Luciana Rossi[1*], Vittorio DelloÔOrto[1], Simona Vagni[1], Vittorio Sala[2], Serena Reggi[3], Antonella Baldi[4].

Protective effect of oral administration of transgenic tobacco seeds against verocytotoxic *Escherichia coli* strain in piglets

[1] Università di Milano. Department of Veterinary Sciences and Technology for Food Safety. Via Celoria 10, 20133 Milan, Italy.
[2] Università di Milano. Department of Veterinary Science and Public Health. Via Celoria 10, 20133 Milan, Italy.
[3] Plantechno S.r.l. Via Staffolo, 60; 26040 Vicomoscano, Cremona, Italy.
[4]Università di Milano. Department of Veterinary Sciences and Technology for Food Safety. Via Trentacoste 2, 20134 Milan, Italy.

*Corresponding author
Luciana Rossi
e.mail: luciana.rossi@unimi.it
phone number: +39-0250315756
fax number:+39-0250315746

Abstract (150 words)

The use of transgenic plants as delivery system for antigenic proteins is attractive for its simplicity and increases likelihood for local immune response at sites of infection. The aim of this study was to evaluate the protective effect of oral administration of tobacco seeds, expressing the FedA, the major protein of the F18 adhesive fimbriae, and B subunit of verocitoxin, against verocytotoxin-producing *E. coli* (VTEC) strain in piglets. Forty-three early weaned piglets, were randomly divided into 4 experimental groups: 3 test groups and a control. Treatment groups orally received a bolus, with different dose of tobacco seeds on 0, 1, 2, 14 days post primary administration. After challenge, with $1*10^{10}$ CFU of O138 *Escherichia coli* strain, piglets showed clinical scores 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 conclusion, oral administration of recombinant tobacco seeds expressing antigenic proteins against VTEC strains can induce a protective effect against challenger strain in piglets.

Keywords
Plant vaccine- pig- F18 fimbriae- Verocytotoxin- *E.coli*- tobacco seeds

Introduction

The verocytotoxin-producing *E. coli* (VTEC) group is composed by *E. coli* strains capable of producing toxins very similar to the one produced by *Shigella dysenteriae*. In human the Shiga toxins produced by *E. coli* may cause anything from uncomplicated diarrhoea to haemorrhagic colitis, which can progress into haemolytic uremic syndrome, composed of a micro-angiopathic haemolytic anaemia, thrombocytopenia and severe acute renal failure requiring intensive care (Ruggenti and Remuzzi, 2012). In pig livestock VTEC strains, belonging to serogroups O138, O139, and O141 (Nagy and Fekete, 2005), are responsible for important economic losses and may cause different disorders such as Oedema disease (OD), a systemic disease that usually occurs in piglets shortly after weaning (mainly within the first 2 weeks post-weaning) and causes high morbidity, mortality and reduction of growth rates. Clinical signs of VTEC infections include palpebral oedema, neurological symptoms (i.e. ataxia, paralysis), recumbency, sudden death. 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 most important symptoms are related to 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, responsible for binding the toxin to the cell surface. 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 VTEC strains colonize the ileum via host-specific F18 fimbriae that allow the bacteria to adhere to F18 receptors on small intestinal enterocytes (Coddens *et al.*, 2009). The F18 fimbria has been also indicated as an important virulence factor related to OD, 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). 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, 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 (Cox et al., 2002). 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 (Streatfield et al., 2006).
The use of transgenic plants as delivery system for antigenic proteins is attractive for its simplicity and increases likelihood for local immune response at sites of infection. Plant as edible vaccine present many potential advantages related to the management of intensive livestock because they can be administered in the feed without having to restrain the animals, which is both less stressful for the animals and reduces labour costs in terms of the multiple injections of traditional vaccines no longer being needed (Pinotti et al., 2003; Widgorovitz et al., 1999).
In this study the attention was focalized on tobacco seeds expressing the FedA, the major protein of F18 fimbriae, 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. Previous studies demonstrated that the Vt2e-B and FedA fimbrial genes were stably incorporated into tobacco plant genome by being transcribed through the nuclear apparatus of the plant for specific expression in the seeds, and that these genes are inherited by the next generation (Rossi et al.,2003a). Mice can be stimulated to produce mucosal antibodies after oral administration of recombinant tobacco seeds for the expression of F18 and VT2e-B antigens (Rossi et al.,2003b; Rossi et al., 2013a). Moreover, tobacco seeds, unlike the leaves, do not contain significant levels of nicotine and, once included in diets for weaned piglets, they showed a good palatability (Rossi *et al.* 2007) and can use as alternative protein source (Rossi et. al 2013b). For these reasons, the aim of this study was to evaluate whether oral administration transgenic tobacco seeds expressing antigenic proteins from verocitoxic E.coli strain can have a protective effect against a subsequent O138 *Escherichia coli* challenge in piglets.

Materials and methods

Animals, facilities and feeding

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 Aujeszkyôs 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 of the University of Milan.
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.

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

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

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 ñEvaluation of immunogenity of tobacco seeds expressing antigenic protein to OD in weaned pigletsò, February 17th, 2011).

Production and Chemical analyses of tobacco seeds

Six homozygous transgenic lines of tobacco (*Nicotiana tabacum L., cv. Xanthi*), three harbouring respectively the Vt2e-B gene and three the FedA subunit of F18 fimbriae gene, previously obtained (Rossi et al., 2003a; Rossi et al., 2013a), were cultivated in a greenhouse in homogeneous environmental conditions (at 15°C night and 25°C day temperature). In particular, a total of eighty F18 positive plants and one hundred and forty VT2eB positive plants, derived from the selected homozygous transgenic plants were cultivated in a grennhouse of Plantechno s.r.l. in a period of 14 months. Each plant grew in individual vessel filled with peat of the same humidity and nutrientôs content. No differences were observed in relation to the yield of seeds among the experimental lines of tobacco plants (about 7-15g of seed per plant). A total amount of 500 grams of transgenic tobacco seeds transformed for the expression of FedA, the major protein of the F18 adhesive fimbriae (F18TS), and 1,900 grams of transgenic tobacco seeds transformed for the expression of VT2eB (VT2eBTS), expressing about 0.6mg of heterologous protein per gram of seed were used as experimental treatments. Wild type tobacco seeds were administered to controls.
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 (Rossi et. al., 2011).
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).

Groups and treatments

Piglets were randomly divided into four experimental groups. Treatment 1 group (T1) was composed of 12 piglets, receiving 20 grams of milled transgenic tobacco seeds (10 grams VT2eBTS and 10 grams F18TS). Treatment 2 group (T2) was composed of 9 piglets, receiving 10 grams of milled VT2eBTS. Treatment 3 group (T3) was composed of 10 piglets, receiving 25 grams VT2eBTS. Control group (CG) was composed of 12 piglets, receiving 20 grams of wild type milled tobacco seeds.
Piglets were fed tobacco seeds on 0,1,2 and 14 days post primary administration (dppa). Day 0 was considered the 5th day after the weaning. Tobacco seeds were individually administered mixed with chocolate and water in palatable boluses. Three hours before the oral administration up to 3 hours after it, animals were deprived of food. About 10-15 min before each administration 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).

Experimental infection

O138 Escherichia coli strain, isolated from weaned piglet that had died because of OD was used as challenge strain. The challenge strain was evaluated, in order to verify the presence of VT2e and F18 genes, by PCR assay using the following experimental conditions.
The volume of all reaction mixture was 50 µl, with 5 µl of template (bacterial DNA) added to PCR mixture. Primers 5ô ï ggATCCATgAAAAgACTAgTgTTTATTTCTTTTg and 3ô gAgCTCTTACTTgTAAgTAACCgCgTAAgC were used for f18 adhesive fimbriae gene detection. Cycling conditions consisting of a hot star at 94°C for 3 min followed by 94°C for 1 min, 56°C for 1min 20secs, 72°C for 1 min 30 sec, and a ýnal extension step of 72°C for 5 min.

Primers 5ôï ggATCCATgAAgAAgATgTTTATAgCgg and 3ôgAgCTCTTAgTTAAACTTCACCTgggCAA were used for B subunit of VT2e toxin gene detection. Cycling conditions consisting of a hot star at 94°C for 3 min followed by 95°C for 1 min, 50°C for 1min, 72°C for 1 min 30 sec, and a ýnal extension step of 72°C for 5 min. PCR assays was carried out with GeneAmp thermal cyclers (Applied Biosystems, Carlsbad, CA) for 25 cycles.

About one week after booster administration (22 dppa), the animals were orally challenged with O138 *Escherichia coli* strain grown in broth medium. 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).

Data and sample collection

Blood was collected from the jugular vein of each animal on 0, 7, 14, 21, 25, 29 and 36 dppa. 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 dppa to determine total IgA levels in the immunization phase, as described below. From the day of challenge until day 14 post-challenge (36 dppa), rectal temperature was daily recorded and the clinical signs of the disease according to point scale score described by Tsukahara et al. (2006). 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 diarrhea (Rossi et al., 2012).

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

Antibodies immunoglobulin A (IgA) and immunoglobulin G (IgG) quantification in faeces and serum samples

In order to separate antibodies from faecal content, 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 ī 20°C until use.

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 . Total IgA and IgG concentrations in serum and faecal samples were evaluated through Pig ELISA IgA and IgG quantitation set (Bethyl Laboratories, Texas). Microtitration 96-well plates were coated for 1 h at ambient temperature (20-25°) with 100ε l of a solution

containing 1 εl of affinity purified anti-Pig IgA (or IgG) coating antibody diluted in 100 εl of coating buffer. After aspiration of the antibody solution and after washing the plate, the wells were blocked with 200 εl of Blocking Solution for 30 minutes and then washed five times with 300 εl of Wash Solution. To each well 100 εl of standard or sample were added and incubated at room temperature for 1 hour. 100 εl of diluted HRP detection antibody were added to each well and incubated at room temperature for 1 hour. After washing 100 εl 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 εl 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 ñCurva Expert 1.3ò software. The concentrations determined were expressed as nanograms of IgA or IgG per 1 ml.

Microbiological analysis of faecal samples

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 oxidationï 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).

Statistical analysis

Data were analysed using the GLM procedure (SAS System, Version 9.2; SAS Institute, Inc., Cary, NC). For the performance data, Average Daily Gain (ADG), Average Daily Feed Intake (ADFI) and Gain-to-Feed Ratio (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.

Results

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.

Table 2 ï Analytical composition of tobacco 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

PCR assay, on different clones of O138 *Escherichia coli* strain, confirmed the presence of virulence factors: the VT2e toxin and the F18 adhesive fimbriae genes. PCR products putatively encoding the B-subunit of

VT2e and F18 fimbriae were identified on agarose gel (1.5% - 0.9%) as bands with a length of 270 and 519 basepairs, respectively (fig. 1,2).

Fig. 1: Agarose gel (1,5%) one-dimensional electrophoresis of PCR products for the detection of VT2e-B gene from genomic DNA of O138 *Escherichia coli* challenge strain.
Lane 1: marker 250 pb; lanes 2ï 6: positive samples; lane 7: negative sample from non pathogenic BL21 *Escherichia coli* strain.

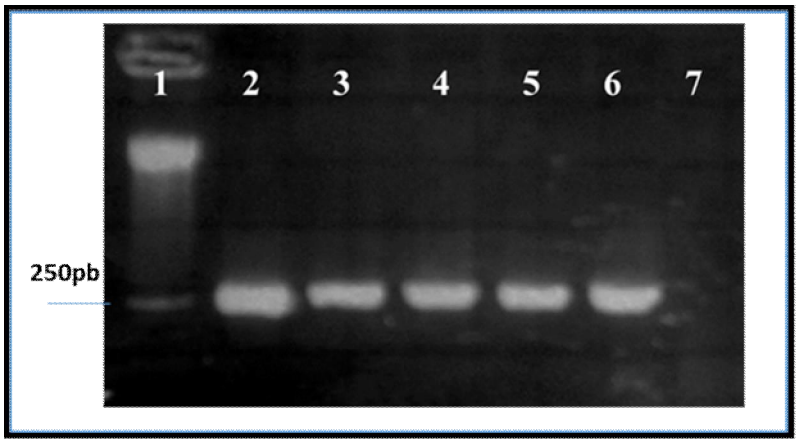
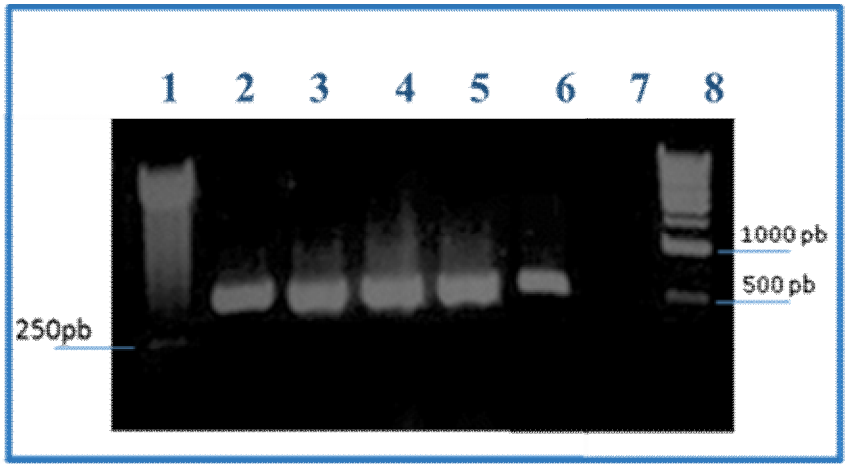


Fig 2: Agarose gel (0.9 %) one-dimensional electrophoresis of PCR products for the detection of F18 gene from genomic DNA of O138 *Escherichia coli* challenge strain.
Lane 1: marker; lanes 2ï 6: positive samples; lane 7: negative control represented by genomic DNA from non pathogenic BL21 *Escherichia coli* strain; lane 8: marker 1kpb.



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).
From the day after challenge, clinical signs related to VTEC infection were observable in infected piglets. Clinical and faecal scores related to VTEC infection, 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.

Table 3 ï 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.

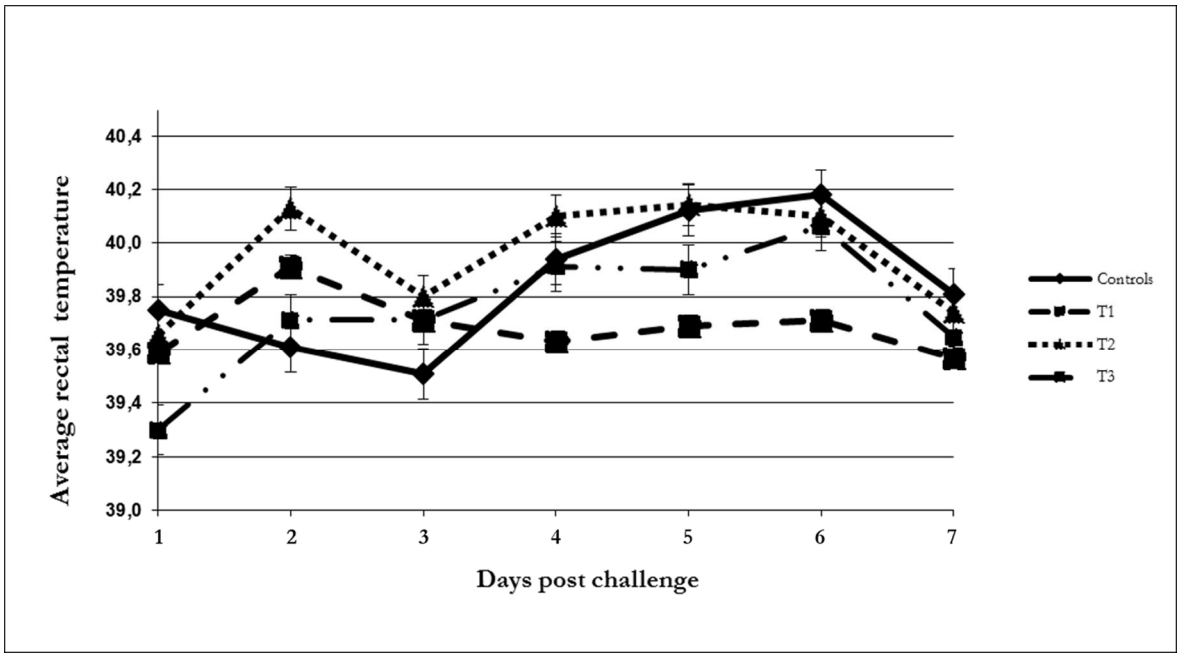
Respiratory score: 0=normal; 1=slightly quick; 2= quick.
Oedemal score in palpebra: 0=normal; 1=mild; 2=severe.
Epiphora score: 0=normal; 1=mild; 2=severe.
Vitality score: 0=good; 1=loose; 2=bad.
Faecal score: 0=normal faeces; 1=soft consistency 2=mild diarrhoea; 3=severe diarrhoea.
Total score is the sum of the average daily scores from day 22 to day 30 (post-challenge period).
One-way ANOVA was used for multiple comparisons. Means in a column without common letters are significantly different (P<0.05)

		Days (post challenge)									**Total score Challenged piglets**	Total score Unchallenged piglets
Scores	**Group**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**		
Respiration	**Contr**	0,6	0,4	0,6	0,7	0,4	0,4	0,7	0,5	0,5	**4,8[a]**	0.2
	T1	0,3	0,2	0,3	0,2	0,1	0,2	0,3	0,3	0,0	**1,9 [b]**	0.1
	T2	0,3	0,1	0,3	0,3	0,3	0,1	0,4	0,4	0,1	**2,4 [b]**	0.2
	T3	0	0,1	0,1	0	0,1	0,3	0,4	0	0,0	**1,0 [b]**	0.1
Palpebral edema	**Contr**	0,6	1,1	1,0	1,0	0,9	1,0	0,6	0,8	1,0	**8,0 [a]**	0
	T1	0,2	0,2	0,4	0,1	0,6	0,4	0,0	0,0	0,0	**1,9 [c]**	0
	T2	1,0	0,6	0,9	0,3	0,0	0,6	0,0	0,3	0,7	**4,3 [b]**	0
	T3	0,8	0,5	0,5	0,3	0,1	0,3	0,1	0,1	0,0	**2,6 [c]**	0.1
Epiphora	**Contr**	0,2	0,4	0,9	1,0	0,5	0,5	0,4	0,8	0,4	**5,1 [a]**	0.3
	T1	0,3	0,3	0,2	0,2	0,2	0,3	0,1	0,2	0,6	**2,4 [b]**	0.1
	T2	0,1	0,4	0,6	0,3	0,0	0,3	0,1	0,1	0,3	**2,3 [b]**	0.1
	T3	0,0	0,3	0,5	0,0	0,0	0,3	0,1	0,0	0,0	**1,1 [b]**	0.8
Vitality	**Contr**	0,9	0,6	0,6	0,8	0,7	0,6	0,4	0,6	0,5	**5,7 [a]**	0
	T1	0,5	0,2	0,0	0,1	0,0	0,1	0,0	0,0	0,0	**0,9 [b]**	0.1
	T2	0,6	0,4	0,1	0,1	0,3	0,4	0,4	0,0	0,1	**2,6 [b]**	0.2
	T3	0,6	0,5	0,4	0,1	0,1	0,1	0,1	0,0	0,0	**1,9 [b]**	0.5
Faecal score	**Contr**	1,1	1,2	1,8	1,3	0,8	0,7	1,1	1,1	0,5	**9,5 [ab]**	7.3
	T1	1,0	1,3	1,0	0,8	0,5	0,4	0,5	0,4	0,3	**6,1 [b]**	4.3
	T2	1,6	2,0	1,9	1,0	1,3	1,6	1,9	1,1	0,7	**13,0 [a]**	6.0
	T3	1,8	1,9	1,9	1,3	1,3	1,4	1,4	1,0	0,6	**12,4 [a]**	3.0

Neurological signs and death were not observed in any pigs throughout the experiment. T1 group presented the lowest values on a daily basis, showing a maximum average daily score (0.6) on day 5 post-challenge. 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 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, $P<0.05$).
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 ($P<0.05$). 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 (fig.4).

Figure 4 ï 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.



However, average temperature and hematocrit percentage did not show significant differences among experimental groups.
Piglets, at the beginning of the experiment (day -5), 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 (table 4).

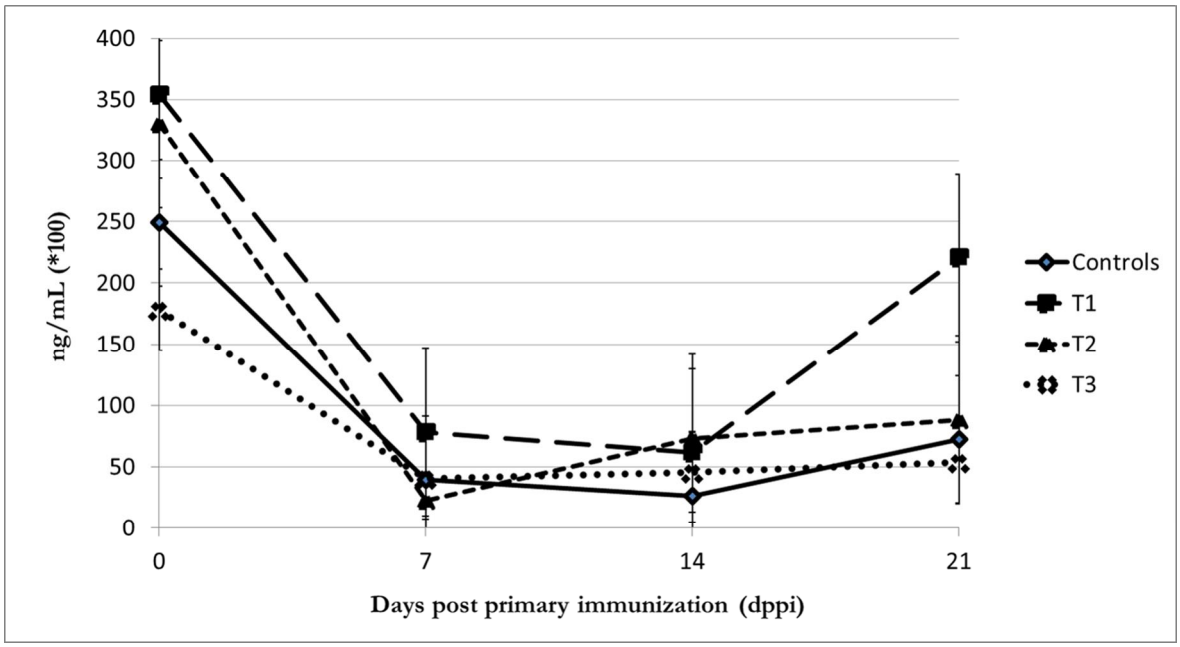
Table 4 ï Growth performance of experimental groups after E. coli infection. VTEC challenge was performed on day 21.T1: piglets orally administered 10g F18TS and 10g VT2e-BTS; T2: piglets orally administered 10g of VT2eBTS; T3: piglets orally administered 25g of VT2eBTS; Control: piglets receiving wild type tobacco seeds.Items were described in challenged (+) and unchallenged (-)animals.Values with different superscript letters in the same row indicate significant difference (P<0.05).BW: body weight; ADG: average daily gain; ADFI: average feed intake; G:F: Gain-to-Feed Ratio.

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 average daily gain (ADG) and average dialy feed intake (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).
In the first post-challenge period (21-25dppa) infected piglets in all experimental groups showed a reduced average daily gain 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.
The performance of T1 not infected group (Average Daily Feed Intake 21-28: 497 g; Average daily gain 21-28: 357±21 g; Gain-to-Feed Ratio 21-28: 0.72) were very similar to those of T1 infected 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.

Item	Control	T1	T2	T3	P	Challenge
BW						
day 21, kg	11.79±0.871	13.39±0.571	14.61±0.442	12.09±0.619	-	+
day 28, kg	13.62±1.20	16.08±0.543	16.54±0.858	13.93±0.903	-	+
day 21, kg	14.53±1.87	13.45±2.26	10.30±1.70	14.40±3.61		-
day 28, kg	17.75±3.46	15.95±2.05	12.80±1.84	18.55±4.60		-
ADG, g						
d 21 to 25	131±64	228±64	214±76	128±72	0.613	+
d 25 to28	433±104	593±104	355±124	442±116	0.496	+
d 21 to 28	261±55	384±55	274±66	263±61	0.352	+
d 21 to 25	356±293	300±50	150±12	481±18	-	-
d 25 to28	533±16	433±16	633±50	742±208	-	-
d 21 to 28	432±160	357±21	357±14	593±99	-	-
ADFI, g						
d 21 to 25	390±22[a]	463±24[b]	487±26[b]	416±24[ab]	0.31	+
d 25 to28	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	+
d 21 to 25	501± 32	432±45	434±27	625±24	-	-
d 25 to28	681±39	607±39	563±35	826±40	-	-
d 21 to 28	568±34	497±42	483±33	701±35	-	-
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±0.9	0.466	+
d 21 to 25	0,71±0.08	0,69±0.21	0,35±0.12	0,77±0.42	-	-
d 25 to 28	0,78±0.7	0,71±0.16	1,12±0.13	0,90±0.11	-	-
d 21 to 28	0,76±01	0,72±0.09	0,74±0.23	0,85±0.13	-	-

Overall, the T1 challenged group, that showed a better clinical presentation after challenge, showed also the best performance data, considering Average Daily Gain (ADG), Average Daily Feed Intake (ADFI) and Gain-to-Feed Ratio (G:F), among the infected groups in the post-challenge period (21-28 dppi).
From the moment of primary oral administration (0 dppi) to 7 dppi a decline of total IgA levels was observed. One week following the fourth administration (booster immunization), the day before challenge (21 dppi), mean IgA faecal levels were increased and were higher in the T1 group, compared to the others (22,000±13,000 ng/ml *vs* control group: 7,200±3,000 ng/ml) (fig.3).

Figure 3 ï 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.



With regard to IgG levels in the faeces, statistically significant differences among experimental groups were not detected. 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).
Total IgA and IgG serum levels monitored in the pre-challenge period showed no significant differences among experimental groups.

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.

Discussion and Conclusions

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 ógrowth checkô, 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. VTEC strains are important causes of diarrhoea, growth retardation and mortality in piglets during the first weeks after weaning. Up to now, no vaccine protecting piglets against these infections is available and treatment relies upon the use of antimicrobials. Before 2006, antibiotic growth-promoters 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.

This study was focused on the evaluation of a protective effect of oral administration transgenic tobacco seeds expressing antigenic proteins from verocitoxic *E.coli* strain, was to set-up experimental immunization. The main problem related to the oral administration of antigens is the degradation in the gastrointestinal tract. The production of antigens in seeds 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 igested (Bailey, 2000). Neverthless the oral administration of whole transgenic tobacco seeds in newly-weaned piglets raises doubts about an effective exposure of the antigen to the sites of activation of the mucosal immune system. So according to a previous study (Rossi et al., 2011) the use of milled transgenic tobacco seeds would appear more appropriate for animals with immature digestive capacity.

Moreover oral delivery of antigens can 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. In the immunisation phase, the amount of antigen used for oral administration 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). 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: FedA, the major protein of the F18 adhesive 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.

O138 *Escherichia coli* challenge strain showed important virulence characteristics, represented by F18 adhesive fimbriae gene and verocitoxin gene, confirmed by PCR analyses. Neverthless *Escherichia coli* infection are multifactorial disorders and experimental infection with *E. coli* alone is not sufficient to reproduce the syndrome as observed in the field (Rossi et al., 2012). In this study, factors predisposing the piglets to the clinical signs were introduced into the experimental challenge model, including an iperproteic diet and 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 ñhigh-riskò 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 *Escherichia coli* diseases.

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).

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

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 FedA). This immunization strategy appeared more effective in preventing the development of clinical signs after challenge with O138 *E. coli* strain.

As expected, 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 (Average Daily Gain, Average Daily Feed Intake and Gain-to-Feed Ratio) 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 average daily gain and feed intake of the group orally immunized with both VT2eB and FedA antigens.
The use of the multicomponent (T1) vaccine again appeared more effective in preventing the negative impact of challenge with O138 *E. coli* strain. 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.
In our experiment (Figure 4), from the moment of primary oral administration (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). 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). 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 specific, but also aspecific, local immune response activity in this group (Fritz at al. , 2012). Althought 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 we didnq observe differences statistical significant.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). In conclusion, the results of this study suggest that oral administration of transgenic tobacco seeds expressing FedA,the major protein of the F18 adhesive fimbriae, 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. Transformed tobacco plants for seed-specific expression of genes encoding FedA protein and VT2e-B subunit of shiga-like toxin may represent a promising non-invasive method of vaccinating swine via their feed.

Aknowledgments
Acknowledgments and Special Thanks thanks Prof. Corrado Fogher for his ideas and essential support. We also wish to thank our technicians, in particular Antonio Crotti and Diego De Nisi, for their valuable contribution in field and in laboratory.

References

Aarestrup FM, Duran CO, Burch DGS (2008) Antimicrobial resistance in swine production. Animal Health Res Rev 9(2):135-148

Bailey M, Plunkett FJ, Rothkotter HJ, Vega-Lopez MA, Haverson K, Stokes CR (2001). Regulation of mucosal immune responses in effector sites. Proc Nutr Soc 60: 427ï 435

Bertschinger HU, Gyles CL (1994) Oedema disease in pigs. In C. L. Gyles (ed.), *Escherichia coli* in domestic animals and humans. CAB Intern, Wallingford, Oxon, United Kingdom: 193ï 219

Bianchi AT, Scholten JW, Moonen Leusen BH, Boersma WJ (1999) Development of the natural response of immunoglobulin secreting cells in the pig as a function of organ, age and housing. Develop Comp Immunol 23: 511ï 520

Bosworth BT, Samuel JE, Moon HW, O'Brien AD, Gordon VM, Whipp SC (1996) Vaccination with genetically modified Shiga-like toxin IIe prevents edema disease in swine. *Infec Imm 64(1): 55-60*

Butler JE, Lager KM, Splichal I, Francis D, Kacskovics I, Sinkora M, Wertz N, Sun J, Zhao Y, Brown WR, DeWald R, Dierks S, Muyldermans S, Lunney JK, McCray PB, Rogers CS, Welsh MJ, Navarro P, Klobasa F, Habe F, Ramsoondar J (2009) The piglet as a model for B cell and immune system development. Vet Immun 128(1/3):147-170

Chen X, Gao S, Jiao X, Liu X (2004. Prevalence of serogroups and virulence factors of *Escherichia coli* strains isolated from pigs with postweaning diarrhoea in eastern China. Vet Microb 103(1/2):13-20

Coddens A, Diswall M, Angström J, Breimer ME, Goddeeris B, Cox E, Teneberg S (2009) Recognition of blood group ABH type 1 determinants by the FedF adhesin of F18-fimbriated *Escherichia coli*. J boil Chem 284: 9713ï 9726

Deng Z, Zhang J, Wu G, Yin Y, Ruan Z, Li T, Chu W, Kong X, Zhang Y, Fan Y, Liu R, Huang R (2007) Dietary supplementation with polysaccharides from Semen cassiae enhances immunoglobulin production and interleukin gene expression in early-weaned piglets. J Sci Food and Agric 87(10): 1868-1873

Franz J, Corthier G (1981) Measurement of porcine faecal IgA, IgG and IgM levels by a competitive enzyme-linked immunosorbent assay. Cli Exper Immunol, 43(3): 645-649

Frenyo VL, Pethes G, Antal T, Szabo I (1981) Changes in colostral and serum IgG content in swine in relation to time. Vet Res Comm 4(4): 275-282

Fritz JH, Rojas OL, Simard N, McCarthy DD, Hapfelmeier S, Rubino S, Robertson SJ, Larijani M, Gosselin J, Ivanov I, Martin A, Casellas R, Philpott DJ, Girardin SE, McCoy KD, Macpherson AJ, Paige CJ, Gommerman JL (2012) Acquisition of a multifunctional IgA+ plasma cell phenotype in the gut. Nature 481:199-205

Gannon VPJ, Gyles CL, Friendship RM (1988) Characteristics of verotoxigenic *Escherichia coli* from pigs. Canadian J Vet Res 52(3): 331-337

Gannon VPJ, Gyles CL (1989). Characteristic of Shiga-like toxin produced by *Escherichia coli* associated with porcine edema disease. Vet Microbiol 24: 89ï 100

Imberechts H, Greve H, Lintermans P (1992) The pathogenesis of edema disease in pigs. A review. Vet Microbiol 31(2-3): 221-233.

Jensen GM, Frydendahlb K, Svendsen O, Jorgensen CB, Cirerac S, Fredholm M, Nielsen JP, Moller K (2006). Experimental infection with *Escherichia coli* 0149: F4ac in weaned piglets. Vet Microbiol 115:243-249.

Lamphear B , Streatfield SJ, Jilka JM, Brooks CA, Barker DK, Turner DD, Delaney DE, Garcia M, Wiggins B, Woodard SL, Hood EE, Tizard IR, Lawhorn B, Howard JA (2002) Delivery of subunit vaccines in maize seed. J Control Rel 85(1/3): 169-180

MacLeod DL, Gyles CL, Wilcock BP (1991) Reproduction of edema disease of swine with purified Shiga-like toxin-II variant. Vet Pathol 28(1): 66-73

Madec F, Bridoux N, Bounaix S, Cariolet R, Duval-Iflah Y, Hampson DJ, Jestin A (2000) Experimental models of porcine post-weaning colibacillosis and their relationship to post-weaning diarrhoea and digestive disorders as encountered in the field. Vet Microbiol 72: 295-310

Nagy B, Fekete P (2005) Enterotoxigenic *Escherichia coli* in veterinary medicine. Int J Med Microbiol 295:443-454

Pinotti L, Rossi L, Rebucci R, Dellô Orto V, Baldi A (2003) The safety of GM crops. Evaluation criteria (part 1).Tecnica Molitoria 38-45

Reggi S, Marchetti S, Patti T, De Amicis F, Cariati R, Bembi B, Fogher C (2005) Recombinant human acid beta-glucosidase stored in tobacco seed is stable, active and taken up by human fibroblasts. Plant Molecul Biol 57(1): 101-13

Rippinger P, Bertschinger HU, Imberchts H. Nagy B. Sorg I. Stamm M. Wild P, Witting W (1995). Designations of F18ab and F18ac for related fimbrial types F107, 2134P and 8813 of *Escherichia coli* isolated from porcine post-weaning diarrhoea and from oedema disease. Vet Microbiol 45, 281ï 295

Rossi L, Baldi A, Dell'Orto V, Fogher C (2003a). Antigenic recombinant proteins expressed in tobacco seeds as a model for edible vaccines against swine oedema. Veterinary Research Communications, 27 (Suppl. 1): 659-61

Rossi L, Di Giancamillo A, Reggi S, Domeneghini C, Baldi A, Sala V, Dellô Orto V, Coddens A, Cox E, Fogher C (2013a). Expression of porcine verocytotoxic Escherichia coli antigens in tobacco seeds and evaluation of gut immunity after oral administration in mouse model J Vet Sci 14(3), 263-270.

Rossi L, Fusi E, Baldi G, Fogher C, Cheli F, Baldi A, Dellô Orto V (2013b) Tobacco Seeds By-Product as Protein Source for Piglets. Open J Vet Med 3, 73-78.

Rossi L, Reggi S, Di Giancamillo A, Domeneghini C, Pinotti L, Fogher C, Baldi A (2003b). Oral administration of tobacco seeds expressing antigenic proteins in mice Balb-C: a model of edible vaccines for oedema disease. Italian Journal of Animal Science, 2 (Suppl.1): 7-9.

Rossi L, Reggi S, Vagni S, Fogher C, Baldi A (2011) Evaluation of gastric degradability of antigenic protein expressed in tobacco seeds. IJAS s1: 19

Rossi L, Selmini G, Cheli F, Fusi E, Fogher C (2007) Potential use of tobacco seed cake in piglets diet. Large Animal Rev 13(5):211-215

Rossi L, Vagni S, Polidori C, Alborali GL, Baldi A, Dellô Orto V (2012) Experimental induction of *Escherichia coli* diarrhoea in weaned piglet. OJVM 2: 1-8

Ruggenti P, Remuzzi G (2012) Thrombotic microangiopathy: *E. coli* O104:H4 German outbreak: a missed opportunity. Nature Rev Nephrol 8, 558-560

Sarrazin E and Bertshinger HU (1997) Role of fimbriae F18 for actively acquired immunity against porcine enterotoxigenic *Escherichia coli*. Vet Microbiol, 54: 133ï 144

Snoeck V, Cox E, Verdonck F, Joensuu JJ, Goddeeris BM (2004) Influence of porcine intestinal pH and gastric digestion on antigenicity of F4 fimbriae for oral immunisation. Vet Microbiol 98(1): 45-53

Snoeck V, Peters IR, Cox E (2006) The IgA system: a comparison of structure and function in different species. Vet Res 37: 455ï 467

Streatfield SJ, Jilka JM, Hood EE, Turner DD, Bailey MR, Mayor JM, Woodard SL, Beifuss KK, Horn ME, Delaney DE, Tizard IR, Howard JA (2001) Plant-based vaccines: unique advantages. Vaccine 19(17/19): 2742-2748

Streatfield SJ (2006) Mucosal immunization using recombinant plant-based oral vaccines. Methods, 38: 150-157

Thompson CL, Wang B, Holmes AJ (2008) The immediate environment during postnatal development has long-term impact on gut community structure in pigs. ISME J 2:739ï 748

Ushida K, Kameue C, Tsukahara T, Fukuta K, Nakanishi N (2008) Decreasing traits of fecal immunoglobulin A in neonatal and weaning piglets. J Vet Med Sci 70(8): 849-852

Verdonck F, Tiels P, Gog KV, Goddeeris BM, Lycke N, Clements J, Cox E (2007) Mucosal immunization of piglets with purified F18 fimbriae does not protect against F18 +*Escherichia coli* infection. Vet Immunol Immunopat 120(3/4): 69-79

Widgorovitz A, Carrillo C, Dus Santos MJ, Trono K, Peralta A, Gómez MC, Ríos RD, Franzone PM, Sadir AM, Escribano JM, Borca MV (1999) Induction of a protective antibody response to foot and mouth disease virus in mice following oral or parenteral immunization with alfalfa transgenic plants expressing the viral structural protein VP1. Virol 255, 347ï 353