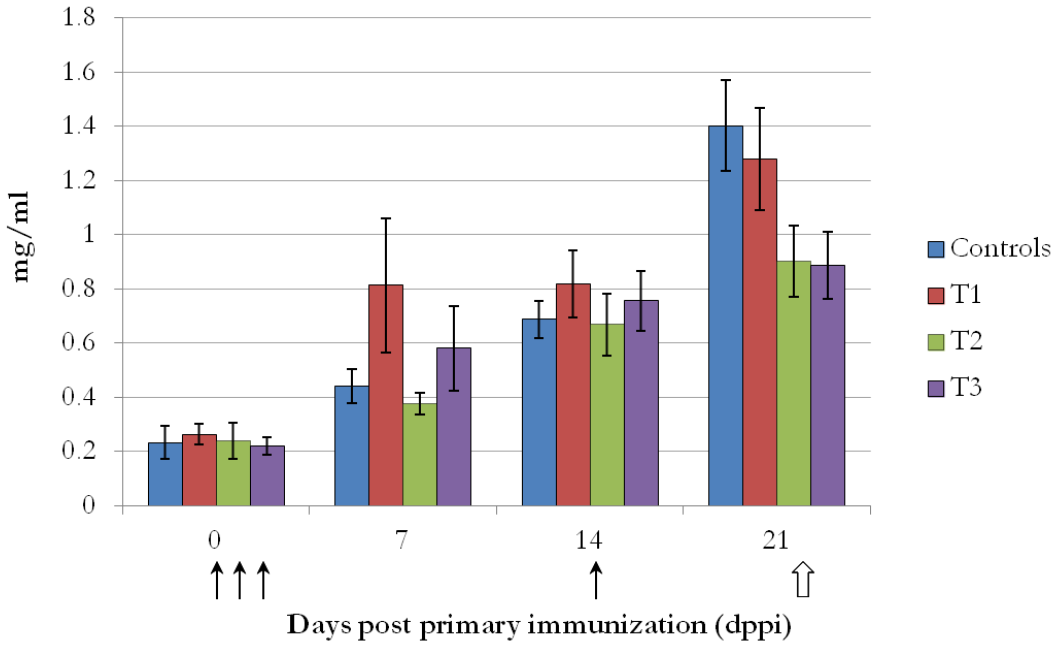

Figure 7 – Mean serum IgA levels of piglets orally immunized with transgenic tobacco seeds (T1, T2, T3) and with wild type tobacco seeds (Controls) in the pre-challenge period. SE of each point is indicated. Black arrows represent immunization and white arrow the VTEC challenge.

5.4.6 Microbiological analysis of faecal samples

None of the piglets presented faecal shedding of hemolytic *E. coli* strains before challenge.

Microbiological evaluation of faecal samples showed presence of hemolytic *E. coli* strains one day after challenge (23 dppi), in almost half of the subjects of each group (41.67% of piglets in control group, 50% in T1 group, 33.33% in T2 group and 40% in T3 group). The microbiological analysis confirmed the effect of the challenger strain on faecal shedding of hemolytic *E. coli* strains. The detection of the *E. coli* strain from the faeces did not invariably correspond to the presence of diarrhoea; this finding is consistent with the clinical features of the disease; in fact diarrhoea is not considered a typical clinical sign of OD.

From 23 dppi to 28 dppi, in some subjects of each group a steady but intermittent faecal shedding of hemolytic *E. coli* strains was observed.

5.4.7 Clinical and faecal score

Clinical signs related to OD, such as palpebral oedema, epiphora, loss of vitality and respiratory problems, in all challenged groups from the day after challenge until day 9 post-challenge are shown in Table 3. Neurological signs and death were not observed in any pigs throughout the experiment. The results of clinical examination showed that experimental infection, while having caused no mortality or neurological symptoms, was effective in reproducing the typical symptoms of the disease.

In particular, focusing on palpebral oedema, more closely related to the disease than other symptoms, the control group presented the highest average daily scores, showing an average score >0.9 from day 2 to day 6 post-challenge. T1 group presented the lowest values on a daily basis, showing a maximum average daily score (0.6) on day 5 post-challenge.

As to vitality, considered to be symptom of general well-being, was better in treated piglets compared to controls on a daily basis. Once again, subjects in T1 group showed a better state of health and were more reactive to stimuli.

For each clinical sign, the average total score, i.e. the sum of average daily scores from day 1 to day 9 post-challenge, was significantly higher in the control group compared to orally immunized groups (T1, T2 and T3, $P<0.05$). This may indicate a protective effect of vaccines containing VT2eB against systemic symptoms induced by toxin VT2e.

A slightly different situation was found when evaluating faecal scores; in this case, T1 group showed scores significantly better compared to T2 and T3 groups ($P<0.05$). This may indicate that vaccination with both antigens probably conferred a protective effect at gastrointestinal mucosal level as well.

Overall, a better clinical presentation was observed after challenge in piglets of T1 group, immunized with transgenic tobacco seeds containing both antigens

(VT2eB and F18). This immunization strategy appeared more effective in preventing the development of clinical signs after challenge with O138 *E. coli* strain.
From day 9 onwards, in all challenged groups the average clinical and faecal score returned within the range of normality. A slight increase in average temperature was measured in all groups after challenge (Figure 8). However, average temperature and hematocrit percentage did not show significant differences among experimental groups.

		Days after challenge									Total score
Scores	Group	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**(d1-9)**
Respiration	Control	0.6	0.4	0.6	0.7	0.4	0.4	0.7	0.5	0.5	**4.8^{a}**
	T1	0.3	0.2	0.3	0.2	0.1	0.2	0.3	0.3	0.0	**1.9^{b}**
	T2	0.3	0.1	0.3	0.3	0.3	0.1	0.4	0.4	0.1	**2.4^{b}**
	T3	0.0	0.1	0.1	0.0	0.1	0.3	0.4	0.0	0.0	**1.0^{b}**
Palpebral edema	Control	0.6	1.1	1.0	1.0	0.9	1.0	0.6	0.8	1.0	**8.0^{a}**
	T1	0.2	0.2	0.4	0.1	0.6	0.4	0.0	0.0	0.0	**1.9^{c}**
	T2	1.0	0.6	0.9	0.3	0.0	0.6	0.0	0.3	0.7	**4.3^{b}**
	T3	0.8	0.5	0.5	0.3	0.1	0.3	0.1	0.1	0.0	**2.6^{c}**
Epiphora	Control	0.2	0.4	0.9	1.0	0.5	0.5	0.4	0.8	0.4	**5.1^{a}**
	T1	0.3	0.3	0.2	0.2	0.2	0.3	0.1	0.2	0.6	**2.4^{b}**
	T2	0.1	0.4	0.6	0.3	0.0	0.3	0.1	0.1	0.3	**2.3^{b}**
	T3	0.0	0.3	0.5	0.0	0.0	0.3	0.1	0.0	0.0	**1.1^{b}**
Vitality	Control	0.9	0.6	0.6	0.8	0.7	0.6	0.4	0.6	0.5	**5.7^{a}**
	T1	0.5	0.2	0.0	0.1	0.0	0.1	0.0	0.0	0.0	**0.9^{b}**
	T2	0.6	0.4	0.1	0.1	0.3	0.4	0.4	0.0	0.1	**2.6^{b}**
	T3	0.6	0.5	0.4	0.1	0.1	0.1	0.1	0.0	0.0	**1.9^{b}**
Faecal score	Control	1.1	1.2	1.8	1.3	0.8	0.7	1.1	1.1	0.5	**9.5^{ab}**
	T1	1.0	1.3	1.0	0.8	0.5	0.4	0.5	0.4	0.3	**6.1^{b}**
	T2	1.6	2.0	1.9	1.0	1.3	1.6	1.9	1.1	0.7	**13.0^{a}**
	T3	1.8	1.9	1.9	1.3	1.3	1.4	1.4	1.0	0.6	**12.4^{a}**

Table 3 – Average score of clinical signs of OD in challenged piglets.
VTEC challenge was performed on day 0.
T1: piglets orally immunized with 6 mg of F18 and 6 mg of VT2e-B; T2: piglets orally immunized with 6 mg of VT2e-B; T3: piglets orally immunized with 15 mg of VT2e-B; Control: piglets receiving wild type tobacco seeds.
Respiratory score: 0=normal; 1=slightly quick; 2= quick.
Oedemal score in palpebra: 0=normal; 1=mild; 2=severe.
Epiphora score: 0=normal; 1=mild; 2=severe.
Vitality score: 0=good; 1=loose; 2=bad.
Faecal score: 0=normal faeces; 1=soft consistency 2=mild diarrhoea; 3=severe diarrhoea.
Total score is the sum of the average daily scores from day 1 to day 9 post-challenge.
One-way ANOVA was used for multiple comparisons. Means in a column without common letters are significantly different ($P<0.05$).

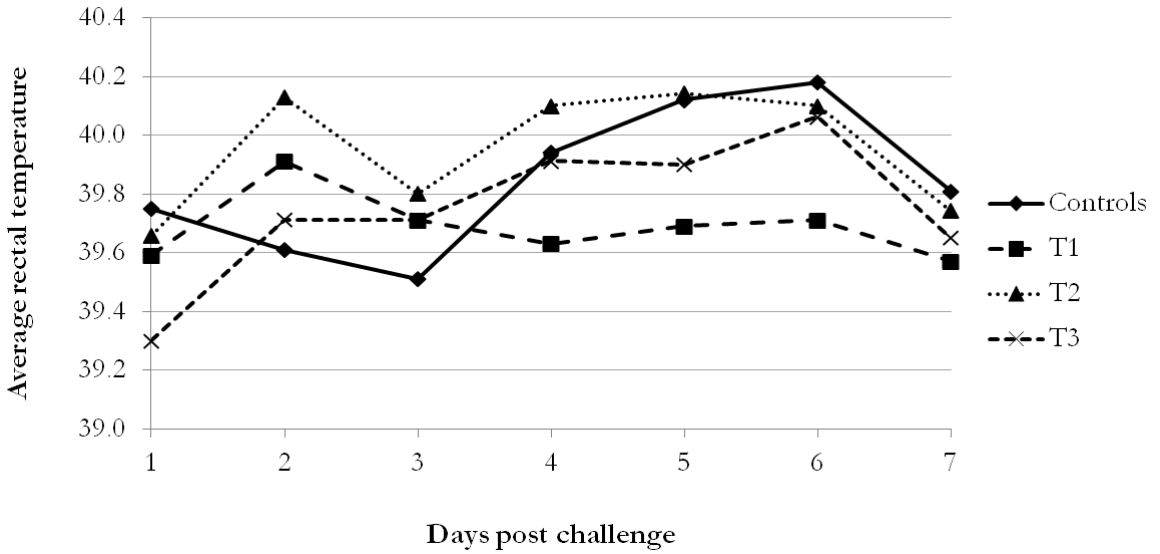

Figure 8 – Average rectal temperature in challenged groups. VTEC challenge was performed on day 0. T1: piglets orally immunized with 6 mg of F18 and 6 mg of VT2e-B; T2: piglets orally immunized with 6 mg of VT2e-B; T3: piglets orally immunized with 15 mg of VT2e-B; Control: piglets receiving wild type tobacco seeds.

5.4.8 Zootechnical performances

Piglets were early weaned at 20±2 days to ensure the sensitivity upon experimental infection and, at the beginning of the experiment (day -5), the animals had an average individual body weight of 5.5 ± 0.27, 5.5 ± 0.39, 5.7 ± 0.15, 5.5 ± 0.5 kg (lsmeans±SE) for control, T1, T2 and T3 group, respectively.

During the immunization period (0-21 dppi), after an initial phase of adaptation to the solid diet, in which feed intake and weight gains were low (from day -5 to day 0), the ADG and ADFI were in line with the standards of growth of the piglets in this phase, with no statistically significant differences among the experimental groups (ADG,g 0-21 dppi: control: 308±29; T1: 346±29; T2: 358±34; T3: 310±32; ADFI,g 0-21 dppi: control: 386±24; T1: 413±22; T2: 527±34; T3: 396±16). As shown by previous studies (Rossi *et al.* 2007), these data confirm that tobacco seeds intake and oral immunization had no effect on the zootechnical performances.

Since OD is responsible for significant economic losses due to high mortality and reduction of growth rates of infected piglets, the zootechnical parameters were measured during the post-challenge period to assess whether oral immunization could reduce the negative impact of *E. coli* infection on the production parameters.

As expected, the zootechnical parameters were clearly affected by experimental infection, especially in the phase immediately following challenge (21-25 dppi, Table 4). In fact, in this period infected piglets in all experimental groups showed

Item	Control	T1	T2	T3	P
BW day 21, kg	11.79±0.871	13.39±0.571	14.61±0.442	12.09±0.619	-
BW day 28, kg	13.62±1.20	16.08±0.543	16.54±0.858	13.93±0.903	-
ADG, g					
d 21 to 25	131±64	228±64	214±76	128±72	0.613
d 25 to 28	433±104	593±104	355±124	442±116	0.496
d 21 to 28	261±55	384±55	274±66	263±61	0.352
ADFI, g					
d 21 to 25	390±22^{a}	463±24^{b}	487±26^{b}	416±24^{ab}	0.031
d 26 to 28	535±36^{ab}	596±40^{b}	516±43^{ab}	467±40^{a}	0.180
d 21 to 28	444±24	513±27	497±29	435±27	0.131
G:F g/g					
d 21 to 25	0.33±0.12	0.54±0.13	0.49±0.15	0.26±0.13	0.438
d 25 to 28	0.81±0.20	0.86±0.23	0.30±0.26	0.95±0.23	0.305
d 21 to 28	0.58±0.08	0.70±0.09	0.49±0.10	0.59±0.09	0.466

Table 4 – Growth performance of experimental groups after *E. coli* infection.
VTEC challenge was performed on day 21.
T1: piglets orally immunized with 6 mg of F18 and 6 mg of VT2e-B; T2: piglets orally immunized with 6 mg of VT2e-B; T3: piglets orally immunized with 15 mg of VT2e-B; Control: piglets receiving wild type tobacco seeds.
Values with different superscript letters in the same row indicate significant difference (P<0.05).

a reduced ADG compared to pre-challenge period, which was more evident for control groups and T3 group compared to others.
In addition, during 21-28 dppi, non infected control and T3 groups showed higher ADFI (control: 568 g[1] T3: 701 g[1]) and non infected control, T2 and T3 groups showed higher ADGs (control: 432±160 g; T2: 357±14 g; T3: 593±100 g) compared to the corresponding infected groups. These data confirm the negative impact of experimental infection on zootechnical performances.
It is noteworthy that the performance of T1 not infected group (ADFI 21-28: 497 g[1]; ADG 21-28: 357±21 g; G:F,g 21-28: 0.72) were very similar to those of T1 infected group, suggesting that *E. coli* infection had no effect on ADG and FI of this group.
Comparing the performance of infected groups among them, shortly after challenge (21 -25 dppi) T1 and T2 groups showed higher ADGs than the other groups and significantly higher ADFI compared to control (P<0.05).
During the following period (25-28 dppi) an increased ADG and ADFI was observed in all infected groups, to a greater extent in group T1.
Overall, the T1 challenged group, that showed a better clinical presentation after challenge, showed also the best performance data (ADGs, FI, G:F) among the

[1] SE is not indicated since FI in this case was measured at one pen level

infected groups in the post-challenge period (21-28 dppi). The use of the multicomponent vaccine again appeared more effective in preventing the negative impact of challenge with O138 *E. coli* strain.
This vaccination strategy probably provided a specific immunocompetence that was activated upon the exposure to the wild-type pathogen. This did not prevent the exposure to the pathogen and, therefore, to the related stress, but allowed to overcome the infection faster and better.

5.4.9 Histological evaluations

Histological examination showed signs of mild enteritis in 3 out of 5 challenged control piglets (60%). None out of the six challenged and unchallenged piglets belonging to T1, T2 and T3 groups presented alterations in their intestinal structure.
Figure 9 shows the histological sections of ileal samples of a challenged control group piglet (Figure 9a) and of a challenged T1 piglet (Figure 9b). In Figure 9a, erosion of the apical villus surface and mild subepithelial hemorrhagic infiltration are evident. There are also connective oedema and lymphocytic infiltrate in the connective tissue of the axis of the villi. The Peyer's patches are strongly activated toward the lumen. In Figure 9b, the epithelium is normal; hemorrhagic or inflammatory infiltrates are not present; signs of oedema are mild, and the Peyer's patches show no signs of activation.

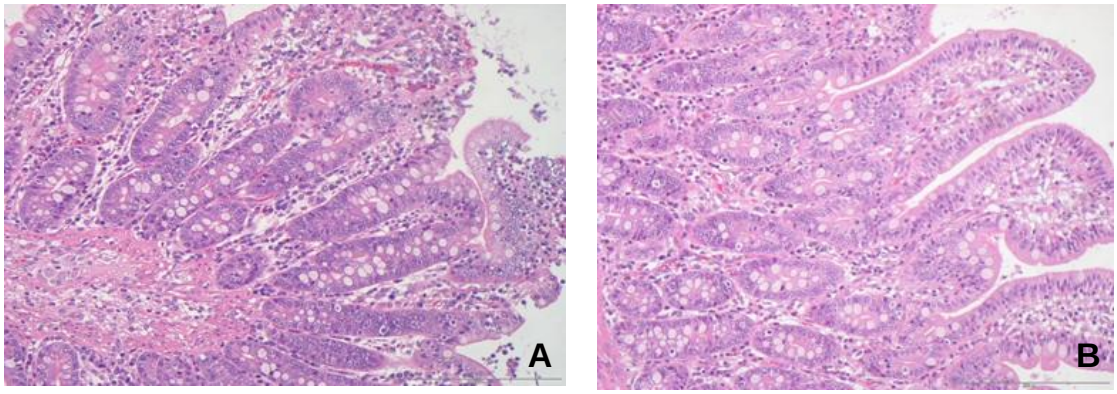

Figure 9 – Histological sections of ileal samples. (A) challenged control group piglet and (B) challenged T1 group piglet.

5.5 Conclusions

In the present study two proteins actively involved in different parts of the pathogenic process of OD were chosen as antigens for oral immunization of weaned piglets: the F18 fimbriae, the primary pathogenic factor, responsible for

the adhesion of *E. coli* strains to enterocyte receptors; and the B subunit of VT2e, responsible for receptor binding capacity of the toxin and consequent systemic damage.

Our results showed, one week following booster immunization (21 dppi), a higher faecal IgA titre in T1 group, receiving both antigens, compared to others, which is probably related to a higher local immune response activity in this group.

The experimental infection, while having caused no mortality or neurological symptoms, was effective in reproducing the typical symptoms of the disease and led to intestinal colonization by *E. coli* strains, as demonstrated by the faecal shedding of hemolytic *E. coli* in almost half of the subjects of each group, as detected one day after challenge (23 dppi).

All groups orally immunized with VT2eB antigen (T1, T2, T3) showed mild clinical signs of OD compared to the control group, and the group orally immunized with both VT2eB and F18 antigens (T1) proved also a better faecal score compared to others.

Clinical examination after challenge thus suggested a possible protective effect of immunization with VT2eB antigen against systemic symptoms induced by toxin VT2e, as well as a protective effect at gastrointestinal mucosal level from immunization with F18 antigen.

Overall, a better clinical presentation was observed after challenge in piglets of T1 group, immunized with transgenic tobacco seeds containing both antigens (VT2eB and F18). This immunization strategy appeared more effective in preventing the development of clinical signs after challenge with O138 *E. coli* strain.

The zootechnical parameters were clearly affected by the experimental infection, especially in the phase immediately following challenge (21-25 dppi), confirming the negative impact of the experimental VTEC infection on zootechnical performances.

The T1 challenged group, with a better clinical presentation after challenge, showed also the best performance data (ADGs, FI, G:F) among the infected groups during post-challenge period (21-28 dppi). Performance of this group was also very similar to that of the corresponding non infected group, suggesting that *E. coli* infection had no effect on ADG and FI of the group orally immunized with both VT2eB and F18 antigens.

The use of the multicomponent vaccine again appeared more effective in preventing the negative impact of challenge with O138 *E. coli* strain.

In conclusion, the results of this study suggest that oral administration of transgenic tobacco seeds expressing F18 and VT2eB antigens may induce a protective effect against O138 *E. coli* infection. In particular, better results were

obtained by the use of the multicomponent vaccine, based on two important virulence factors of VTEC strains.
For all measured parameters, no differences were observed between T2 and T3 groups, suggesting that no dose-response effect was shown for the Vt2e-B antigen in our experimental conditions.
Moreover, the oral immunization itself had no negative effects on production, as no significant differences in body weight and daily weight gain have been observed between control and immunized piglets.
Transformed tobacco plants for seed-specific expression of genes encoding F18 fimbriae and VT2e-B subunit of shiga-like toxin may represent a promising non-invasive method of vaccinating swine via their feed.

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CHAPTER 6

General Discussion

6. General discussion

The weaning transition is a particularly complex period, during which the piglets are confronted by multiple stressors. The immediate effect of weaning is a dramatic reduction in feed intake and a consequent 'growth check', which continues to represent a major source of production losses in commercial piggeries. Weaning also causes alterations in intestinal architecture and function, predisposing to diarrhoea and intestinal infections, particularly from *E. coli* strains. Post-weaning diarrhoea (PWD) and Oedema Disease (OD) are important causes of diarrhoea, growth retardation and mortality in piglets during the first weeks after weaning. PWD is caused by F4+ and/or F18+ enterotoxin-producing *E. coli* (ETEC), while OD is the result of an infection with verotoxin-producing F18+ *E. coli* (VTEC). Up to now, no vaccine protecting piglets against these infections is available and treatment relies upon the use of antimicrobials. Before 2006, AGP were widely used, added to feed for piglets from birth to weaning, with the aim of improving the composition of intestinal microflora in piglets and of reducing enteric infections. Due to the fact that the use of antibiotics as growth promoters has been banned in the EU, intensive research has focused on the development of alternative strategies for prevention of these infections in weaned piglets. The main theme of this research concerns the study of novel strategies for the control of these two important diseases, focusing in particular on the use of feed additives and immunogenic products derived from engineered plants.

Using an appropriate disease model represents an effective approach to evaluate substitute products for antibiotics in the prevention of diarrhoea *in vivo*. However, a criticism often directed at disease challenge models to induce diarrhoea after weaning is that incidence and severity of the diarrhoea observed are often less than what experienced in commercial herds. For this reason, our first study was addressed to set up experimental conditions to simulate the outbreak of diarrhoea through a controlled *E. coli* challenge.
In our study, factors predisposing the piglets to PWD were introduced into the experimental challenge model, including stress factors (i.e. related to weaning, transport and group formation), iperproteic diet and 30 mL 10% bicarbonate solution orally administered with purpose to neutralize gastric acid and to increase gastric survival rate of the challenger strain. The diet administered during the first three days, containing a high level of crude protein, was intended to be a "high-risk" diet, since several pathogens preferentially ferment proteins and high amounts of crude protein in the diet of newly weaned piglets have been identified as one of the predisposing factors of post-weaning colibacillosis. Moreover, the high level of CP was due to the presence in the feed of soybean

meal, an ingredient that seems to favour the occurrence of PWD. The tested experimental conditions with *E. coli* challenge affected faecal consistency ($P<0.001$) and faecal colour. This study showed that the a single dose of 10^8 CFU was able to reproduce diarrhoea in 96.67% of the challenged piglets. The challenger strain, hemolytic and verocytotoxic in most of faecal isolates showed high virulence, related to the onset of severe diarrhoea. The rate of mortality (10%) in the challenged group was comparable with the mortality normally occurring in the field. The measurement of zootechnical parameters evidenced a depressed FI, which persisted for about five days after the challenge and a subsequently reduced ADG, as encountered in the field. These findings support the view that, being post-weaning diarrhoea multifactorial, other factors have to be combined with the oral challenge to reproduce the disease under experimental conditions.
The experimental protocol described in this study could be used for further studies, in order to evaluate nutritional strategies to prevent or control *E. coli* diarrhoea in weaned piglets.

The two subsequent experimental works are part of a project in which transgenic tobacco plants have been engineered for the seed-specific expression of antigens against verotoxin-producing *E. coli* (VTEC) strains and evaluated as edible vaccine in weaned piglets.
Oral administration of whole transgenic tobacco seeds as edible vaccine for newly-weaned piglets raises doubts about an effective exposure of the antigen to the sites of activation of the mucosal immune system. The use of milled transgenic tobacco seeds would appear more appropriate for animals with immature digestive capacity, however there are no data available concerning the gastric degradability of antigenic proteins expressed in both whole and milled tobacco seeds in weaned piglets. For this reason, the second trial investigated the effect of gastric fluid derived from weaned piglets on VT2e-B antigenic protein expressed in whole and milled tobacco seeds, in the perspective of their use for immunization trials on piglets.
Our results confirmed that natural encapsulation of antigenic proteins within plant cells offers protection against digestion in the stomach.
The ability of specific polyclonal rabbit serum to recognize the antigen even after 3 hours of incubation with porcine gastric fluid indicate that the residual amount of transgenic proteins after digestion of both milled and whole seeds appears sufficient for their use in immunization trials on piglets.
This study represented a preliminary step for the subsequent one, which was designed to evaluate the use of milled transgenic tobacco seeds expressing antigenic proteins of VTEC strains as edible vaccine in piglets.

In the last study, two proteins actively involved in different parts of the pathogenic process of OD were expressed in tobacco seeds and used as antigens for oral immunization of weaned piglets: the F18 fimbriae, the primary pathogenic factor responsible for the adhesion of *E. coli* strains to enterocyte receptors, and the B subunit of VT2e, responsible for receptor binding capacity of the toxin and consequent systemic damage.

Our results showed, one week following booster immunization, a higher faecal IgA titre in T1 group, receiving both antigens, compared to others, which is probably related to a higher local immune response activity in this group.

All groups orally immunized with VT2eB antigen (T1, T2, T3) showed mild clinical signs of OD compared to control group and the group orally immunized with both VT2eB and F18 antigens (T1) showed also a better faecal score compared to others. Clinical examination after the challenge thus suggested a possible protective effect of immunization with VT2eB antigen against systemic symptoms induced by the toxin VT2e and a protective effect at the gastrointestinal mucosal level of immunization with F18 antigen.

Overall, a better clinical presentation was observed after challenge in piglets of T1 challenged group, immunized with transgenic tobacco seeds containing both antigens (VT2eB and F18).

The T1 group, with a better clinical presentation after challenge, showed also the best performance data (ADGs, FI, G:F) among the infected groups during post-challenge period (21-28 dppi). Performance of this group was also very similar to that of the corresponding non infected group, suggesting that *E. coli* infection had no effect on ADG and FI of the group orally immunized with both VT2eB and F18 antigens. The use of the multicomponent vaccine again appeared more effective in preventing the negative impact of challenge with O138 *E. coli* strain.

In conclusion, the results of this study suggest that oral administration of transgenic tobacco seeds expressing F18 and VT2eB antigens may induce a protective effect against O138 *E. coli* infection. In particular, better results were obtained by the use of the multicomponent vaccine, based on two important virulence factors of VTEC strains.

For all measured parameters, no differences were observed between T2 and T3 groups, suggesting that no dose-response effect was shown for the Vt2e-B antigen in our experimental conditions.

Moreover, the oral immunization itself had no negative effects on production, as no significant differences in body weight and daily weight gain have been observed between control and immunized piglets.

Transformed tobacco plants for seed specific expression of genes encoding F18 fimbriae and VT2e-B subunit of shiga-like toxin may represent a promising non-invasive method of vaccinating swine via their feed.

CHAPTER 7

Summary

7. Summary

There is currently a great interest in developing alternative feeding strategies for animal health maintenance and disease prevention, in order to reduce the negative impact of removing antibiotic growth promoters (AGP) within the EU (EC Regulation 1831/2003), while minimizing the therapeutic use of antibiotics. The main theme of this research concerns the management of some health problems that frequently occur during the weaning of piglets, focusing in particular on the use of feed additives and immunogenic products derived from engineered plants. Three different trials were designed to study novel strategies for the control of two important diseases, caused by infection with *Escherichia coli* strains, typically occurring during the weaning phase of the piglet, specifically *E. coli* diarrhoea and Oedema Disease (OD).

E. coli diarrhoea is a multifactorial disease responsible for heavy economic losses in pig production. The study of alternative strategies for the prevention of *E. coli* diarrhoea is limited by its variable incidence in the field and by the difficulty to reproduce the disease after experimental infection. For these reasons, the aim of the first study was to set up experimental conditions to simulate the outbreak of diarrhoea through a controlled *E. coli* challenge. Thirty-five healthy piglets, weaned at 33±2 days, from a selected farm, were divided into three experimental groups: control group (CG) including 5 piglets, infected group 1 (IG1) including 10 piglets and infected group 2 (IG2) including 20 piglets. One day after arrival, the piglets of IG1 and IG2 were orally inoculated with $3.7*10^8$ CFU of E. coli O149 strain, while CG received 5 mL of sterile physiological saline. The animals were fasted for 3 hours before and after challenge and 30 mL of 10% NaHCO3 solution were individually administered 15 minutes before challenge. The IG and CG groups were fed with a diet containing a high level of protein (CP: 28%) for 3 days after infection. The health status, faecal score (0:normal; 1:soft; 2:liquid; 3:watery) and faecal colour (3:brown; 2:green; 1:yellow) were individually recorded daily for 20 days after challenge for IG1 and CG and for 2 days after challenge for IG2. Polymerase chain reaction, serotyping and biochemical identification were established for the evaluation of *E. coli* strains from faecal samples. The effects of the challenge were analyzed by multivariate repeated measures. Diarrhoea was observed in 96.67% (58.6% severe; 41.4% mild) of all infected piglets and occurred on average 1.3 days after challenge. The CG group presented one piglet with a transient mild diarrhoea. The *E. coli* challenge significantly affected the consistency and the colour of faeces ($P<0.001$). A significant correlation ($r=-0.89$; $P<0.001$) between faecal colour and consistency was found. 70% (14/20) of infected piglets with severe diarrhoea shed *E. coli* O149 in their faeces. The

O149 challenger strain was detected in 17 out of 30 (56.7%) infected piglets two days after experimental infection, and 15 out of 17 isolated O149 *E. coli* strains (88%) were also haemolytic. Zootechnical parameters did not show significant differences. These findings support the view that, being post-weaning diarrhoea multifactorial, other factors have to be combined with the oral challenge to reproduce the disease under experimental conditions. We concluded that the experimental protocol described in this study can be used in the evaluation of nutritional strategies to prevent or control *E. coli* diarrhoea in weaned piglets.

The two subsequent experimental works are part of a wider project, still in progress in our Department, in which transgenic tobacco plants have been engineered for the seed-specific expression of antigens against verotoxin-producing *E. coli* (VTEC) strains responsible for OD. Novel strategies are required to control VTEC infections, considering that at present no vaccines are available and the treatment relies upon the use of antibiotics, which may contribute to the increase of antimicrobial resistance. In this context, plant-vaccines have considerable potential and represent a promising strategy for mucosal vaccination in terms of low costs, safety, storage, transportation and for the production of specific antibodies in the mucosa, where the major pathogens gain access to the body. In the present studies two different lines of tobacco seeds were previously transformed *via agroinfection* for the expression of two antigenic proteins of VTEC strains: the F18 fimbria, responsible for the adherence of the bacteria on small intestinal enterocytes, and the B subunits of verotoxin 2e (VT2e), responsible for binding the toxin to specific receptors on the cell surface.

One of the most important issues related to the oral delivery of plant-based vaccines is the potential for antigen degradation in the gastrointestinal tract. For this reason, a preliminary trial was designed to evaluate the effect of swine gastric fluid, derived from weaned piglets, on the VT2e-B antigenic protein expressed in whole and milled tobacco seeds. Samples of transgenic tobacco seeds, both milled and whole, were incubated with porcine gastric fluid, at 38° C in a Dubnoff Shaker for 1, 2 and 3 hours. After gastric fluid removal, by centrifugation and washing with PBS, the samples were homogenized in the presence of a protein extraction buffer. Western blot was performed on representative samples of extracted proteins, quantified by the Bradford method, using rabbit polyclonal serum. The Vt2e-B specific signals were observed on SDS-page in all samples derived from transgenic tobacco seeds. Nevertheless, from 0 h to 3 h, a progressive reduction of intensity of the Vt2e-B specific signal was observed. No significant differences were detected on the reduction of signal intensity between samples derived from whole and milled tobacco seeds. Based on the results obtained, we could conclude that the residual amount of

transgenic proteins after digestion of both milled and whole seeds appears sufficient for their use in immunization trials on piglets.

Within the same project, the last trial was designed to evaluate the use of transgenic tobacco seeds expressing antigenic proteins of VTEC strains as edible vaccine in piglets. A total of 43 weaned piglets (20±2 days) were randomized into 4 groups. Three immunized groups (T1, T2, T3) orally received a bolus of tobacco seeds (TS) mixed with chocolate on days 0, 1, 2, 14 of the trial. In particular, the T1 group received 10 grams of TS-F18+ and 10 grams of TS-VT2e-B+, the T2 group received 10 grams of TS-VT2e-B+ and the T3 group received 25 grams of TS-VT2e-B+. Control Group (CG) received 20 grams of wild type TS. The amount of transgenic protein was estimated about 0.6 mg/gram of whole TS. In this immunization phase faecal and blood samples were collected weekly to evaluate IgA and IgG amounts by ELISA assays. On day 22, the piglets were orally challenged with $1*10^{10}$ CFU of O138 *Escherichia coli* strain, using the experimental protocol described in the first trial. Faecal score, body temperature and clinical signs related to OD (palpebral oedema, epiphora, neurological and respiratory symptoms, vitality) were determined, daily for 15 days after challenge, for each piglet through specific point scales. Zootechnical performances and haematocrit percentages (HT) were evaluated during the experimental period. T1, receiving both the antigens, showed a higher level of IgA in faeces than other groups on day 21 (22,000±13,000 ng/ml *vs* control group: 7,200±3,000 ng/ml). No differences were observed among groups in relation to the total IgG titre in faeces and total IgA and IgG levels in serum. For each clinical sign, the average total score (the sum of the average daily score from day 1 to day 9 post-challenge) was significantly higher in the control group compared to orally immunized groups ($P<0.05$) and the latter showed a faster recovery than CG. In the same period (day 1 to 9 post-challenge), T1 showed a significantly higher consistency of faeces compared to T1 and T2 ($P<0.05$). No differences were observed in body temperature and HT. After challenge (day 21 to 25), the average daily feed intake ($P<0.05$) and the average daily gain were higher in T1 and T2 than CG. For all measured parameters, no differences were observed between T2 and T3, suggesting that no dose-response effect was shown for the Vt2e-B antigen in our experimental conditions. In conclusion, oral administration of recombinant tobacco seeds expressing antigenic proteins against VTEC strains can induce an increase of mucosal antibodies and a protective effect against the challenger strain in piglets. This represents a promising non-invasive method of vaccinating swine via their feed.

CHAPTER 8

Appendix

Review papers

8. Appendix. Review papers

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Vitamin E Bioavailability: Past and Present Insights

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ABSTRACT

Over the past decades several studies have investigated the importance of an adequate vitamin E status to sustain both animal health and production in dairy cows. Cow milk is considered as a remarkable source of bioactive components promoting human health, which has renewed interest in the effects of vitamin E supplementation on its nutritional value, sensory quality and shelf life. Thus, defining relative bioavailability, utilisation and transfer into milk of different vitamin E formulations is particularly important to assess the adequate levels of supplementation for animal health and milk quality. In nature vitamin E is present under one isomeric form, RRR α-tocopherol; when α-tocopherol is synthesized chemically, a racemic mixture of 8 *possible isomers of α-tocopherol in equimolar concentrations is produced* (*all-rac α-tocopherol*). *The different stereoisomers have different biopotencies in humans and livestock*; *the conversion factor between RRR and all-rac vitamin E was estimated by early studies on the basis of the rat foetal resorption bioassay, and then extended to other species. Recent advances on the distribution of vitamin E stereoisomers in plasma and tissues have highlighted the need to formulate new conversion factors in dairy cows as well as in humans. On account of this, the present article aims to consider past and recent data related to vitamin E in dairy cow nutrition.*

Keywords*: *Vitamin E, Dairy Cows, Bioavailability, Milk

1. Introduction

Vitamin E is one of the most important components of cellular antioxidant systems. In nature it is present under eight different forms, four tocopherols (α-, β-, γ-, δ) and four tocotrienols (α-, β-, γ-, δ), of which α-tocopherol is the most bioactive. As an antioxidant, α-tocopherol is able to prevent free-radical mediated tissue damage, and thus to prevent or delay the development of degenerative and inflammatory diseases; in such a role it has been extensively investigated in many species, humans included. About dairy cows, several reviews have discussed extensively on this topic over the past decades [1-4]. According to different surveys vitamin E supplementation helps to enhance animal health and production and, when dietary selenium is adequate, it significantly reduces the incidence of intramammary infections (IMI) and clinical mastitis [5]. Vitamin E also seems to be crucially involved in immune system function so that supplementation with supra-nutritional levels of the vitamin, at least in some instances, results in improved immune responses [4]. These benefits, particularly in a context of much-reduced use of fresh forage (vitamin E-rich) in dairy cow nutrition, have led to a substantial increase in recommended intake levels for this animal [6]. Vitamin E supplementation is a common practice in animal nutrition, and

increasing the feed content of synthetic or natural vitamins is the best way to maintain adequate plasma levels for animal health.

Objective of this paper is to consider bioavailability, bioactivity and transfer into milk of different vitamin forms. First, we discuss the new insights on the utilisation of vitamin E isomeric forms and formulations, and then we consider the dietary role of vitamin E to improve animal health and milk quality.

2. Vitamin E Bioavailability and Utilisation

2.1 Natural versus Synthetic Vitamin E

When α-tocopherol is synthesised chemically, a racemic mixture of all 8 possible isomers of α-tocopherol in equimolar concentrations is produced (synthetic form, also referred as *all-rac* α-tocopherol and historically labeled dl-α-tocopherol), with four stereoisomers showing a 2R configuration (RRR, RRS, RSS, RSR, having R configuration at position 2´ of the phytyl tail) and four stereoisomers possessing a 2S configuration (SRR, SSR, SRS, and SSS, having S configuration at position 2´ of the phytyl tail, **Figure 1**). Among possible different forms of α-tocopherol, RRR α-tocopherol is the only isomeric form of vitamin E produced by plants (natural form, historically labeled d-α-tocopherol) and is therefore the only one naturally present in feedstuffs.

Since the free forms are easily oxidized, more stable forms - such as acetate and succinate esters of α-tocopherol (RRR α-tocopheryl acetate, RRR α-tocopheryl succinate, *all-rac* α-tocopheryl acetate, *all-rac* α-tocopheryl succinate) - have been synthesized to be used as feed additives. The ester forms are very stable to *in vitro* oxidation; however, they need to be hydrolysed in the animal gut to free tocopherol which exerts its activity *in vivo* [1]. The acetate ester of *all-rac* α-tocopherol (*all-rac* α-tocopheryl acetate) is the most common form of vitamin E supplementations, due to its cost and stability in animal feeds.

The different stereoisomers of vitamin E have different biopotencies, with the RRR form having a greater activity than the *all-rac* form. The terms *biopotency* or *bioactivity* usually refer to the amount of a nutrient associated with some measured physiological endpoints, e.g. prevention of specific deficiency symptoms [7]. The relative activity of RRR and *all-rac* vitamin E was estimated by the United States Pharmacopeia (USP) [8] based on the rat foetal resorption bioassay and the USP conversion factor of 1.36 equating RRR to *all-rac* α-tocopheryl acetate was extended to other species. Recently, the FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed) confirmed the conversion factors stated by USP in 1979 for livestock, reporting that

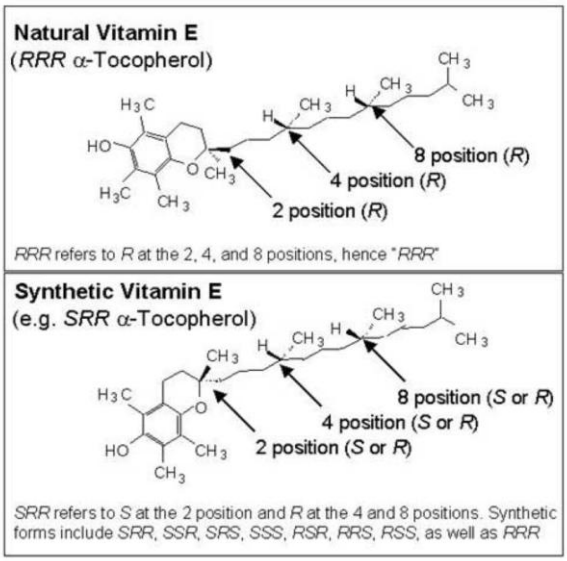

Figure 1. Natural and synthetic vitamin E.

Table 1. Factors for converting International Units of vitamin E to α-tocopherol to meet recommended intake in humans.

Chemical form	USP Conversion Factors[a] [8, 15]		IOM Conversion Factors[b] [14]
	IU/mg	*mg/IU*	*mg/IU*
all-rac α-tocopheryl acetate	1.00	1.00	0.45
all-rac α-tocopherol	1.10	0.91	0.45
RRR α-tocopheryl acetate	1.36	0.74	0.67
RRR α-tocopherol	1.49	0.67	0.67

a.The USP conversion factors are based on rat foetal resorption assays conducted in the 1940s.
b.The amounts of acetate compounds are adjusted for their different molecular weights relative to α-tocopherol, by dividing the mg of the vitamin E compound by its molecular weight (α-tocopheryl acetate = 472; α-tocopherol = 430) and multiplying by the molecular weight of α-tocopherol (430). The activities of the synthetic α-tocopherol compounds have been divided by 2 because the 2S stereoisomers contained in synthetic α-tocopherol are not maintained in the blood.
Source: [14]

all-rac α-tocopheryl acetate, RRR α-tocopherol and RRR α-tocopheryl acetate are efficacious in all animal species and have different biopotencies: one International Unit (IU) of vitamin E is defined as 1 mg *all-rac* α-tocopheryl acetate, as 0.74 mg of RRR α-tocopheryl acetate and as 0.67 mg of RRR α-tocopherol [9].

It is noteworthy that in humans the Institute of Medicine (IOM) redefined the USP conversion factors on the basis of studies indicating that the 2S stereoisomers of *all-rac* α-tocopherol were not maintained in human plasma [10-12] or in tissues [13] and published new conversion factors (**Table 1**), limiting the active form of vitamin E to the 2R stereoisomeric forms of α-tocopherol, which represent 50% of *all-rac* α-tocopheryl acetate and 100% of RRR α-tocopheryl acetate formulations [14].

Some recent comprehensive reviews of literature data suggest that bioavailability of RRR α-tocopheryl acetate should be reconsidered for livestock animals as well, and that new conversion factors, in line with human studies, are needed [15-16]. A suggestion deriving from a deeper understanding of bioavailability concerning stereoisomeric forms of vitamin E, achieved by studying their distribution in plasma and tissues of various animal species by analytical methods based on chiral HPLC procedures [15].

Distribution of α-tocopherol isomers in plasma and milk has been recently studied when feeding *all-rac* or RRR forms to periparturient dairy cows. Meglia *et al.* [17] showed that RRR α-tocopherol is the predominant form (more than 86% of the total) found in plasma of cows fed 1000 IU/day of either supplemental *all-rac* α-tocopheryl acetate, RRR α-tocopheryl acetate, or RRR α-tocopherol. Weiss *et al.* [18], after supplementation of 2500 IU/day of vitamin E from *all-rac* α-tocopheryl acetate or RRR α-tocopheryl acetate, found concentrations of α-tocopherol in cow plasma ranging from 1.2 to 1.4 times greater for RRR treatment than for *all-rac* treatment.

In both studies, very low concentrations of 2S isomers were detected in plasma of cows fed *all-rac* α-tocopheryl acetate. As the 2S isomers represent 50% of the isomers present in the *all-rac* α-tocopherol, such data suggest that 1 g of *all-rac* α-tocopheryl acetate is equivalent to 0.5 g of RRR α-tocopheryl acetate in dairy cows.

A study evaluating plasma concentration and distribution of α-tocopherol stereoisomers after intramuscular injection of 2.5 g *all-rac* α-tocopheryl acetate [19] evidenced difference in turnover rate of different stereoisomers in cow plasma: the 2S stereoisomers had showed the faster disappearance from blood compared to the other forms; however, considering the plasma concentration of stereoisomers one day after

Table 2. Effect of supplemental vitamin E (2500 IU) on concentration of total α-tocopherol in blood neutrophils and neutrophil function.

	Control	RRR	All-rac	SE
α-tocopherol in neutrophils (ng/10^6 cells)[1]	2.71[a]	5.84[b]	4.62[b]	0.61
Phagocytosis[2]	5.08[a]	4.92[a]	5.68[b]	0.18
Kill, %[3]	86	84	84	1.5
Kill index[4]	3.94[a]	3.72[a]	4.34[b]	0.14

[a, b]Means in same row with different superscripts differ ($P<0.05$)
[1]neutrophils were collected from cows at 3 DIM; [2]number of bacteria (dead or alive) within a neutrophil; [3]percentage of phagocytized bacteria that are dead; [4] (positive/100) x (kill/100) x bacteria/neutrophil. Source: [18]

injection, the 2S forms showed also a faster increase related to their initial level, when compared to the other forms. Based on plasma concentrations at 10 days after injection, Dersjant-Li and Peisker [20] calculated a higher relative bioavailability of RRR α-tocopheryl acetate over *all-rac* α-tocopheryl acetate. However, considering that plasma distribution of stereoisomers is different over time, sampling time (e.g. day 1 or day 5) chosen to determine the relative bioavailability is crucial. As a general rule, the ratio of bioavailability, being not constant but variable [21], should be estimated considering the plasma profile over time (and relative area under the concentration curve, AUC) rather than on a single timepoint.

Methodologies used in the evaluation of α-tocopherol forms bioavailability frequently diverge according to different studies, making it difficult to compare sometimes dissimilar results [20]. To estimate true relative bioavailability of RRR over *all-rac* α-tocopheryl acetate, it is also important to consider the contribution given by α-tocopherol originating from basal ingredients, consisting only of RRR form and responsible for the basal plasma level.

Bioactivity underpinning different bioavailability of vitamin E isomers is still under debate. In fact, the RRR form, while being more bioavailable, does not appear to affect neutrophil phagocytosis and killing. In the previously-mentioned study, Weiss *et al.* [18] found that, although cows fed on supplemental RRR α-tocopherol showed higher concentration of α-tocopherol in neutrophils, phagocytosis was greater in neutrophils from cows supplied with supplemental *all-rac* vitamin E (**Table 2**).

Not only its chemical form, but also different formulations and delivery systems can greatly affect vitamin E bioavailability [22-24]. A formulation can have a major influence on intestinal availability of vitamin E, which in dairy cows may be limited by ruminal degradation. Encapsulation technologies made it possible to formulate products that deliver specific nutrients to small intestine absorption sites by protecting them from degradation within the rumen. Baldi *et al.* [23] compared the pharmacokinetic parameters of vitamin E after intraruminal administration of oil-based, silica-adsorbed and microencapsulated preparations and found that *all-rac* α-tocopheryl acetate adsorbed on silica or microencapsulated resulted in different pharmacokinetic profiles and in a greater relative bioavailability than the oil-based preparation. Bontempo *et al.* [24], after oral administration of α-tocopherol in oil, encapsulated in liposomes or cyclodextrin, found that encapsulated preparations had a longer persistence in blood and a slightly greater availability than the oil-based preparation.

In general, it has been observed (**Table 3**) that plasma total response (AUC) increases linearly with increasing dose, while the maximal plasma α-tocopherol reached does not increase to the same extent.

2.2 Vitamin E Interactions with Other Nutrients

Vitamin E interacts with other nutrients that can influence its bioavailability. Several papers have examined this topic deeply, as reported below. Vitamin E absorption is closely tied to the digestion and absorption of fats, thus the type and amount of fat in the gut has an important influence on the absorption efficiency of the vitamin. Mid-lactation cows receiving 25, 125 or 250 IU of vitamin E and

Table 3. Pharmacokinetic parameters of α-tocopherol after intraruminal administration of dl-α-tocopherol or dl-α-tocopheryl acetate in oil-based (O) and microencapsulated (M) preparations.

	dl-α-tocopherol		dl-α-tocopheryl acetate	
	O	*M*	*O*	*M*
Maximum concentration (μg/ml)	4.86±0.49	5.03±0.39	4.08±0.21	3.29±0.14
Final concentration (μg/ml)	1.97±0.47	2.61±0.33	2.58±0.30	2.66±0.22
Area under curve (μg/ml/h)	496±54.0	547±26.7	465.4±38.7	620.25±108.5

Data are means±SE. Source: [4]

diets containing no supplemental fat or 2.25% added fat from roasted soybeans or tallow, showed a rate of increase of plasma α-tocopherol 1.9 greater when fat was supplemented [25]. Nevertheless, Baldi *et al*. [26] showed that when 0.2 kg/day of calcium-soaps (relative to isoenergetic starch) were fed to early lactation dairy cows in addition to vitamin E, this had no effect on the vitamin E status. These different results can be explained in two ways: first, the amount of fat in the basal diet may affect vitamin E absorption efficiency and, hence, plasma levels; second, absorption might also be affected by differences in the rate of digesta flow through the gut at different stages of lactation.

Dairy cows receiving 20 g/day of rumen-protected choline showed higher plasma concentrations of α-tocopherol than control cows during the first 30 days of lactation (2.46 *vs.* 1.85 μg/ml), suggesting that choline supply can reduce the physiological drop of vitamin E in the transition period [27]. The mechanism by which choline maintains vitamin E levels in this period has not been completely understood yet. Anyway, as α-tocopherol is preferentially incorporated in to nascent VLDLs, a reasonable hypothesis is that choline, a vital component of lipoproteins, influences vitamin E status by promoting VLDL formation [28].

Bioactivity of vitamin E is closely related to the supplementation of adequate dietary levels of other nutrients involved in the cell antioxidant system. In particular, as an integral component of the enzyme glutathione peroxidase (GSH), selenium is important to maintain proper levels of the components of the tissue defence mechanisms against free-radical damage and its metabolic function is closely linked to vitamin E. When vitamin E exerts its antioxidant activity it is converted into tocopheroxyl radical that needs to be converted back to tocopherol in order to prevent α-tocopherol free-radical related damage. Regeneration of tocopherol involves multiple reactions and interacting molecules [29], including vitamin C and the selenium-containing enzyme GSH.

Several studies have shown that contemporary administration of vitamin E and selenium may result into a synergistic enhancement of both immune response and disease resistance in domestic animals, particularly in ruminants [30, 31]. In dairy cows, selenium nutritional requirement is 0.3 mg/kg dry matter [6] and selenium supplements are available in form of inorganic mineral salts, such as sodium selenite or selenate and organic forms, such as selenium-enriched yeasts. Effects of selenium supplementation on immune function and bovine mammary gland health have been recently reviewed by Salman *et al*. [32] and scientific evidence related to the effect of oral supplementation of organic and inorganic forms on milk selenium concentration in cattle have been summarised by a systematic review and meta-analysis [33].

3. Vitamin E Function and Activity

3.1 Vitamin E and Mammary Gland Health

Based on health and immune function in cows, vitamin E intake has been generally considered adequate when α-tocopherol plasma levels are above 3–3.5 μg/ml. In order to maintain these blood values, non-grazing dairy cows are suggested to be fed on 1000 international units (IU) of supplemental vitamin E daily during the dry period and 500 IU during lactation [6].

It is established that plasma vitamin E levels fall significantly around parturition in dairy cows so that it is difficult to maintain levels considered adequate for their health in this period.

During the periparturient period, dairy cows experience alterations in immune responsiveness and a consequent greater susceptibility to various metabolic and infectious diseases including mastitis, metritis and retained foetal membranes. Several components of the host defence system are impaired during this period, in particular a decreased activity of neutrophils has been associated with increased susceptibility to mastitis [34, 35].

Several studies reported positive effects of supplementation with supranutritional levels of vitamin E on the immune system, specifically a higher killing ability, superoxide production and u-PA activities of neutrophils and improved macrophage function [36-42].

Benefits of vitamin E and selenium supplementation in this critical period on mammary gland health have been registered since 1984 [43] and most of the studies published over the past decades clearly indicate that adequate dietary vitamin E and selenium intakes, above normal nutritive requirements and supplemented during the dry period, reduce milk SCC and influence the prevalence and severity of IMI in dairy herds [26,

42, 44, 45]. A recent meta-analysis [5] of 34 papers published between 1984 and 2003 that addressed the relationship between vitamin E and udder health, confirmed that different levels of supplementation, up to a maximum of 4000 IU during the dry and early lactation periods, were associated with lower IMI, somatic cell counts (SCCs) and clinical mastitis. On average, vitamin E supplementation was associated with a 14% reduction in the risk of IMI, with a reduction of milk SCC by a factor of 0.70 and a 30% decrease in the risk of occurrence of clinical mastitis.

Other studies showed no positive effects of vitamin E supplementation on udder health. LeBlanc *et al.* [46] found no association between serum α-tocopherol concentration after injection of 3000 IU of vitamin E one week before expected calving and the risk of mastitis at or soon after calving. Similarly, a study [47] performed in European commercial dairy herds with a high incidence of veterinary-treated clinical mastitis did not find any significant effect of extra daily supplementation of 1610 mg of RRR α-tocopheryl acetate around calving on udder health or on other cow health problems.

A recent experiment conducted on five farms with historically high rates of mastitis in the Netherlands reported for the first time higher incidences of clinical and subclinical mastitis in cows receiving a daily supplement of 3000 IU of vitamin E during the dry period, compared to cows receiving a daily supplement of 135 IU [48]. The mean vitamin E level at dry off for the cows involved in this study was 2 to 3 times higher than starting levels for cows in other studies [46, 49] and in the opinion of the authors this is the most reasonable explanation for the results observed. In this study, an initial blood vitamin E level at dry off above 6.42 μg/ml was considered a risk factor for developing clinical mastitis. However in another study with average blood vitamin E levels equal to or higher than 6 μg/ml during the periparturient period, a positive rather than negative effect of supplementation on the immune function and SCC/ml levels in milk was found [41].

The plasma concentration of α-tocopherol at dry off is obviously a critical factor in determining the positive effect of vitamin E supplementation on the incidence of mastitis. Additionally, different levels of oxidative stress may explain the variation in results from different studies. As previously discussed, the importance of establishing the levels of other nutrients interacting with vitamin E has to be considered to determine whether supplementation will be beneficial or not [29, 50]. Finally, differences in the conclusions from different studies may be explained by differences in farm management, differences in the way vitamin E is supplemented (oral v. i.m. injection), and even in the type of microorganism causing the disease.

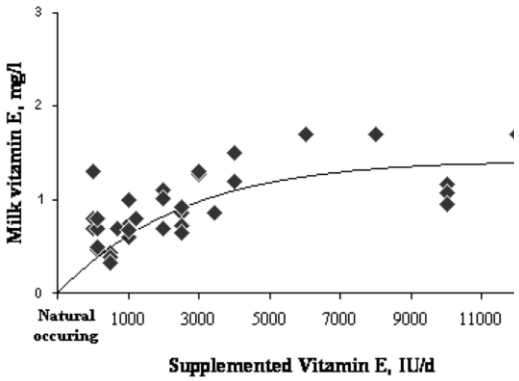

Figure 2. Milk content of vitamin E in relation to levels of vitamin E supplementation in dairy cows. Data from [17, 18, 25, 26, 57, 58, 59].

3.2 Vitamin E and Milk Quality

It is well known that vitamin E supplementation has a positive effect not only on animal health, but also on milk quality. Results from most studies [26, 39, 42] strongly suggest that high levels of vitamin E supplementation (2000-3000 UI/cow per day) during the periparturient period are effective in reducing milk SCC by about 29% in treated cows.

Furthermore, as an antioxidant vitamin E helps to slow lipid peroxidation and to maintain oxidative stability and flavour of milk. Several studies reported a positive effect of vitamin E on oxidative stability of milk [51-53]. Bovine milk is susceptible to auto-oxidation when the level of vitamin E falls below 20μg/g of fat [54] and this leads to the development of an "oxidised flavour" (OF), described as cardboard-like, metallic or tallow-like. The fatty acid profile of milk fat is a major factor in the development of OF, and milk with a high concentration of polyunsaturated fatty acids, as linoleic or linolenic acid, is more likely to develop OF [55]. Recently, there has been a considerable interest in developing specific animal feeding regimes in order to increase the polyunsaturated

lipid content of milk, and in particular the content of fatty acids thought to benefit human health, such as conjugated linoleic acids. Milk with a high content of these lipids may benefit human health, but at the same time is more vulnerable to oxidation. In this context, increasing the vitamin E content of milk may represent a useful way to protect lipids from peroxidation and to maintain nutritional and organoleptic qualities of milk. It has also been shown that the uptake of plasma vitamin E by the mammary gland of dairy cows increases when diets enriched in polyunsaturated fatty acids are fed [56].

Available data on the amount of supplemental vitamin E required to ensure oxidative stability of milk with a high unsaturated fat content are not conclusive. Weiss [55] suggested supplementing at least 3000 IU of vitamin E per day, when OF is a problem. By contrast, Slots *et al.* [57] found that supplementation of the feed with 2600 and 3400 IU of *all-rac* α-tocopheryl acetate for 16 days did not improve oxidative stability of milk.

Although several studies [25, 26, 55] indicated that transfer of vitamin E from feed to milk is low, around 1.6-2.2%, supra-nutritional supplementation to transition cows can increase the vitamin E content of milk (**Figure 2**). Vitamin E supplementation at 2000 UI/day from 14 days before expected calving to 7 days after was found to produce an increase of vitamin E content of milk of about 40%, compared to supplementation at 1000 UI/day [26].

The rather low transfer efficiency of α-tocopherol in milk could also be dependent on the form of vitamin E supplemented. In the previously-mentioned studies [17, 18], the distribution of α-tocopherol stereoisomers in milk in relation to the supplementation of natural and synthetic vitamin E to periparturient dairy cows has also been investigated. Data from these studies showed that concentrations of α-tocopherol in milk was 1.24 to 1.43 times greater for cows fed the RRR supplement compared to cows fed the *all-rac* supplement. Moreover, irrespective of dietary treatment, α-tocopherol with natural stereochemistry (RRR) was by far the most predominating form in milk of cows (at least 86% of the total, [17]). Similarly, Slots *et al.* [57] reported that after supplementation with 2600 IU and 3400 IU of *all-rac* α-tocopheryl acetate, only the four 2R stereoisomers were excreted into the milk, with the RRR isomer dominating over others (84.3% and 88% of the isomers, respectively).

A preferential uptake or transfer of the RRR form from plasma into milk is also suggested by recent studies investigating the effect of farming system type on vitamin E content of milk. Even in the absence of feed supplementation with synthetic vitamins, milk and milk products from organic farms were found to contain more or as much vitamin E than conventional milk [60-62] and concentrations of the 2R stereoisomers in milk were not significantly different between high- and low-input conventional systems and organic low-input system [63].

High levels of vitamin E are effective in maintaining milk quality and safety in general, and a further advantage in increasing vitamin content of milk by animal nutrition rather than fortification is that it contributes to animal health safeguard, a primary factor in determining safety, quality and health benefits of food of animal origin [64].

4. Conclusion and Implications

Defining the relative bioequivalence between RRR and *all-rac* forms is fundamental to determine proper recommended intakes of different supplemental forms of vitamin E on the basis of α-tocopherol conversion factors, in dairy cows as in humans.

In literature, recent data clearly show that bioavailability of RRR α-tocopheryl acetate relative to *all-rac* α-tocopheryl acetate in dairy cows is 2:1. Nonetheless, the use of bioavailability studies in order to determine the ratio of potency of the different vitamin E stereoisomers is currently under debate. In the absence of clinical endpoints, no studies have been performed in cattle and pigs, as well as in humans, to assay the potency of RRR relative to *all-rac* α-tocopherol so far. On one hand, being bioavailability a precondition for any biological activity, some authors based the determination of utilisation efficiency and efficacy of vitamin E stereoisomers on bioavailability studies. Other authors disagree with such inference arguing that, as RRR and *all-rac* are not chemically identical, any ratio of bioavailability calculated in this way cannot be a valid substitute for the ratio of potency. Indeed, as discussed above, a greater bioavailability of the RRR form does not necessarily correspond to a higher bioactivity, as evidenced by no effect of this form shown on

neutrophil function. Furthermore, an essential requisite frequently not considered in vitamin E feeding trials for the estimation of the true relative bioavailability of RRR over *all-rac* α-tocopheryl acetate, is the correction of α-tocopherol intake originating from basal ingredients.

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Proprietà nutraceutiche del latte e salute del suinetto

Abstract

Gli effetti benefici del latte vanno oltre il suo apporto bilanciato di principi nutritivi ideale per i fabbisogni digestivi e metabolici del neonato. Le conoscenze scientifiche hanno evidenziato, nel corso degli anni, un ruolo "funzionale" del latte, grazie alla presenza di diverse componenti bioattive. Tali sostanze possono anche essere definite "nutraceutiche" per i loro effetti protettivi e benefici sullo stato di salute, sia nel neonato che nelle successive fasi di accrescimento.

Introduzione

In passato, l'osservazione di una maggiore morbosità e mortalità dovuta a patologie gastroenteriche e respiratorie nei neonati alimentati artificialmente rispetto a quelli alimentati con latte materno ha suggerito un rilevante ruolo protettivo del latte contro le patologie infettive neonatali, che è stato in primo luogo attribuito alla presenza di anticorpi secretori. Tuttavia, nel corso degli anni, diversi macro/micronutrienti presenti nel latte, e i prodotti della loro parziale digestione, hanno dimostrato di svolgere funzioni protettive essenziali per lo sviluppo del neonato.

Fra le componenti native del latte, specifiche proteine e peptidi, nucleotidi, acidi grassi essenziali, vitamine, enzimi e oligosaccaridi svolgono funzioni "extranutrizionali", quali attività di modulazione della microflora intestinale, di regolazione del trofismo intestinale, di protezione da agenti patogeni intestinali o di immunomodulazione (Severin and Wenshui 2005, Bauman *et al.* 2006). La molteplicità di queste sostanze, unitamente alla loro azione sinergica sull'ecosistema intestinale e sulla maturazione del sistema immunitario, conferisce al latte un ruolo fisiologico "funzionale", fondamentale per il mantenimento della salute del neonato. La caratterizzazione e la comprensione delle funzioni biologiche di tali sostanze rappresenta una base interessante per lo sviluppo di nuovi agenti profilattici e terapeutici utili durante le fasi iniziali dello svezzamento, al fine di sostenere la crescita e le performance degli animali e ridurre l'incidenza di problematiche sanitarie, garantendo così una maggiore continuità nel passaggio critico dall'alimentazione lattea a quella solida.

Peptidi immunomodulatori

Il suinetto alla nascita per la protezione immunitaria, lo sviluppo e la sopravvivenza dipende completamente dall'assunzione di fattori immunitari specifici e aspecifici tramite il colostro e il latte materno. Il sistema immunitario mucosale è quasi completamente assente, le Placche di Peyer sono costituite da

follicoli primordiali circondati da pochi linfociti T e quasi nessuna cellula immunitaria è presente a livello di villi e cripte intestinali. Lo sviluppo dell'immunità mucosale avviene in diverse settimane, attraverso una serie di stadi definiti, durante i quali il sistema immunitario è esposto ad un'ampia gamma di antigeni associati agli alimenti, a batteri commensali e a microrganismi patogeni (Bailey *et al.* 2001, Lalles *et al.* 2007).
I primi mesi di vita neonatale rappresentano un periodo critico per lo sviluppo del sistema immunitario, che deve acquisire la capacità di sviluppare tolleranza agli antigeni alimentari e di generare un'adeguata risposta agli antigeni patogeno-derivati (immunocompetenza).
Allo svezzamento, il sistema immunitario del suinetto è da considerarsi ancora immaturo e lo stress sociale, ambientale, nutrizionale associato allo svezzamento, soprattutto se praticato ad un'età precoce, può compromettere le difese immunitarie e predisporre all'insorgenza di patologie di varia natura.
Numerose ricerche hanno dimostrato che il latte, che rappresenta la principale fonte proteica per il neonato, oltre alle proteine maggiori contiene un ampio gruppo di proteine e di peptidi presenti in forma latente, che sembrano essere coinvolti nello sviluppo e nella maturazione funzionale del sistema immunitario mucosale (Politis and Chronopoulou, 2008).
Le proteine del latte sono soggette a proteolisi ad opera di proteasi native come il sistema plasmina/plasminogeno, nello stomaco durante la digestione, nell'intestino ad opera di enzimi di derivazione batterica o durante i processi di produzione di prodotti fermentati o di formaggi (Baldi *et al.* 1996, Politis 1996, Fantuz *et al.* 2001). La demolizione enzimatica delle proteine del latte genera una serie di peptidi dotati di diverse proprietà biologiche antimicrobiche, ipertensive, antitrombotiche e di immunomodulazione. Peptidi bioattivi possono derivare dall'idrolisi di tutte le maggiori proteine del latte, tuttavia due specifiche aree della β-caseina (residui aminoacidici 60-70 e 191-202) rappresentano "zone strategiche" di cleavage enzimatico che permettono di ottenere diversi peptidi con proprietà multifunzionali (Clare and Swaiswood, 2000). Nel primo periodo di vita tali peptidi sembrano rivestire un ruolo essenziale nello sviluppo della tolleranza alimentare nei confronti degli antigeni presenti negli alimenti e nell'induzione della risposta immunitaria nei confronti di antigeni patogeno-derivati, fondamentale per evitare la ben nota suscettibilità dei neonati alle infezioni (Hill 1987).
Diversi studi hanno dimostrato la capacità di tali peptidi di stimolare o sopprimere l'attività del sistema immunitario (Baldi *et al.* 2005), modulandone alcune proprietà come il processo di maturazione delle cellule fagocitarie. L'attività di immunomodulazione di due peptidi derivati dalla β-caseina è stata testata da studi in vivo (Politis e Chronopoulou, 2008) e in vitro (Chronopoulou *et al.* 2006) su monociti, macrofagi e neutrofili ematici ottenuti da suinetti allo

svezzamento. Utilizzando come parametri la produzione di anioni superossido, fondamentale per l'azione citotossica nei confronti dei patogeni (figura 1) e il sistema urochinasi attivatore del plasminogeno (uPA), i peptidi testati hanno dimostrato un'azione di regolazione dell'attività delle cellule fagocitarie durante le prime due settimane post svezzamento. Al contrario, nessuno dei peptidi testati ha avuto effetti sull'attività delle cellule fagocitarie isolate un mese più tardi, a 5-6 settimane dallo svezzamento. La spiegazione più plausibile di questo è che tali peptidi siano efficaci nella fase che coincide con l'immaturità del sistema immunitario (1-2 settimane dopo lo svezzamento) e che perdano le loro proprietà immunomodulatorie un mese più tardi, quando presumibilmente il sistema immunitario dovrebbe aver raggiunto la sua piena maturazione funzionale. I risultati degli studi condotti negli ultimi anni lasciano pertanto ipotizzare che il ruolo in vivo di alcuni peptidi presenti nel latte sia quello di modulare e guidare lo sviluppo della risposta immunitaria in sede intestinale nel neonato, fino al raggiungimento della sua piena maturazione.

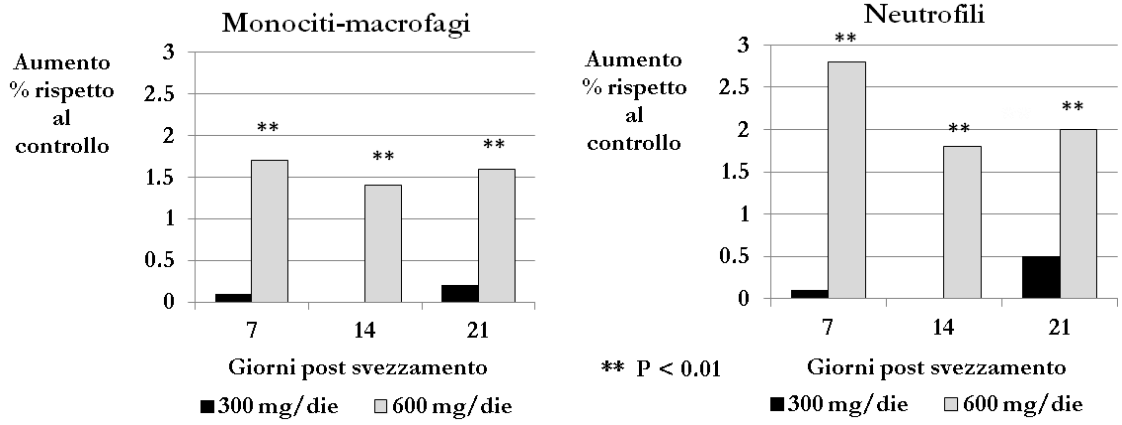

Figura 1 – Effetto della somministrazione di peptidi della caseina a suinetti svezzati sulla produzione di anioni superossido da parte di monociti-macrofagi e neutrofili (da Politis e Chronopoulou, 2008, modificato).

Lattoferrina

Tra le proteine biologicamente attive presenti nel latte, negli ultimi anni la lattoferrina ha attratto l'interesse della ricerca in quanto efficace strumento di difesa dalle infezioni e dagli stress correlati allo svezzamento.

Appartenente alla famiglia delle transferrine, la lattoferrina è una proteina multifunzionale coinvolta in diverse funzioni fisiologiche, quali la regolazione dell'assorbimento intestinale del ferro, i processi di difesa immunitaria aspecifica nei confronti di virus e batteri, i processi antinfiammatori. Possiede inoltre proprietà antiossidanti e anticarcinogene.

Studi in vitro e in vivo hanno dimostrato un'attività antimicrobica ad ampio spettro della lattoferrina nei confronti di batteri patogeni (Nuijens *et al.* 1996) e

un supporto prebiotico per microrganismi benefici come i bifidobatteri e i lattobacilli (Griffiths *et al.* 2003).
L'attività antimicrobica della lattoferrina nei confronti di batteri Gram positivi e Gram negativi, quali *Escherichia coli* (Dionysius *et al.* 1993) e Bacillus *spp.* (Oram and Reiter 1968) è stata ampiamente documentata da studi sia in vitro che in vivo. In uno studio condotto da Pecorini *et al.* (2005), l'attività antibatterica di lattoferrina ricombinante porcina è stata testata su 5 ceppi di *E. coli* isolati da suinetti sotto scrofa con diarrea. I test in vitro hanno mostrato un effetto inibitorio della lattoferrina ricombinante sulla crescita batterica del 30% alla concentrazione di 0,1 mg/ml (figura 2).

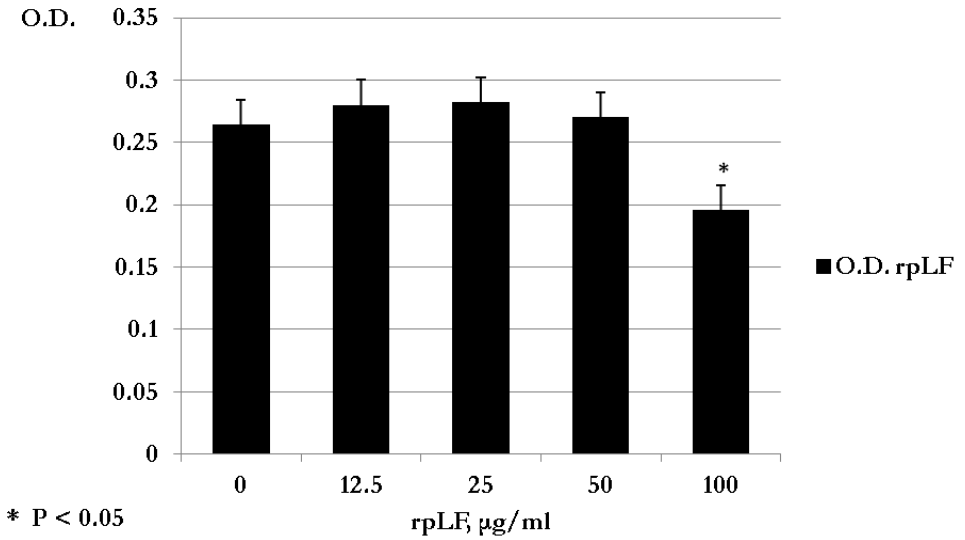

Figura 2 – Attività antibatterica di lattoferrina ricombinante porcina (rpLF) su ceppi di *E. coli* isolati da suinetti sotto scrofa con diarrea (Pecorini et al., 2005).
Alla concentrazione di rpLF nel terreno di coltura di 100 µg/ml l'attività antibatterica è stata pari al 30% rispetto al controllo (0 µg/ml).

L'effetto antibatterico della lattoferrina si esercita principalmente attraverso due meccanismi: il primo è da ricondursi alla sua capacità di legare il ferro, limitando l'impiego di questo nutriente da parte dei microrganismi e inibendo così la crescita batterica (effetto batteriostatico); il secondo è da collegarsi ad un'interazione diretta della molecola con gli agenti infettivi, attraverso il legame dei suoi aminoacidi con molecole anioniche presenti sulla superficie di alcuni batteri, virus, funghi e parassiti, che ne provoca la lisi cellulare (effetto battericida) (Gonzalez-Chavez *et al.* 2009).
Oltre all'attività antimicrobica, diversi studi hanno dimostrato un'azione di stimolazione selettiva della lattoferrina sulla crescita di batteri intestinali benefici appartenenti ai generi lattobacillus e bifidobacterium. In un recente studio (Pecorini *et al.* 2005) è stata valutata la capacità di lattoferrina bovina, lattoferrina

umana e lattoferrina ricombinante porcina di stimolare la crescita di 4 ceppi di lattobacilli. Già a basse concentrazioni tutte e tre hanno stimolato la crescita di *Lactobacillus casei spp casei*, mentre non hanno avuto alcun effetto sulla crescita degli altri ceppi, a dimostrazione che l'attività prebiotica della lattoferrina è ceppo-dipendente (figura 3).

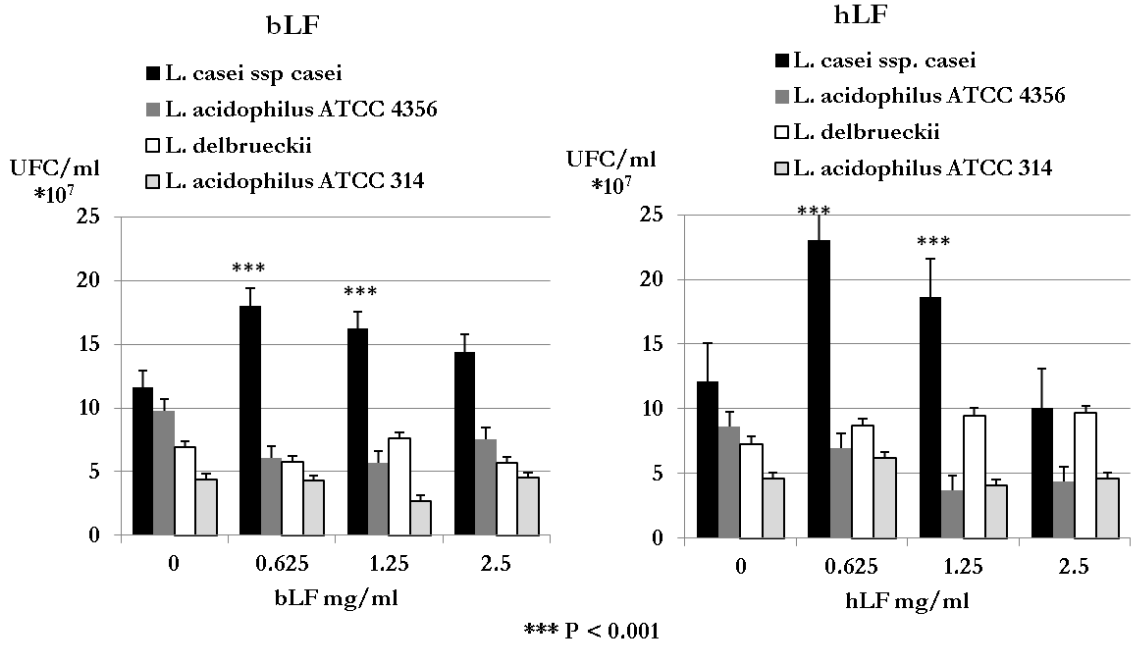

Figura 3 – Effetto della lattoferrina bovina (bLF) e della lattoferrina umana (hLF) sulla crescita dei lattobacilli (Pecorini *et al*, 2005).
hLF e bLF alle concentrazioni di 0.625 e 1.25 mg/ml hanno stimolato positivamente (P<0.001) la crescita di *L. casei spp. casei*. Gli altri ceppi non sono stati influenzati dal trattamento.

L'impiego di lattoferrina nella fase di svezzamento ha dimostrato effetti positivi sulle performance di crescita dei suinetti (Wang *et al.* 2006) e sull'incidenza di diarrea, analoghi a quelli rilevati in seguito a trattamento con antibiotici (Shan *et al.* 2007). Inoltre, come già dimostrato nell'uomo e in altre specie animali, Shan *et al.* (2007) hanno osservato anche nel suinetto in svezzamento un miglioramento dei parametri immunitari, evidenziato dall'aumento dei livelli sierici di IgG, IgA, IgM, C4, IL-2.
Attualmente, la lattoferrina può essere prodotta attraverso l'isolamento dal latte e dal colostro di diversi mammiferi o come lattoferrina ricombinante attraverso sistemi di espressione batterici, fungini o virali.

CLA immunomodulatori

La frazione lipidica è una delle componenti del latte più studiata per quanto concerne i composti bioattivi ad azione nutraceutica. Recentemente, l'interesse

per le applicazioni nutraceutiche dei prodotti derivati dal latte si è focalizzato sui coniugati dell'acido linoleico (CLA), un gruppo di acidi grassi poliinsaturi, isomeri posizionali (9,11; 10, 12; o 11,13) e geometrici (cis o trans) dell'acido linoleico, per i quali sono stati dimostrati numerosi effetti benefici per la salute, sulla base di studi sperimentali su modelli animali, inclusi effetti anticarcinogenici, antiaterogenici, antidiabetici e antiadipogenici (Belury 2002). È possibile che diversi isomeri dell'acido linoleico possiedano un'attività biologica, tuttavia gli effetti fisiologici dei CLA attualmente noti sono prodotti dagli isomeri cis-9, trans-11 e trans-10, cis-12, i più studiati e i più impiegati in ricerca come integratori nutrizionali, sottoforma di preparazioni di sintesi chimica contenenti uguali quantità dei due isomeri.
Diversi studi in vitro e in vivo hanno dimostrato proprietà immunomodulatorie dei CLA nei roditori, volatili, suini e nell'uomo (O'Shea *et al.* 2004). Studi recenti hanno dimostrato un effetto benefico dei CLA sullo sviluppo della competenza immunitaria nel suinetto. L'integrazione alimentare di CLA nel suinetto produce un aumento della proliferazione linfocitaria e all'aumentare del livello di integrazione (0,67, 1,33 e 2%) un incremento lineare della percentuale di linfociti CD8+ nel sangue periferico (Bassaganya-Riera *et al.* 2001), migliorando la protezione immunitaria contro i virus e attenuando le alterazioni immunologiche e infiammatorie prodotte da virus immunosoppressivi quali circovirus suino tipo 2 (Bassaganya-Riera *et al.* 2003).
Inoltre, la somministrazione di CLA (0,5 e 1%) a suinetti svezzati migliora alcuni parametri immunitari aspecifici, come i livelli ematici di IgG e lisozima (Corino *et al.* 2002).
Il latte di scrofa è normalmente privo di CLA, se questi non vengono somministrati con la dieta (Jahreis *et al.* 1999). I CLA assunti con la dieta vengono secreti nel colostro e nel latte con un'efficienza di trasferimento che può variare rispettivamente dal 53 al 63% e dal 55 al 69% (Bee 2000). Bontempo *et al.* (2004) hanno rilevato la presenza di CLA solo nel colostro di scrofe a cui erano stati somministrati CLA (0,5%) in tarda gestazione e nella successiva lattazione. La somministrazione di CLA alle scrofe ha anche influenzato la composizione acidica della componente lipidica del colostro, determinando un aumento degli acidi eicosenoico e eicosatrienoico e una riduzione degli acidi palmitoleico e γ-linolenico. Inoltre, è stato osservato un aumento significativo delle IgG nel colostro e un effetto positivo sui parametri immunologici sia della scrofa che del suinetto. In particolare, la somministrazione di CLA ha prodotto un aumento significativo dei livelli sierici di lisozima e di IgG nelle scrofe (circa 1,5 volte superiori rispetto al controllo) e nei suinetti (quasi 2 volte rispetto al controllo, figura 4).

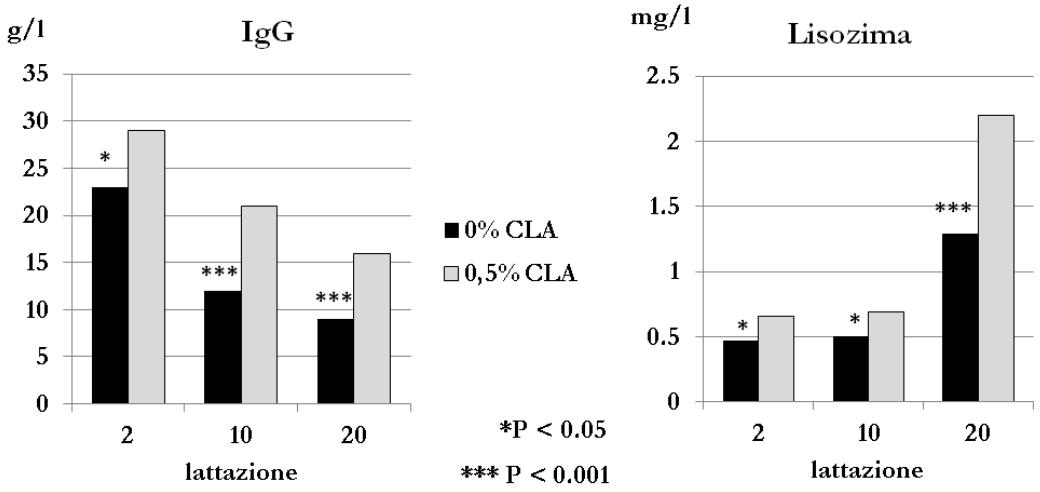

Figura 4 – Effetto della somministrazione di CLA alle scrofe sulle variabili immunologiche nel siero dei suinetti (Bontempo et al., 2004).

Gli effetti immunomodulatori dei CLA sono stati confermati da uno studio successivo (Corino *et al.* 2009), che ha indagato l'effetto della durata della somministrazione di CLA alle scrofe (0,5% da 7 giorni prima a 7 giorni dopo il parto e 0,5% da 7 giorni prima del parto allo svezzamento) sulla crescita e sui parametri immunitari delle scrofe e dei suinetti. In entrambi i gruppi trattati con CLA, livelli di IgG, IgA e IgM nel colostro (figura 5) e i livelli sierici di IgG nei suinetti allo svezzamento (figura 6) erano significativamente superiori rispetto al controllo.

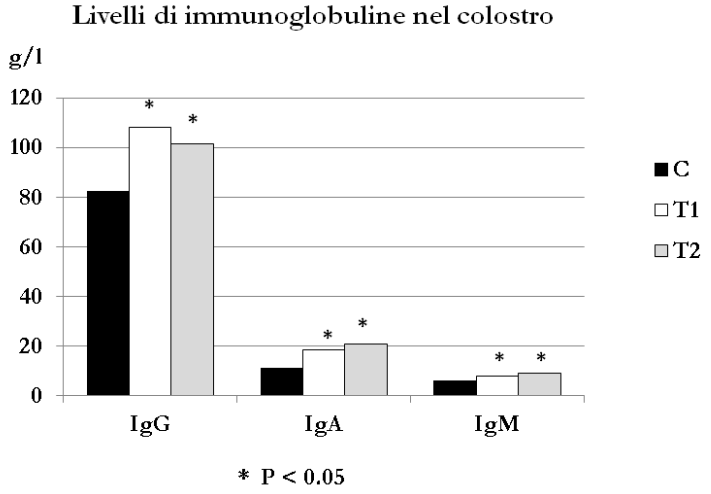

Figura 5 – Effetto della somministrazione di CLA alle scrofe sui livelli di immunoglobuline nel colostro (Corino *et al.*, 2009)

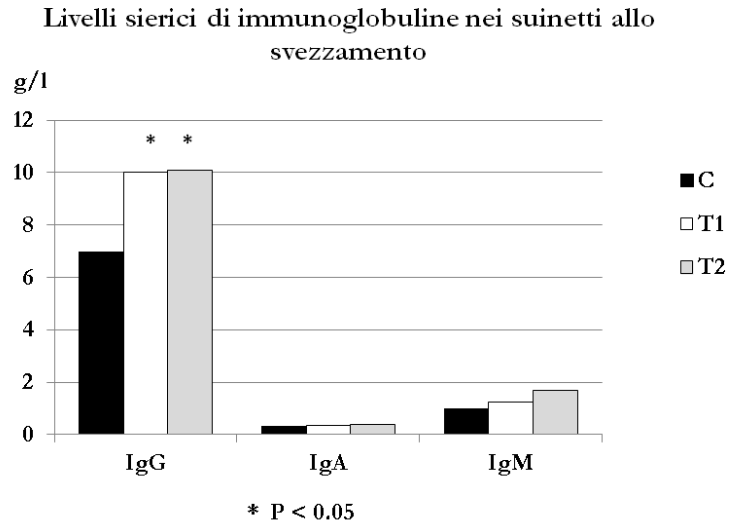

Figura 6 – Effetto della somministrazione di CLA alle scrofe sui livelli sierici di immunoglobuline nei suinetti allo svezzamento (Corino *et al.*, 2009)

Un'adeguata assunzione di IgG attraverso il colostro è di grande importanza per il suinetto neonato dal momento che l'immunità passiva rappresenta il suo principale strumento di difesa dalle infezioni; inoltre, la capacità del suinetto di sintetizzare IgG sembra positivamente correlata alla quantità di IgG materne assorbite (Rooke and Bland 2002) e i parametri immunologici del colostro sembrano influenzare la capacità immunitaria dei suinetti anche dopo lo svezzamento (Krakowski *et al.* 2002). Il miglioramento dei parametri immunitari osservato in seguito alla somministrazione di CLA assume quindi particolare importanza negli animali giovani, che non hanno ancora sviluppato una risposta immunitaria specifica, in cui queste sostanze sembrano agire come "attivatori" del sistema immunitario.
In virtù dei loro effetti benefici sul miglioramento della risposta immunitaria, nonché dei risultati ottenuti da studi che suggeriscono un'attività antinfiammatoria a livello di mucosa intestinale (Hontecillas *et al.* 2002), l'impiego dei CLA nell'allevamento suino è tuttora oggetto di studi in quanto interessante alternativa all'impiego di antibiotici ad uso auxinico (Bassaganya-Riera *et al.* 2004, Corino *et al.* 2009).

Oligosaccaridi e glicani

I glicani sono carboidrati complessi presenti nel latte come oligosaccaridi o come glicoconiugati, glicolipidi, glicoproteine, mucine e glicosamminoglicani.
Dopo il lattosio, i glicani più rappresentati nel latte umano sono gli oligosaccaridi. La frazione oligosaccaridica è quantitativamente superiore alla frazione lipidica e simile a quella proteica (5-20 g/L) e rappresenta circa il 10%

del contenuto calorico del latte umano (Newburg 2009). Nel latte bovino, la concentrazione è molto più bassa (0.7-1.2 g/L nel colostro, Veh *et al.* 1981). Gli oligosaccaridi fucosilati, che costituiscono il 70% degli oligosaccaridi nel latte umano, sono assenti nel latte bovino, che è invece più ricco di oligosaccaridi sialilati (Tao *et al.* 2008).
Questi composti recentemente hanno acquisito un crescente interesse scientifico e commerciale in relazione alle loro proprietà prebiotiche e antinfettive, evidenziate nel latte umano.
I primi studi riguardanti l'attività prebiotica della componente oligosaccaridica del latte risalgono agli anni 70, quando è stato dimostrato che un glicano isolato dal latte umano, definito "bifidus factor", era in grado di stimolare la crescita di *Bifidobacterium bifidum*, uno dei microrganismi più rappresentati nell'intestino dei neonati (Gyorgy *et al.* 1974). Da allora sono stati isolati e caratterizzati più di un centinaio di oligosaccaridi presenti nel latte umano, molti dei quali hanno dimostrato un'attività prebiotica specifica. Questi composti infatti resistono ai processi digestivi nel tratto gastroenterico superiore e arrivano intatti all'intestino, dove agiscono come substrati per la crescita di bifidobatteri e lattobacilli, stimolando selettivamente la crescita della microflora non patogena.
I potenziali effetti benefici della componente oligosaccaridica del latte umano sono stati studiati nel corso degli anni con particolare enfasi per i loro effetti prebiotici, tuttavia recentemente sono stati osservati diversi altri meccanismi grazie ai quali questi composti possono apportare beneficio alla salute del neonato. Circa il 90% degli oligosaccaridi ingeriti, infatti, può essere ritrovato intatto e non metabolizzato nelle feci del neonato, il che suggerisce altre funzioni, oltre a quella di mantenere la desiderata composizione della microflora intestinale (Bode 2009).
Studi condotti nell'ultimo decennio hanno identificato nel latte umano un crescente numero di glicani bioattivi, ognuno dei quali si è dimostrato in grado di inibire l'attività solo di uno specifico patogeno o di una famiglia di patogeni e di inattivare tossine, come la tossina termostabile (ST) di *E. coli* (Newburg *et al.* 2005, tabella 1).
Si ritiene che l'attività protettiva degli oligosaccaridi verso agenti infettivi intestinali sia legata in primo luogo ad una selettiva capacità di inibizione del legame tra agenti patogeni e cellule intestinali. La maggior parte dei patogeni intestinali infatti utilizza come epitopi specifici glicani esposti sulla superficie cellulare per identificare e legarsi alle cellule target nella fase iniziale del processo patogenetico. Alcuni di questi epitopi sono presenti anche nella componente oligosaccaridica del latte umano e questo ha suggerito che i glicani presenti nel latte possano agire come analoghi solubili, in grado di legarsi a specifici patogeni e di inibire in tal modo il loro legame ai recettori delle cellule intestinali dell'ospite (Newburg 1999) (figura 7). *Campylobacter jejuni*, ad esempio, aderisce

Glicoconiugato	Patogeno	Meccanismo	Concentrazione(*)
GM1	*Vibrio cholera* *Campylobacter jejuni*	Legame con tossina	180 µg/L
GM3	*E. coli* enteropatogeno	Legame con tossina	13 mg/L
Gb3	Shiga toxin	Legame con tossina	100-150 µg/L
Condroitin solfato	Virus immunodeficienza umana	Legame con virus	6 mg/L
Lactaderina	Rotavirus	Legame con virus	100 µg/L
Mucina	*E. coli*	Legame con patogeno	1 g/L
Glicopeptidi mannosilati	*E. coli* enteroemorragico	Legame con tossina	60 mg/L
Oligosaccaridi	*Streptococcus pneumoniae*	Legame con patogeno	0.2-10 g/L
	E. coli enteropatogenico	"	
	Listeria monocytogenes	??	3 g/L
	Clostrium spp.	Prebiotico	3 g/L
	Campylobacter jejuni	Legame con patogeno	
	Rotavirus	Prebiotico	
Oligosaccaridi fucosilati	*Campylobacter jejuni*	Legame con patogeno	1-25 mg/L
	Vibrio cholera	Legame con patogeno	1-25 mg/L
	E. coli enterotossina	Legame con tossina	40 µg/L
Glicoproteine associate a macromolecole	Norovirus *Pseudomonas aeruginosa*		370 mg/L
Sialillactosio	Cholera toxin *E. coli* *P. aeruginosa* Virus influenza Poliomavirus *Helicobacter pylori*		200 mg/L

Tabella 1 – Latte umano: glicoconiugato con attività di inibizione dei patogeni (adattato da Newburg *et al.*, 2005; Séverin and Wenshui, 2005).
(*) Concentrazione della frazione attiva o componente del latte

all'epitopo H-2, espresso sulla superficie delle cellule epiteliali intestinali. Questo legame è inibito da oligosaccaridi presenti nel latte umano, in particolare dal 2-fucosillattosio, la cui struttura è analoga all'epitopo H-2 (Ruiz-Palacios *et al.* 2003). L'incidenza di diarrea da Campylobacter nei neonati allattati al seno è

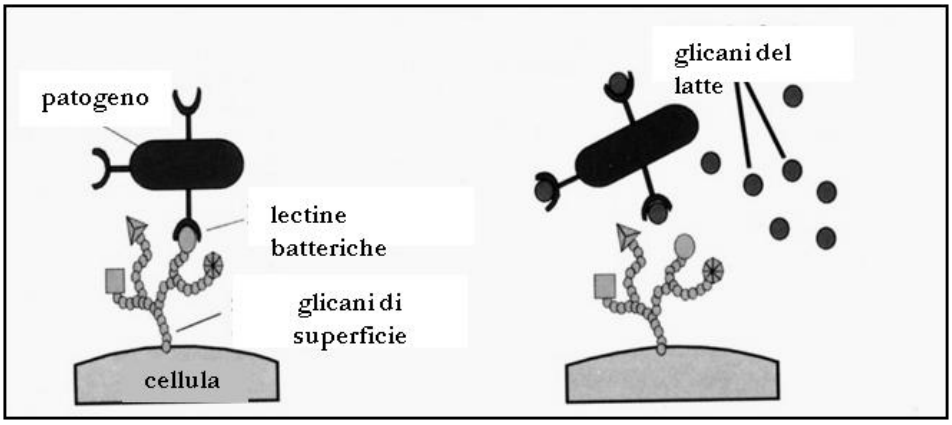

Figura 7– Meccanismo d'azione degli oligosaccaridi presenti nel latte umano (adattato da L. Bode, 2009).
La maggior parte dei patogeni intestinali esprime proteine (lectine) che legano i glicani esposti sulla superficie cellulare per identificare e aderire alle cellule target nella fase iniziale del processo patogenetico. I glicani presenti nel latte, strutturalmente simili ai glicani esposti sulla superficie delle cellule epiteliali intestinali, possono funzionare da analoghi ligandi le lectine batteriche bloccando in tal modo l'adesione dei batteri alle cellule intestinali.

infatti inversamente correlata alla quantità di 2-fucosillattosio presente nel latte materno (Morrow *et al.* 2004). L'effetto antibatterico degli oligosaccaridi presenti nel latte umano è stato dimostrato anche per la diarrea da calicivirus (Morrow *et al.* 2004), rotavirus (Yolken *et al.* 1992) e per le infezioni da enterotossina termostabile di *E. coli* (Newburg *et al.* 2004). Studi recenti hanno inoltre riportato un effetto protettivo degli oligosaccaridi presenti nel latte umano anche nei confronti delle infezioni respiratorie del neonato (Stepans *et al.* 2006), dimostrando così che l'effetto protettivo dei glicani potrebbe estendersi ad altre mucose oltre a quella gastroenterica.
Agendo come prebiotici, modulatori delle funzioni mucosali o inibitori dei patogeni, nel loro complesso i glicani costituiscono uno dei principali componenti del sistema di difesa immunitario del latte umano. Anche il latte delle altre specie animali contiene glicani, alcuni dei quali in quantità apprezzabili, che potrebbero svolgere un'azione protettiva dai patogeni importante nel neonato. L'isolamento e la caratterizzazione delle diverse frazioni oligosaccaridiche del latte in relazione alla specie animale potrebbe rappresentare un promettente approccio allo sviluppo di nuovi additivi per la profilassi e la terapia delle patologie gastroenteriche e respiratorie del neonato.

Glutamina e nucleotidi

Lo svezzamento del suinetto è caratterizzato da un'immediata, seppure transitoria, drastica riduzione dell'assunzione di alimento, responsabile di denutrizione e calo della crescita. Questo a sua volta comporta alterazioni

dell'architettura e della funzionalità intestinale responsabili di disordini digestivi e spesso di diarrea. Tra le più significative alterazioni dell'anatomia dell'intestino tenue si riscontra una riduzione del 20-30% del peso della mucosa associata all'atrofia dei villi intestinali (Pluske *et al.* 1997, Lalles *et al.* 2004).
Diverse sostanze di tipo nutraceutico sembrano esercitare rilevanti effetti sullo sviluppo di un corretto metabolismo cellulare a livello enterocitario. La glutamina è un aminoacido considerato "condizionatamente essenziale", in quanto pur essendo normalmente presente negli alimenti in quantità adeguate ai fabbisogni, in particolari condizioni di stress quali lo svezzamento, il suo apporto alimentare può risultare insufficiente. È indispensabile per la crescita delle cellule e dei tessuti in rinnovamento come gli enterociti, in quanto costituisce il substrato ossidativo più importante per le cellule a rapida moltiplicazione ed è una fonte importante di atomi carboniosi per la gluconeogenesi. In virtù del suo ruolo chiave nel mantenere la struttura e la funzionalità intestinale, è stata oggetto di notevole interesse come supplemento nutrizionale mirato alla salute della mucosa intestinale (Domeneghini *et al.* 2006a). Alcuni studi hanno dimostrato un ruolo importante della glutamina nel mantenimento dell'integrità mucosale in risposta a traumi o infezioni (Souba *et al.* 1990) e in relazione a condizioni di stress associate ad atrofia della mucosa gastroenterica (Remillard *et al.* 1998).
I nucleotidi, come costituenti degli acidi nucleici, regolano la sintesi proteica e il loro apporto è essenziale per i tessuti in rapida attività proliferativa con un elevato tasso di turnover cellulare, come la mucosa intestinale dell'animale giovane. Come la glutamina, intervengono nella modulazione dei fenomeni di proliferazione cellulare e di apoptosi in vitro (Schlimme *et al.* 2000).
Il suinetto riceve nucleotidi e glutamina attraverso il latte e il colostro e la concentrazione di nucleotidi nel latte di scrofa risulta essere relativamente costante durante la lattazione (Mateo *et al.* 2004), indicando che il loro apporto è fondamentale nelle prime fasi di vita del suinetto.
L'impiego di tali sostanze riveste interesse non solo nel suinetto lattante ma soprattutto nella fase di svezzamento, quando la riduzione di assunzione di alimento influisce negativamente sulle caratteristiche morfometriche della mucosa intestinale, con una progressiva riduzione dell'altezza dei villi e un incremento della proliferazione cellulare a livello di cripte, che porta alla diminuzione del rapporto altezza dei villi/profondità delle cripte e all'aumento del numero di cellule immature.
Le osservazioni istometriche eseguite sui villi e le cripte intestinali confermano l'ipotesi che tali sostanze siano potenzialmente in grado di ripristinare la riduzione dello spessore della mucosa che si verifica allo svezzamento (Domeneghini *et al.* 2004, 2006b). L'integrazione nella dieta di suinetti svezzati di glutamina (0,5%) si è dimostrata infatti efficace nel mantenere la conformazione

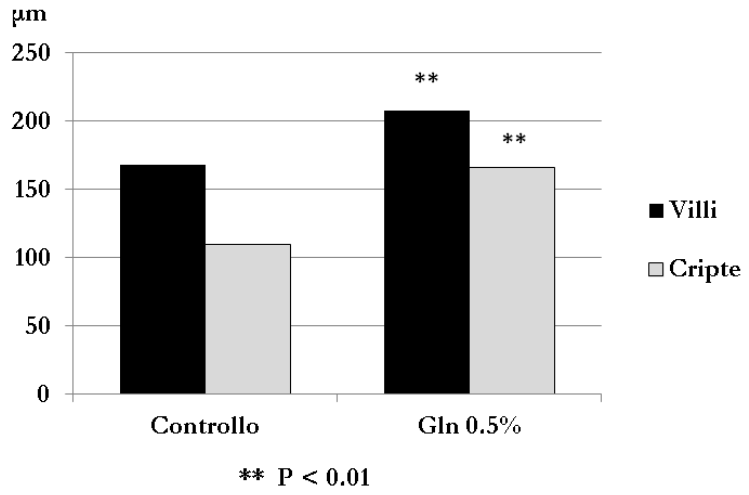

Figura 8 – Somministrazione di L-glutamina a suinetti in svezzamento: effetto sull'altezza dei villi e sulla profondità delle cripte della mucosa ileale (Domeneghini *et al.*, 2006).

e la funzionalità della barriera mucosale, come evidenziato dall'aumento dell'altezza dei villi e della profondità delle cripte della mucosa ileale (figura 8). Inoltre, nei suinetti trattati le cellule linfatiche del GALT hanno mostrato una più elevata attività proliferativa e una minore apoptosi rispetto al controllo, suggerendo una maggiore efficienza delle componenti difensive mucosali conseguente alla somministrazione di glutamina.

Conclusioni

Nel corso degli ultimi anni la ricerca in campo umano ha dimostrato la presenza nel latte di un ampio range di componenti bioattive, con proprietà benefiche non solo per lo sviluppo del neonato, ma anche per la salute e per la prevenzione di patologie in età adulta.

I risultati di tali ricerche, condotte per lo più in vitro o su modelli animali, tra cui il suino in particolare, hanno aperto interessanti prospettive per l'impiego di tali sostanze come supplementi o prodotti alimentari, al fine di effettuare una profilassi nutrizionale alternativa agli antibiotici a uso auxinico nell'allevamento suino. In questo campo, le conoscenze scientifiche aumentano rapidamente e riguardano un crescente numero di sostanze bioattive naturalmente presenti nel latte. In tale contesto vanno anche considerati i risultati di diversi studi che hanno valutato l'impiego di colostro bovino come "promotore di crescita naturale" nella fase di svezzamento, in quanto fonte di componenti bioattive con funzioni antimicrobiche e di promotori della crescita (Boudry *et al.* 2008).

È ancora ampia l'area da esplorare in questo settore della ricerca e per molte di queste sostanze restano da valutare dosi e tempi di somministrazione, anche in funzione dei costi dell'integrazione. Maggiori conoscenze relative alla variazione di concentrazione di tali sostanze nel corso della lattazione, le possibili interazioni, la valutazione di un loro impiego come tali o incluse in una matrice lattea, potranno suggerirne un impiego strategico, in funzione dell'età allo svezzamento, caratteristiche delle diete utilizzate, condizioni sanitarie dell'allevamento, al fine sostenere lo sviluppo del sistema immunitario e la salute dell'intestino durante il processo di svezzamento dei suinetti.

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CHAPTER 9

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