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Evaluation of the prophylactic potential of non-enterotoxigenic *Escherichia coli* (non-ETEC) vaccine immunization and dietary mannan oligosaccharide competitive exclusion benefits against ETEC infections in weaned pigs

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) strains expressing F4 and F18 fimbriae are the most common causative agents of post-weaning diarrhoea (PWD) in pigs. The growing global restriction on the use of antibiotics in food animals has encouraged research into the development of nutritional and feeding strategies as well as vaccination against PWD. The aim of this study was to evaluate the efficacy of a live oral F4ac$^+$F18ac$^+$ non-ETEC vaccine candidate (VAC) to stimulate gut and systemic cellular immunity in 4-week old pigs over 5 weeks following immunization. The

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onset and duration of protective immunity against on-farm occurring PWD, growth performance, diarrhoea scoring and mortality, as well as the phenotypic proportions of immune cells, were determined. Faecal and ileal samples were taken for determining the microbial composition or phenotyping of naïve/memory T cells. Also, the effect of prebiotic supplement mannan oligosaccharide (MOS) in the prevention of small intestinal colonization by ETEC, and its potential adjuvanticity in combination with the vaccine (VAC+MOS) were assessed. The pigs supplemented with MOS or that received VAC had significantly higher body weight (BW) ($P<0.05$) on Day 14, whereas the VAC+MOS treated pigs had significantly lower BW on Day 35. Treatment with VAC+MOS resulted in considerably reduced clinical PWD, in particular the incidence and severity of diarrhoea and mortality. The total bacterial load in the ileum was much lower in the pigs from all 3 principal groups (MOS, VAC, and VAC+MOS) than in the control (CON) group (19×10^7, 17×10^7 and 12×10^7 *vs*. 23×10^8 CFU/mL, respectively) on Day 35. The pigs from the principal groups had significantly higher proportions of tested immune cells ($P<0.05$) on Days 28 and 35. The localization and frequency of naive $CD45RA^+$ and memory $CD45RC^+$ T lymphocytes indicated their different distribution patterns within particular tissue structures, such as the villi, crypts, epithelium, *lamina propria* and areas (interfollicular follicular and Peyer's patches) of ileal mucosa. This may indicate their different functions in intestinal immune responses to intraluminal microbes and their products, vaccinal immunogens and/or immunomodulators/adjuvants. To conclude, active mucosal immunity is needed to protect pigs against PWD. Hence, oral vaccination of pigs against both F4 and F18 ETEC, in combination with prebiotic supplementation represents a sustainable, practical and effective approach in PWD control.

Key words: immunization; *Escherichia coli;* competitive exclusion; mannan oligosaccharide; pigs

Introduction

The largest part of the immune system of animals, including swine, is in the gut. This part is organized as the gut associated lymphoid tissues (GALT), comprising dispersed and aggregated lymphoid and myeloid cells residing in the intestinal mucosa barrier between the organism and the external environment, primarily intraluminal content. These cells are generally divided into functional compartments known as inductive or effector sites. The inductive compartment of the GALT is comprised of the appendix, isolated lymphoid follicles, and Peyer's patches (PP), whereas the *lamina propria* (LP) is generally considered an effector site within the GALT. As such, the GALT provides specific host defence, and encompasses the largest collection of immune cells in the body (BURKEY et al., 2009).The gut must fulfil its primary absorptive function of nutrients, while the GALT must simultaneously effectively discriminate and respond appropriately to a diverse environment of harmful enteric pathogens and their products, but also tolerate harmless dietary antigens or antigens from commensal microbiota. Namely, the mucosal surfaces of the GALT establish a complex cooperation with the intestinal lumen and its contents, which is equally important in order to achieve a homeostatic balance between immune tolerance and immune responsiveness (BAILEY, 2009). Additionally, during the critical periods of birth and weaning, the intestinal $CD4^+$ T cells predominantly residing in the LP of pigs express isoforms of CD45, *i. e.* $CD45RA^+$ and $CD45RC^+$ of either naïve or memory phenotype, which is consistent with antigen recognition and immunological memory, respectively (HAVERSON et al., 1999). Moreover, recent data on the immunohistological and histomorphometrical patterns of the localization/distribution and quantification of both naïve $CD45RA^+$ and memory $CD45RC^+$ T and B cells in the jejunal and ileal mucosa of weaned pigs, intragastrically (*i. g.)* immunized with levamisole adjuvanted vaccine candidate $F4ac^+$ or $F18ac^+$ non-ETEC strains, have demonstrated their key roles when activated with foreign immunogens, resulting in their highly increased numbers shortly following vaccination against a challenge $F4ac^+$ ETEC strain, the causative agent of postweaning diarrhea (PWD) in swine (KOVŠCA JANJATOVIĆ et al., 2009; KOVŠCA JANJATOVIĆ et al., 2010).

PWD is considered to be one of the economically most important diseases in swine husbandry (MELKEBEEK et al., 2013). Enterotoxigenic *Escherichia coli* (ETEC) strains are the main

cause of PWD. The key virulence factors of ETEC in PWD are bacterial fimbrial adhesions and enterotoxins. Adhesions mediate ETEC bacteria's initial attachment to a pig's gut epithelial cells, and subsequent colonization in the pig's small intestines. Enterotoxins, including heat-labile toxin (LT) and heat-stable toxin (ST), disrupt fluid homeostasis in small intestinal epithelial cells, to cause electrolyte-rich fluid hyper-secretion and diarrhoea. Currently there are no effective prevention measures to protect weaned pigs against PWD. Vaccination would be the most practical and effective prevention approach, and vaccines inducing anti-adhesion immunity to block ETEC attachment and colonization, and also antitoxin immunity to neutralize enterotoxicity, are considered optimal against ETEC-associated PWD (ZHANG, 2014). Although progress has been made in past decades in developing effective vaccines against PWD, challenges continue to exist due to the disease complexity and the immunological heterogeneity among ETEC strains. Recent progress in using safe toxoid antigens, toxoid fusion antigens and an MEFA (multiepitope fusion antigen) approach to developing multivalent vaccines for broad protection, however, shows promise for developing new vaccines for effective protection against ETEC-associated PWD (DUAN et al., 2020). Pigs commonly develop diarrhoea 3-10 days after they are weaned. PWD continues to be one of the most important swine diseases which causes death occurring in suckling and weaned pigs worldwide (FAIRBROTHER et al., 2005; LUPPI, 2017). PWD is caused by pathogenic bacteria and viruses, including enterotoxigenic *Escherichia coli* (ETEC), corona viruses (both transmissible gastroenteritis and porcine epidemic diarrhoea virus), and rotaviruses, but ETEC strains are the predominant cause of PWD (ZHANG, 2014). PWD results in weight loss, slow growth and acute death in recently weaned pigs, and this causes substantial economic losses to swine producers worldwide (NAGY and FEKETE, 2005). Currently, there are no effective prevention strategies available to protect against PWD. Oral administration of specific-antibody-containing egg yolk, sow milk or plasma proteins, treatment with dietary or diet supplementary probiotics have been attempted. But these treatments are less or not effective, or not economically practical. Prophylactic treatment with antibiotics may relieve disease burden (HEO et al., 2013), but excessive use of antibiotics is linked to an increase in antimicrobial resistance, and potentially poses a threat to public health and the environment (SMITH et al., 2010). On the other hand, implementation of a ban on the use of antibiotic growth promoters in the European Union (since 2006) and the USA (since 2017) has initiated an increase of PWD outbreaks (ZHANG, 2014). Although a few vaccine products have acquired licenses in a few countries, there are no vaccines currently available to effectively protect against PWD (RUAN et al., 2011; MELKEBEEK et al., 2013). In general, to protect newly weaned pigs, an active intestinal immune response is required, in which the production of antigen-specific secretory IgA plays an important role. The vaccination strategies applied against PWD include oral subunit vaccines based on the concept of fimbriae as colonization factors with a crucial role in the occurrence of ETEC infections (VAN den BROCK et al., 1999). Improvement of the stability and efficacy of this approach opens new possibilities in the development of a combined vaccine against both F4 and F18 fimbrial antigens (VALPOTIĆ et al., 1994; VIJTIUK et al., 2005; BOŽIĆ et al., 2006; KOVŠCA JANJATOVIĆ et al., 2009; KOVŠCA JANJATOVIĆ et al., 2010; FAIRBROTHER et al., 2017). However, these ETEC vaccines are of the live attenuated or the live wild type (comprising non-ETEC and non-pathogenic avirulent *E. coli* strains) for oral delivery. A tripartite fusion protein, EaeG-FedF-LT (192) A2:B of ETEC elicited IgA antibodies in serum, faeces and intestinal washes that neutralize cholera toxin, inhibit adherence of F4 and F18 fimbriae, and protect pigs against clinical signs of PWD after an $F4^+$ ETEC infection (RUAN et al., 2011). The use of fusion protein antigens might be an interesting strategy towards the development of a multivalent vaccine against ETEC-induced PWD (MELKEBEEK et al., 2013). However, such systemic immunization and the duration of the induced serum IgA response should be approached with care, especially when interpreting the results of these parenteral studies.

In order to modulate an intestinal mucosal secretory IgA response, rather than a systemic one, the induction has been performed of specific mucosal immune responses through oral immunization of pigs with a live attenuated ETEC strain expressing multivalent fusion protein antigens (RUAN and ZHANG, 2013), and has protected weaned pigs against challenge ETEC infection. Moreover, a multi epitope fusion antigen (MEFA) strategy has been developed, and a single MEFA antigen successfully constructed expressed by an *E. coli* strain to induce broad immunity and protection against PWD (RUAN et al., 2014).

The growing global concern regarding the increasing risk of antimicrobial resistance, their misuse in food producing animals and their environmental impact, has encouraged researchers to investigate potential alternatives, particularly the development of nutritional and feeding strategies, as well as vaccination protocols, in order to combat PWD due to ETEC infection (LUISE et al., 2019). These strategies also include the use of direct-fed microbial blends containing live and naturally occurring porcine microorganisms developed from the intestinal contents of a healthy pig, comprising single or multiple bacterial strains, for the protection of weaned pigs from an F18 ETEC challenge infection (BECKER et al., 2020). The numerous papers reporting nutritional interventions using a vast variety of natural products as feed additives (SUN and KIM, 2017), include brewer's dried yeast as a source of mannan oligosaccharide (MOS), a prebiotic which also may act as an enteropathogen antiadhesive (WHITE et al., 2002). More recent studies recorded that MOS may selectively stimulate proliferation and the activities of gut-healthy bacteria, significantly improve immunity and intestinal morphology, and reduce the frequency, severity and duration of F4 ETEC infection, and thus indirectly help prevent PWD and promote the growth/health of weaned pigs (NOCHTA et al., 2009; VALPOTIĆ et al., 2016; VALPOTIĆ et al., 2017; VALPOTIĆ et al., 2018). Among the potential alternatives to antimicrobial growth promoters (AGP), MOS has received particular attention (AGAZZI et al., 2020). Via the modulation of gut microbiota, MOS can also improve some morpho-functional aspects of the gut. Despite formally belonging to the class of prebiotics, *i.e.,* non-digestible feed ingredients that promote the growth of beneficial microbiota in the intestines, the mode of action of MOS differs slightly from the other class compounds (HALAS and NOCHTA, 2012). Namely, MOS can modify the microbiota mass and composition through an indirect mechanism known as competitive exclusion (HARVEY et al., 2005), rather than acting as a direct nutrient for the intestinal microbial populations. Specifically, MOS is able to prevent the adhesion of pathogenic bacteria to intestinal epithelial cells by attachment to the mannose-binding proteins expressed on the bacterial fimbriae, including ETEC strains.

The present study was designed to evaluate: (i) the efficacy of a live oral bivalent F4ac$^+$/F18ac$^+$ non-ETEC vaccine candidate to stimulate intestinal and systemic cellular immunity in 4-week-old just-weaned pigs, (ii) the incidence, severity, duration and mortality due to on-farm occurring PWD over 5 weeks following weaning, and, (iii) the growth kinetics of the pigs. Also, the design of the study assesses the potential benefits of prebiotic MOS supplement that may provide (iv) competitive exclusion of the colonization of the small intestine by F4ac$^+$ F18ac$^+$ ETEC, and (v) may exhibit adjuvanticity for the vaccine candidate tested.

Materials and methods

Pigs, housing and feeding. Sixty-four crossbred pigs (Swedish Landrace x Large White x Belgian Pietrain), castrates and females were used, weighing approximately 8.4 kg, the progeny of five litters from 3rd parity sows on a commercial swine farm (Lužani, "Žito", Osijek) in eastern Croatia. The pigs were weaned at 26 days of age, housed, managed and fed a standard weaner diet (without antimicrobials or growth promoters) according to the rearing technology of the farm. Two days after weaning at 28 days of age, the weaners were randomly divided into four groups comprising 16 pigs each, ear-tagged with numbers 1-16 and kept in separate pens in the same rearing facility of the farm, as detailed earlier (VALPOTIĆ et al., 2014). The experiment was conducted throughout a period of 35 days. The animals used in this experiment were

maintained in facilities approved by the Croatian Association for Accreditation of Laboratory Animal Care, and in accordance with the current regulations and standards issued by the Ministry of Agriculture of Croatia. Experimental and animal management procedures were conducted in accordance with the "Directive for the Protection of Vertebrate Animals used for Experimental and other Purposes" (86/609/EEC). The pigs were fed a standard corn-soybean meal diet, formulated as Phase 1 (from Day 0 to Day 21) and Phase 2 (from Day 22 to Day 35 of the experiment) diets, to meet their nutrient requirements (VALPOTIĆ et al., 2016).

Vaccine candidate strains. The recombinant nontoxigenic F4ac$^+$ vaccine candidate strain 2407 (O9: K36: H19: F4ac:LT$^-$ STb) was attenuated, as detailed earlier (CASEY and MOON, 1990). The vaccine candidate F18ac$^+$ non-ETEC strain 2143 (O157: K119: F18ac) was attenuated by a slightly modified culturing procedure, as briefly described earlier (KOVŠCA JANJATOVIĆ et al., 2009), to reduce its toxicity, as previously suggested (GORDON et al., 1992.) Both strains were kindly donated, as acknowledged more recently (KOVŠCA JANJATOVIĆ et al., 2011) and kept in glycerol broth at –80 °C until used.

Experimental design. At 28 days of age (or Day 0 of the experiment) the pigs were treated as follows: the control group of pigs (CONTROLS) received 60 mL of Trypticase soya broth (TSB) by intragastrical route (*i. g*.) as a placebo. The diet for the pigs from the first principal group (MOS) was supplemented with 0.2% of MOS (Bio-Mos®, Alltech, Nicholasville, KY, USA), whereas the diet for the pigs from the second principal group (VACCINE) that were *i. g*. immunized with 10^{10} colony forming units (CFU) mL of F4ac$^+$ F18ac$^+$ non-ETEC vaccine candidate, in 60 mL of TSB, was not supplemented with MOS. The third group of principal pigs (VACCINE + MOS) received bivalent non- ETEC vaccine *i. g.* and were fed a diet supplemented with MOS. The experiment was conducted over a period of 35 days (or until 63 days of age), and the pigs were monitored daily and weighed/sampled (for peripheral blood samples and rectal swabs/intestinal content) at seven-day intervals, starting on Day 0 before the treatments.

Growth changes. The pigs were weighed at weekly intervals during the experiment, and changes in their body weight were recorded. The changes in body weight within the treated groups of pigs were calculated on the basis of differences between either body weight at the beginning of the experiment (Day 0 = 100% of body mass) or average group body weight on Days 7, 14, 28 and 35 of the experiment, in comparison to average body weight of the pigs from the non-treated CONTROLS.

Diarrhoea scoring and mortality rate. The pigs were monitored daily for diarrhoea and/or other clinical signs of gut disorders (such as anorexia and weight loss), and the incidence and severity of diarrhoea were recorded. The severity of diarrhoea was scored as follows: 0 = normal faeces, 1 = soft faeces, 2 = fluid faeces, and 3 = projectile diarrhoea. Pigs with scores of either 2 or 3 were defined as diarrheic. After collection, we summarized the obtained data and calculated a diarrhoea severity score (DSS), which represented the sum of diarrhoea severity over the course of the 35 days of the experiment. Apart from morbidity due to diarrheal disease, other mortality was also monitored, and dead pigs were necropsied and examined for gross pathology changes.

Bacterial faecal shedding and intestinal colonization. In order to monitor gut health, we also determined the bacterial species/serovars isolated from rectal swabs and ileal content. The rectal swabs (r. s.) were taken from all the pigs per group on Days 0, 14 and 35 of the experiment, for isolation and serotyping of the enteric bacteria. On either Day 0 or Day 35 of the experiment two pigs per group were euthanized by intracardial injection of 0.3 mL/kg of T61 preparation (Hoechst®, München, Germany), and sampled for bacteriology and immunohistology. Immediately following euthanasia, a 10 cm segment of the mid ileum, with digestive content, was first ligated and then taken for counting of intraluminal bacteria. For determination of the total number of *E. coli* cells in 1 mL of the ileal content (CFU/mL) the samples were diluted in serial dilutions up to 10^{10} in saline, and each dilution was plated onto selected culturing plates (WINN et al., 2006). In order to isolate and count *E. coli* bacteria, 1 mL of each dilution was added into two

Petri dishes, into which Tryptone-bile glucuronic medium was poured (TBX; contains bile salts No. 3 and 5-bromo-4-chloro-3-indolil β-d-glucuronic acid (BCIG)). Each serial dilution was plated in duplicate, and after 24 h of incubation at 37 °C the grown colonies were counted on an automatic computer-assisted counter, and the number of CFU per mL calculated as detailed earlier (VALPOTIĆ et al., 2014). For identification and serotyping, one loop (0.1 mL) of the ileal content was plated onto a blood agar base with 5% of defibrinated sheep blood (Blood Agar Base No. 2 OXOID CM 271) and XDL agar. The most common fimbrial antigens of *E. coli* F4, F5, F6 and F18 were serotyped using Minca medium by rapid slide agglutination with specific commercial antisera (Denka Seiken, Japan). The haemolytic isolates of *E. coli* were identified by plating the ileal content onto TSB with 5% of defibrinated sheep blood with esculine. The identification of the haemolytic isolates of *E. coli* bacteria from the r. s. and their further serotyping was performed using the same procedure as for those isolated from the ileal content (VALPOTIĆ et al., 2014).

Analyses of peripheral blood immune cell proportion kinetics. At the same weekly intervals blood samples (1 mL) were taken from 7 of 16 pigs from each group (ear-tagged with numbers 1–7) from the *vv. cava cranialis* into glass tubes (Beckton Dickinson Plymouth, UK), with ethylenediaminetetraacetic acid (EDTA) as the anticoagulant (Sigma, St. Louis, USA) for flow cytometry (FCM) analyses. The monoclonal antibodies (mAbs) reactive with swine leukocyte surface molecules, *i.e*. the cluster of differentiation (CD) antigens and fluorescent dye conjugates that we used for identification/quantification of total $CD45^+$ lymphoid cells, as well as for $CD4^+$ and $CD8^+$ T or $CD21^+$ B cell subsets have already been described (VALPOTIĆ et al., 2014; ANDRIŠIĆ et al., 2020). Single cell suspensions (100 µL) were prepared in triplicate (comprising 10,000 cells each), incubated with mAbs (50 µL), and processed as previously described (BOŽIĆ et al. 2002). The fluorescence of the mAb-labelled porcine lymphoid cells was quantified using a Coulter EPICS-XL flow cytometer (Beckman Coulter Miami FL, USA), as detailed earlier (VALPOTIĆ et al., 1994). The isotype-matched mouse immunoglobulins were used to detect nonspecific fluorescence in the control cell suspensions.

Immunohistological analyses of ileal $CD45RA^+$ and $CD45RC^+$ T cells. Immediately following euthanasia on either Day 0 or Day 35 of the experiment, five specimens taken from the mid ileum of each of two pigs per group (either 4-5 cm from 4-week-old pigs or 7-8 cm from 9-week-old pigs, respectively), were fixed in 10% neutral-buffered formalin (pH 7.0 and 7.6) for 24 h until used for immunohistology analysis. After fixation, the ileum specimens were dehydrated, embedded in paraplast (Sigma, Sherwood Medical Industries, Saint Louis, MO, USA), cut into 5 µm thick serial sections, and processed for standard hemalaun (Meyer's solution; Kemika, Zagreb, Croatia) and eosin staining. These sections were examined by light microscope (Leitz, Orthoplan, Germany) to identify gut mucosa areas suitable for immunohistological localization/distribution of ileal T cell subsets with naïve ($CD45RA^+$) and memory ($CD45RC^+$) phenotype/function. The paraplast-embedded sections were processed for an indirect immunoperoxidase (IP) method, including staining with the avidin/biotin complex (ABC) technique, as detailed earlier (LACKOVIĆ et al., 1997; VALPOTIĆ et al., 2014). Primary mAbs MIL13 and MIL5 (kindly donated by professors Karin Haverson and Chris Stokes from the School of Veterinary Sciences, University of Bristol, UK) reactive with either porcine $CD45RA^+$ naive or $CD45RC^+$ memory T cells, respectively, and secondary polyclonal Abs conjugated with horseradish peroxidase were used to study *in situ* identification and distribution patterns of these cells residing in the ileal mucosa of weaned pigs, as described previously (VALPOTIĆ et al., 2014). After drying, the sections were examined by light microscope (Eclipse E600, Nikon, Tokyo, Japan) and the areas selected for immunohistological identification/localization of the tested cell subsets were photographed using a digital camera (DMX1200, Nikon, Tokyo, Japan).

Statistical analyses. Statistical analyses of data were performed using the SAS 9.4 software package (Statistical Analysis Software 2002–2012

by SAS Institute Inc., Cary). The GLIMMIX procedure was used with a generalized linear mixed methodology, binomial distribution and logit link function for lymphocytes and leucocytes. The statistical model included the fixed effects of group and period. The effect of each animal on the repeated measurements over time was included in the model with a compound-symmetry structure. Analysis of variance (PROC GLM) was used to analyse the body weight and statistical model, including the fixed effects of group and period. A multiple comparison test of the least-square means with Tukey correction was performed using the SLICE option to compare each group level within the period. The data are presented as mean ± standard error of the mean (SEM), with the distributions shown in their original scales, and the level of statistical significance was set at $P<0.05$.

Results

Weight gain. The pig groups were compared at baseline for bodyweight (Day 0) which was equally distributed between the treatment groups without any statistically significant differences ($P>0.05$), until Day 28 of the experiment, with the exception of Day 14 (Table 1). Namely, on Day 14 the pigs that were either immunized with bivalent live F4ac$^+$ F18ac$^+$ non-ETEC vaccine candidate (VACCINE) or supplemented with dietary MOS had significantly higher body weight gain ($P<0.05$), as compared to the control pigs. However, the pigs from the VACCINE and the VACCINE + MOS groups had significantly lower body weight gain ($P<0.05$) than the CONTROLS at the end of the experiment. Interestingly, the pigs from the MOS group also had significantly higher body weight gain ($P<0.05$) on Day 35 of the experiment.

Clinical and pathological findings. The pigs were monitored daily for general health, behaviour, appetite, body condition, and faecal consistency. Diarrhoea incidence and severity were scored, and gross pathological findings of the cases of PWD were recorded, as already detailed. The group of pigs that were immunized with a single dose of bivalent live F4ac$^+$ F18ac$^+$ non-ETEC vaccine candidate and were fed with MOS supplement (VACCINE + MOS) had less diarrheic pigs than the other two principal groups that received either VACCINE or MOS (1/16 *vs* 2/16 *vs* 3/16 or for 50% and 66.67%), respectively (Table 2). Also, these pigs had much lower incidence of cases of diarrhoea (1/16 *vs* 5/16 or for 66.67%) than the CONTROLS. All three groups of principals had considerably lower mortality rate (1/16 *vs* 3/16 for 66.67%) as compared to the controls. The CONTROL group of pigs had a much higher total DSS (diarrhoea severity score) than the pigs from the VACCINE + MOS group (13 *vs* 1 or for 92.31%). However, the sum of DSS observed in the two other groups of principal pigs, treated either with MOS or VACCINE, was only slightly lower than that of the CONTROLS (13 *vs* 5 *vs* 3 or for 61.54% and 76.92%). The control pigs

Table 1. Comparative changes in the body weight (BW) of weaned pigs supplemented with dietary mannan oligosaccharide (MOS), perorally immunized with either a live F4ac$^+$ F18ac$^+$ non-ETEC vaccine candidate (VACCINE) against porcine colibacillosis, or with a combination of the two (VACCINE + MOS) on Day 0 (or 4 weeks of age), over the 5 weeks of the experiment.

Group of pigs*	MEAN BW (kg)***					Pooled SEM values
	Day 0	Day 7	Day 14	Day 28	Day 35	
CONTROL**	8.33	9.02	10.30^{A}	15.17	18.74^{A}	0.976
MOS	8.10	9.33	10.97^{AB}	14.92	19.21^{A}	1.237
VACCINE	8.78	9.65	12.07^{B}	14.71	17.65^{B}	1.206
VACCINE + MOS	8.35	9.66	10.44^{A}	14.72	18.02^{AB}	1.195

*Groups comprised 16 pigs each; **Control pigs received *p. o.* 60 mL of TSB as a placebo; ***Values with different superscripts in the same column significantly differ between the groups at $P<0.05$ or lower.

Table 2. The incidence and severity of postweaning diarrhoea, and mortality rate of diarrheic pigs in-feed treated on Day 0 (or 4 weeks of age) with either MOS or *p. o.* with a live F4ac$^+$ F18ac$^+$ non-ETEC vaccine candidate (VACCINE) against porcine colibacillosis, or with a combination of the two (VACCINE + MOS) over the 5 weeks of the experiment

Treatment of pigs	No. of diarrheic pigs/total no. of pigs (%)**	Diarrhoea severity score (DSS)***		Average diarrhoea severity (ADS)****		No. of dead pigs/ total no. of pigs (%)
		Sum of DSS	% difference vs. control	ADS ratio	% difference vs. control	
CONTROL*	5/16 (31.25)	13	/	0.37	/	3/16 (18.75)
MOS	3/16 (18.75)	5	- 61.54	0.14	- 62.16	1/16 (6.25)
VACCINE	2/16 (12.50)	3	- 76.92	0.09	- 75.68	1/16 (6.25)
VACCINE + MOS	1/16 (6.25)	1	- 92.31	0.03	- 91.89	1/16 (6.25)

*Control pigs received *p. o.* 60 mL of TSB as a placebo, and except this group of pigs the VACCINE group of pigs was not supplemented with the MOS during 35 days of the experiment; **Groups comprised 16 pigs each; *** diarrhoea severity score (DSS): 0 = normal faeces, 1 = soft faeces, 2 = fluid faeces or 3 = projectile diarrhoea as summarized during 35 days of the experiment; ****Sum of DSS/35 days.

had a higher ADS (average diarrhoea severity) ratio than the principal pigs (0.37 *vs* 0.14 *vs* 0.09 *vs* 0.03 or for 62.16%, 75.68% and 91.89%). Thus, the pigs from these three principal groups had a generally much lower ADS ratio following 35 days of the experiment as compared to the controls.

Faecal shedding of E. coli isolates and colonization of the ileum. Faecal shedding of haemolytic/nonhaemolytic (Hly/nHly) *E. coli* isolates was more pronounced in the control pigs as compared to the principal groups of pigs, such as MOS, VACCINE and VACCINE + MOS (4/3 *vs*. 2/2, 1/1 and 2/2), respectively, on Day 0 before the treatments (Table 3). 35 days after the treatments, at the end of the experiment, the control pigs had almost the same faecal shedding pattern of Hly/nHly) *E. coli* isolates (4/2) as recorded on Day 0. However, the principal groups of pigs had either totally reduced numbers of Hly *E. coli* isolates 2/2 *vs*. 0/3 (MOS) and 2/2 *vs*. 0/2 (VACCINE + MOS), or the same as recorded on Day 0 (1/1 *vs*. 1/5) in the pigs from the VACCINE group. On Day 35 the total bacterial load in the ileum was much lower in the pigs from all three principal groups (MOS, VACCINE, and VACCINE + MOS) than in the control pigs (19 x 10^7, 17 x 10^7 and 12 x 10^7 *vs*. 23 x 10^8 CFU/mL), respectively) at the end of the experiment.

Intensity and kinetics of circulating immune cell responses. Changes in the proportion of systemic circulating immune cell subsets, as determined by the FCM analyses, were recorded for CD4$^+$ and CD8$^+$ T lymphocytes, CD21$^+$ B lymphocytes and CD45$^+$ lymphoid cells over the 35 days of the experiment (Table 4). In general, the pigs from the principal groups had significantly higher proportions of tested lymphoid cells than the pigs from the control group. This increase was regularly recorded from Day 28 of the experiment, and it continued until Day 35 or the end of the experiment in all principal groups regardless of the treatment applied. However, there was an exception in the VACCINE + MOS group of the pigs, where all four lymphoid cell subsets tested were significantly higher than the CONTROLS ($P<0.05$) as recorded on all the days of sampling following the treatment on Day 0 of the experiment. Also, some minor discrepancies from general trend were observed for CD8$^+$ T cells and CD21$^+$ B cells in the MOS and the VACCINE groups (Table 4). The pigs treated with either VACCINE or VACCINE + MOS had significantly higher CD45$^+$ cells *(*$P<0.05$) from Day 7 to Day 35 of the experiment. Regarding CD4$^+$ T cells, the specific immunization with either F4ac$^+$ F18ac$^+$ non-ETEC vaccine candidate (VACCINE) or with the bivalent vaccine in combination with the MOS supplementation (VACCINE + MOS)

Table 3. Bacterial isolates from rectal swabs (r. s.) and the number of colony forming units (CFU mL) in the ileal samples (i. s.) from weaned pigs supplemented with dietary MOS (from day 0 to 35 of the study, or day 28 to day 63 of life), perorally immunized (at day 0) with either a live F4ac$^+$ F18ac$^+$ non-ETEC vaccine (VACCINE) candidate against porcine colibacillosis, or with a combination of both (VACCINE + MOS) over the 5 weeks of the experiment

Bacterial isolates* (r. s.)/ No. of bacteria (i. s.) at Day of the study**	Treatment of pigs***			
	CONTROL	MOS	VACCINE	VACCINE + MOS
E. coli O157:K119:F18ac	+		+	
E. coli (nHly) i *Enterococcus* spp.	+	+		+
E. coli (nHly)	+	+		+
NF		+	+	+
E. coli O149:K91:F4ac (Hly) and *Enterococcus* spp.	+	+	+	++
Enterococcus spp		+	+++	+
Enterococcus faecium		+		+
E. coli O149:K91:F4ac (Hly)	+ +	+		
E. coli O8:K87:F4ac (Hly)	+			
CFU/mL at Day 0	37 x 10^8	42 x 10^8	22 x 10^8	32 x 10^8
E. coli (nHly)			+++	
Enterococcus faecium	+	++	+	++
E. coli (nHly) I *Enterococcus* spp.	+	++		+
E. coli O149:K91:F4 (Hly)	+			
E. coli O157:K119:F18ac (Hly) and *Enterococcus* spp.	+		+	
E. coli O149:K91:F4 (Hly) i *Enterococcus* spp.	+			
E. coli O8:K87:F4ac (Hly) and *E. faecium*	+			
E. coli (nHly) and E. *faecium*			++	+
E. coli O157:K119:F18ac	+	+		
NF		++	+	+++
CFU/mL at Day 35	23 x 10^8	19 x 10^7	17 x 10^7	12 x 10^7

*Species/serovar of the isolates = no. of positive (+) or negative (-) findings (NF) per pig/group; Hly = haemolytic or nHly = nonhaemolytic; **r. s. and i. s. were taken either from all pigs per group on days 0, 14 and 35 or from 2 euthanatized pigs per group on days 0 and 35 of the study, respectively; *** Groups comprising 16 pigs each and were fed standard weaner diet (CONTROL and VACCINE) or a diet supplemented with 0.2% MOS

stimulated a significantly higher proportion of these cells (P<0.05) from Day 7 to Day 35 of the experiment compared to the values obtained in the CONTROLS (Table 4).

The proportion of CD8$^+$ T cells was significantly higher in the pigs that were treated with either VACCINE (P<0.05) or VACCINE + MOS (P<0.05) than in the CONTROLS for the entire course of the post treatment period, *i. e.* from Day 7 to Day 35 (Table 4). Interestingly, the pigs treated with MOS in their diet had either a significantly decreased proportion of this T cell subset (P<0.05) on Day 14, or it increased from Day 28 to Day 35 (P<0.05) of the experiment. Similar findings were obtained for CD21$^+$ B cells as their proportion significantly increased in the pigs treated with either VACCINE (P<0.05) or VACCINE + MOS (P<0.05) over the same period of the experiment (Table 4). Also, the pigs supplemented with MOS had either slightly, but not significantly, lower values of this cell subset on Day 14 or significantly higher values (P<0.05) from Day 28 to Day 35 of the experiment.

Frequency and distribution patterns of ileal CD45RA$^+$ and CD45RC$^+$ T cells recruitment.

Table 4. The kinetics of the proportion of peripheral blood lymphoid cell subsets of weaned pigs supplemented with dietary MOS (from day 0 to 35 of the experiment, or from day 28 to day 63 of age), *i. g.* immunized (on day 0) with either a live F4ac$^+$ F18ac$^+$ non-ETEC vaccine (VACCINE) candidate against porcine post-weaning diarrhoea (PWD) or with a combination (VACCINE + MOS) over the 5 weeks of the experiment

Treatment of pigs*	Day of the study**	Proportions of peripheral blood lymphoid cell subsets (Mean %) ***			
		CD45$^+$	CD4$^+$	CD8$^+$	CD21$^+$
CONTROL	0	50.46	17.57	11.10	20.30
	7	55.90^A	18.83^A	11.10^A	22.11^A
	14	57.94^A	20.19^A	14.00^A	23.97^A
	28	61.01^A	21.36^A	12.17^A	24.08^A
	35	61.70^A	21.36^A	12.37^A	23.87^A
Pooled SEM values		0.727	0.201	0.580	0.176
MOS	0	50.11	17.14	10.11	20.53
	7	54.06^A	18.92^A	10.79^A	22.01^A
	14	59.66^A	20.86^A	11.93^B	23.86^A
	28	69.24^B	24.23^B	13.85^B	27.69^B
	35	69.55^B	24.20^B	13.77^B	27.66^B
Pooled SEM values		0.704	0.196	0.561	0.168
VACCINE	0	50.62	17.85	10.00	20.20
	7	59.64^B	21.45^B	12.03^B	24.31^B
	14	68.15^B	27.19^B	15.22^{AC}	26.14^B
	28	72.07^C	27.42^C	14.81^C	29.19^C
	35	72.12^C	27.22^C	14.62^C	29.22^C
Pooled SEM values		0.718	0.220	0.610	0.178
VACCINE + MOS	0	50.46	17.57	9.71	20.30
	7	63.77^C	24.13^C	13.11^B	23.51^C
	14	72.71^C	25.54^C	15.50^C	25.90^B
	28	72.96^C	27.50^C	15.41^C	26.51^D
	35	72.91^C	27.42^C	15.44^C	26.54^D
Pooled SEM values		0.662	0.208	0.631	0.177

*Standard weaner diet only (nontreated CONTROL and the VACCINE-treated pigs) or diet supplemented with 0.2% MOS (pigs treated with either MOS or VACCINE + MOS); **Blood sampling days;*** Values marked with different superscripts in the same column differ significantly between the groups at P<0.05 or lower. Groups comprised 16 pigs each on day 0 of the study.

Frequency and distribution patterns of the ileal CD45RA$^+$ naïve and CD45RC$^+$ memory T lymphocytes residing in the corresponding gut mucosal sites of the pigs aged 9 weeks, following exogenous recruitment at 4 weeks of age with either a MOS-supplemented diet or live intragastrically (*i. g.*) applied bivalent non-ETEC vaccine candidate (VACCINE), or with a combination of the two (VACCINE + MOS), as was demonstrated immunohistologically *in situ,* are presented in Figures 1 and 2, respectively. In the CONTROLS, the CD45RA$^+$ naïve T lymphocytes were visible, very sparsely distributed in the villi of the ileal *lamina propria* (LP), more abundantly within the Lieberkühn crypts (LC), and only a solitary one in the submucosa (Fig. 1 a). However, these cells were predominantly observed in Peyer's patches (PP) and were also numerous in the interfollicular

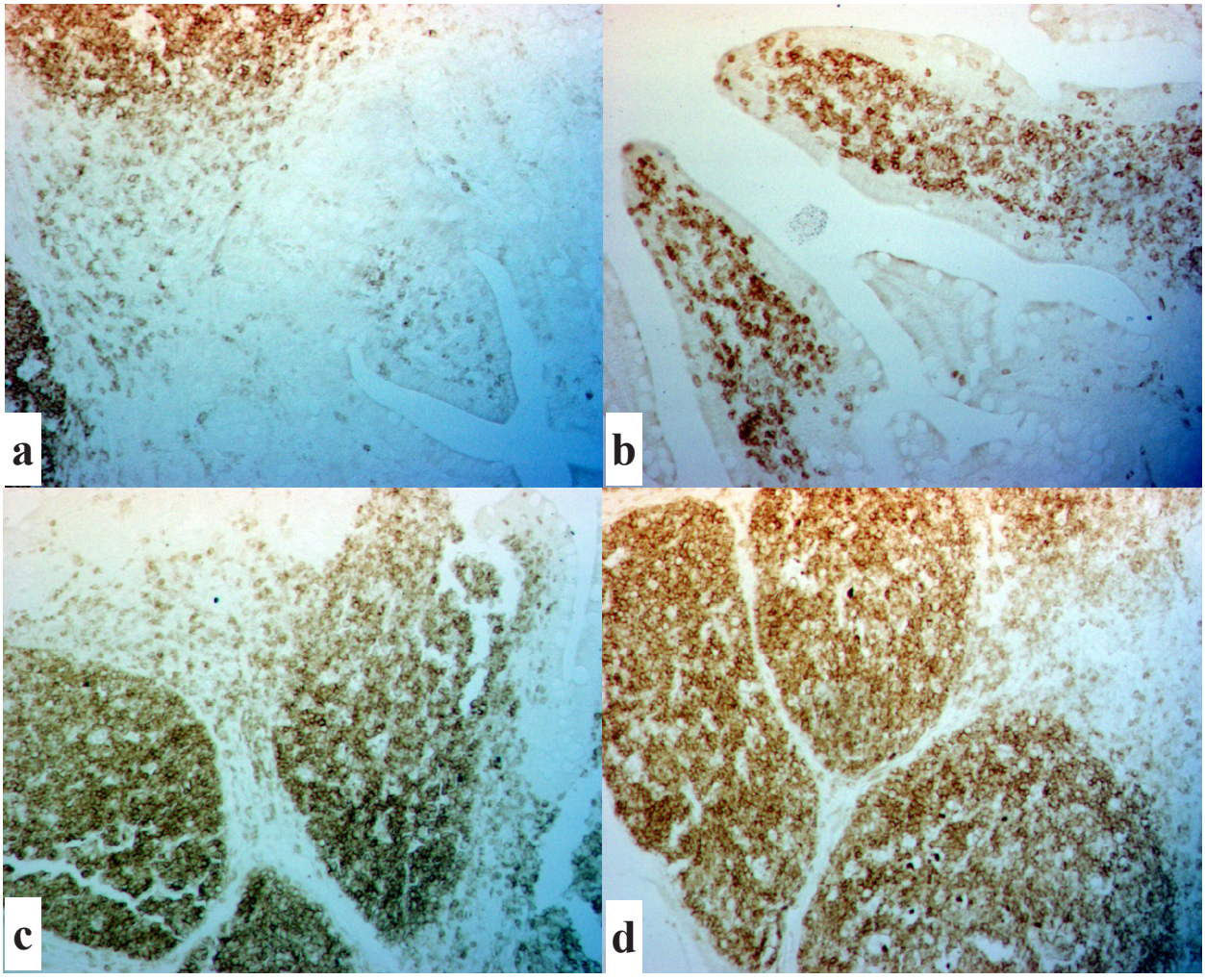

Fig. 1. Identification and localization of CD45 RA$^+$ naive T cells in the ileal villous *lamina propria* and Peyer's patches of 9-week-old pigs from the control group (a) that *i. g*. received 60 mL of TSB as a placebo on Day 0 (28 days of age), and the principal groups of pigs that received either dietary immunomodulator MOS (b), *i. g.* F4ac$^+$ F18ac$^+$ non-ETEC vaccine candidate (c) or a combination of both (d) after 5 weeks of the experiment, as demonstrated by an indirect IP method**;** x 200

areas (IFA). The naïve CD45RA$^+$ T cells in the pigs fed the diet supplemented with MOS were similarly distributed within the villous LP, as was noticed in the CONTROLS. Also, there were slightly more dense patterns of these cells in the IFA and significantly more in the follicular areas (FA) of the ileal PP of the MOS-supplemented pigs, in comparison to those observed in the CONTROLS (Fig. 1 b). It is very likely that the treatment with the VACCINE stimulated moderate recruitment of naïve CD45RA$^+$ T cells within both the IFA and FA of the ileal PP of the pigs 5 weeks following *i. g*. immunization (Fig. 1 c). Also, these cells were numerous and distributed throughout the LP of the intestinal villi, within the LC, and were quite often visible adjacent to the basal membrane of the ileal epithelial cells. However, these cells were rarely observed within the ileal enterocytes. A higher frequency of naïve CD45RA$^+$ cells was observed in the middle of the villous LP and in the IFA and FA of the pigs treated with the VACCINE + MOS combination compared to the CONTROLS (Fig. 1 d). These cells were rarely visible within the layer of ileal epithelial cells.

The distribution patterns of memory CD45RC$^+$ T lymphocytes within the ileal mucosal LP of

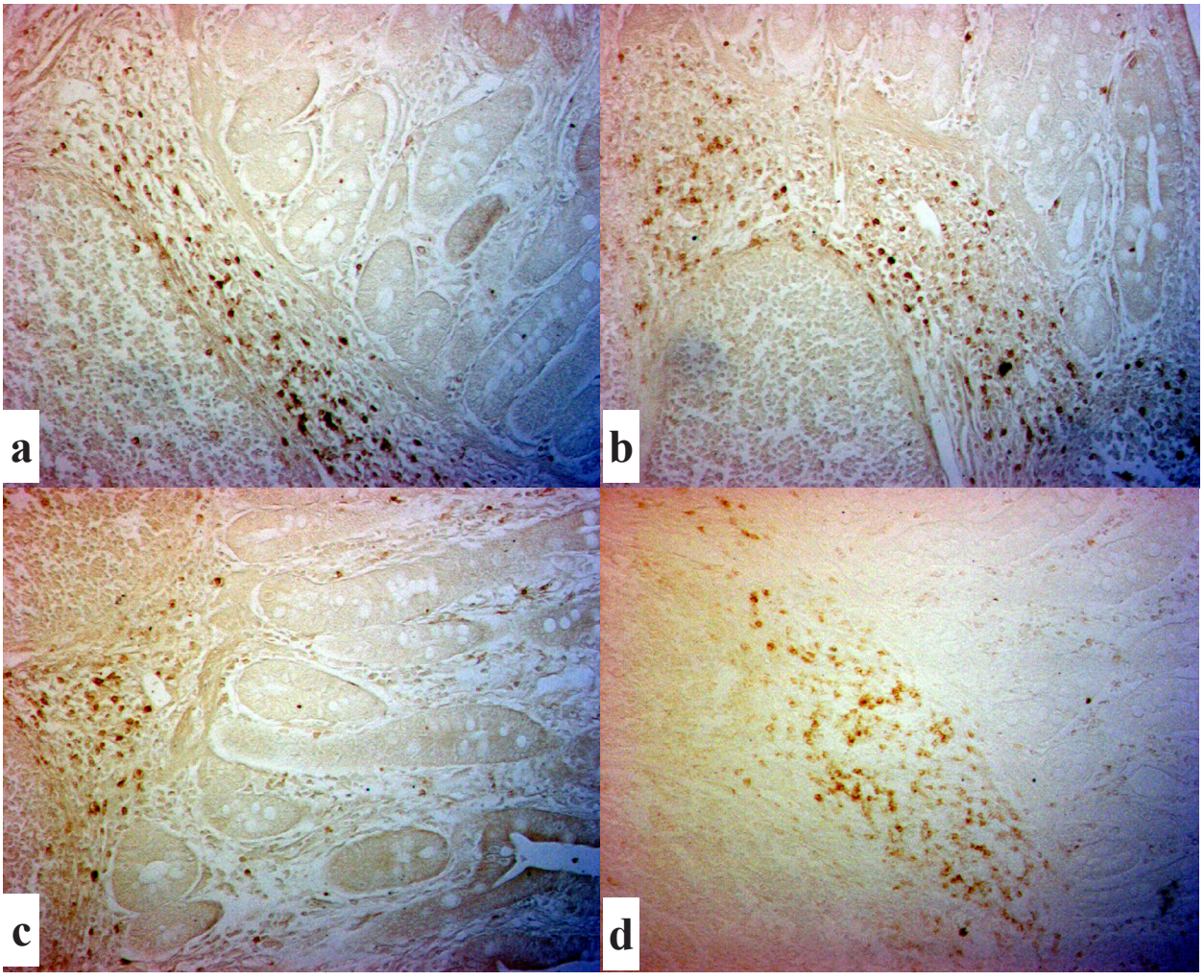

Fig. 2. Identification and localization of CD45 RC^+ memory T cells in the ileal villous *lamina propria* and Peyer's patches of 9-week-old pig from the control group (a) that received on Day 0 (28 days of age) *i. g.* 60 mL of TSB as a placebo, and the treated groups of pigs that received either dietary immunomodulator MOS (b), *i. g.* $F4ac^+ F18ac^+$ non-ETEC vaccine candidate (c) or a combination of these (d) after 5 weeks of the experiment, as demonstrated by an indirect IP method; x 200

the CONTROLS after 5 weeks of the experiment are shown in Fig. 2 a. The memory $CD45RC^+$ T lymphocytes were mostly observed dispersed in the villi and the IFA, but were rather scarce in the PP. These cells were more abundant within the LP of the villi in the MOS-supplemented pigs than in the CONTROLS (Fig. 2 b.). However, memory $CD45RC^+$ T lymphocytes were found to be more numerous in the villous LP of the ileum from the pigs that were *i. g.* vaccinated with the $F4ac^+ F18ac^+$ non-ETEC vaccine candidate (VACCINE), 5 weeks following immunization (Fig. 2 c.). The pigs that received the combination of the VACCINE and MOS on day 0 of the experiment (Fig. 2 d), also had similar distribution/frequency patterns of memory $CD45RC^+$ T lymphocytes after 5 weeks of the experiment. Namely, these cells were predominantly observed within the villous LP and in the IFA, but were less abundant in the FA of the ileal PP. The localization and frequency of naive $CD45RA^+$ and memory $CD45RC^+$ T lymphocytes indicated their different distribution patterns within particular tissue structures (villi, LC, epithelium, LP) and areas (IFA, FA, PP) of the ileal mucosa, which may indicate their different functions in intestinal immune responses to intraluminal

foreign organisms/substances, such as microbes and their antigens, vaccinal immunogens and/or immunomodulators/adjuvants. Moreover, these two T cell subsets should be able to participate simultaneously in tolerogenic responses to harmless dietary antigens.

Discussion

The growing global prohibition on the use of the AGP in food animals has motivated research towards the development of nutritional and feeding strategies, including competitive exclusion modulation by prebiotic MOS, as well as vaccination against ETEC strains, which are the most important causes of PWD in pigs. Herein, the efficacy of a live oral F4ac$^+$ F18ac$^+$ non-ETEC vaccine candidate (VACCINE) was evaluated, with or without the addition of MOS, using a model of stimulation of active intestinal mucosal and systemic cellular immunity to protect weaned pigs against PWD over 5 weeks following the immunization. It is assumed that this approach is a sustainable, practical and more effective in the search for a bivalent, highly immunogenic and safe vaccine, with the supplementary beneficial effects of MOS.

Early growth kinetics and BW gain in weaned pigs are closely associated with gut health and function. Our results showed that the pigs supplemented with dietary MOS or immunized with bivalent live F4ac$^+$ F18ac$^+$ non-ETEC vaccine candidate (VACCINE) had significantly increased body weight ($P<0.05$) on Day 14 of the experiment. However, the pigs from the VACCINE and the VACCINE + MOS groups had significantly lower body weight gain ($P<0.05$) than the CONTROLS on Day 35 the experiment. These findings are in accordance with the beneficial properties of MOS, such as decreased incidence and severity of diarrhoea (ZHAO et al., 2012; VALPOTIĆ et al., 2016) and increased growth performance (MIGUEL et al., 2004). The most significant data on growth in MOS-supplemented pigs were obtained during the first two weeks following weaning (LEMIEUX et al., 2003), which agrees well with our results. Namely, the improved performance or feed efficiency observed seems to be related to the increased disease resistance against pathogenic microbes, such as ETEC, enabling establishment of a low immune status (KIM et al., 2000) and maximizing nutrient utilization for growth, rather than for high activation of the immune system (DAVIS et al., 2002). This is also in agreement with our finding of significantly lower BW gain in both the VACCINE and VACCINE+MOS groups of pigs on Day 35 of the experiment, when the systemic cellular immune status was significantly elevated for 4 out of the 5 weeks after immunization with the F4ac$^+$ F18ac$^+$ non-ETEC vaccine candidate. In general, our results obtained for the development of the clinical symptoms of PWD (diarrhoea) and the related parameters, including its frequency, severity and duration (which were limited by the 5 week period of the experiment), are the most accepted response indices for evaluating ETEC infection (LUISE et al., 2019). These diarrhoea indicators can be assessed using different methods, including evaluation of faecal consistency scores, faecal dry matter and days of diarrhoea. As in our earlier studies, we used a slightly modified faecal score classification according to the Bristol Stool Scale (STOKES et al., 2004), where the faecal consistency score is reduced to 4 levels (0 = normal faeces, 1 = soft faeces, 2 = fluid faeces or 3 = projectile diarrhoea), and scores between 2 and 3 are defined as a clinical sign of diarrhoea (VALPOTIĆ et al., 2013; VALPOTIĆ et al., 2014). Overall, one of the most important aspects is the collection time of the faecal consistency data. Thus, the pigs were monitored daily and taking samples for faecal consistency and diarrhoea score recording started early on Day 0 before any treatments. They were administered 8 hours later. Diarrhoea incidence and severity were scored, and the gross pathological findings of the mortality cases due to PWD were recorded, as detailed earlier. The group of pigs immunized with a single dose of bivalent live F4ac$^+$ F18ac$^+$ non-ETEC vaccine candidate and fed with MOS supplement (VACCINE+MOS) had fewer diarrheic pigs 1/16 (6.25%) than the other two principal groups that received either VACCINE or MOS 2/16 or 3/16 (12.50% or 18.75%, respectively). The immunized pigs which received a dietary MOS supplement also had much lower

incidence of cases of diarrhoea 1/16 (6.25%) than the CONTROLS 5/16 (31.25%) during the course of the experiment. All three groups of principals had a considerably lower mortality rate 1/16 (6.25 %) compared to the CONTROLS 3/16 (18.75%). Our results confirmed the efficacy of a single-dose of live non-pathogenic *E. coli,* of either bivalent F4/F18 or monovalent F4 lyophilized vaccines for oral administration, and for the F18-ETEC and the F4 -ETEC challenges, as reported by NADEAU et al. (2017) and FAIRBROTHER et al. (2017), respectively. However, these authors immunized the pigs 10 days earlier (at 18 days of age) than we did (at 28 days of age), and challenged them at 7 and 21 days post-vaccination. They recorded a significant increase in serum IgM at 7 days post-vaccination and of both serum specific IgM and IgA antibodies at 21 days post-vaccination. Although we analysed proportions of the peripheral blood immune cells in our model system, and obtained quantities of the tested subsets that may act protectively against naturally occurring ETEC infection, we assume that it would be advisable to take into consideration the fact that young pigs reach adult immunocompetence and functional maturity at 7 to 9 weeks of age (BAILEY et al., 2005).

Regarding the post-immunization incidence of morbidity and mortality due to PWD, as well as faecal shedding of the vaccinal strain, our results are comparable to those obtained by NADEAU et al. (2017) as these authors also applied F4/F18 bivalent vaccine, and also successfully protected pigs against PWD. Faecal shedding analyses of both vaccinal non-ETEC strains tested was quantified daily from the samples obtained by taking rectal swabs using standard live bacteria count methods. The presence of the vaccinal F18ac$^+$ *E. coli* strain (O157: K119: F18ac) was determined in the pigs from the CONTROL group and the VACCINE group, and the absence of the vaccinal F4ac$^+$ non-ETEC strain (O9: K36: H19: F4ac, LT$^-$ STb$^-$) was determined before vaccination on Day 0. The latter strain was not identified in rectal swab samples regardless of the group of pigs during the experiment. However, quantitative analyses of CFU per mL of the ileal contents from 2 euthanatized pigs per group on Days 0 and 35 of the study showed the immunogenic and protective potential of the treatments applied. The values for CFU/mL on Day 0 of the study ranged between the groups of pigs from 37 x 10^8 (CONTROLS) to 22 x 10^8 (VACCINE), but after 5 weeks following the treatments these values significantly decreased, as recorded on Day 35 of the study, ranging from 12 x 10^7 (VACCINE + MOS) to 17 x 10^7 (VACCINE). The values obtained for the CONTROL pigs was somewhat decreased on Day 35 (37 x 10^8 *vs* 23 x 10^8 CFU/mL) in relation to Day 0 of the study. This could be ascribed to the impact of their more hygienic rearing conditions (the groups of pigs were housed in separate pens, but in the same nursery unit of the farm), following the vaccination against PWD and/or dietary MOS with their beneficial effects on gut health and immunity as well as prevention of PWD in pigs (VALPOTIĆ et al., 2016; SUN and KIM, 2017; VALPOTIĆ et al., 2018). However, it is certain that the non-treated CONTROL pigs were not protected against potential development of PWD in the same way as the treated groups of pigs, according to the clinical and bacteriological examinations and the parameters obtained. Conversely, it is very likely that in the pigs that either received F4/F18 non-ETEC vaccine candidate, alone or combined with dietary MOS, or who were fed with MOS supplement, active protective systemic and intestinal immunity were induced against naturally occurring ETEC infection and development of PWD. Similar protection has been reported for monovalent F4 vaccine (FAIRBROTHER et al., 2017) and bivalent F4/F18 vaccine (NADEAU et al., 2017) in an either F4-ETEC or F4/F18 ETEC challenge model. It has been suggested that the live bacterial strains in orally applied vaccines, such as the live bivalent F4ac$^+$ and F18ac$^+$ non-ETEC vaccine tested in this study, adhere to the corresponding enterocyte receptors and stimulate rapid production of specific anti-fimbriae antibodies, thus preventing intestinal colonization by pathogenic ETEC strains expressing the same fimbriae (MELKEBEEK et al., 2013).

Besides the abovementioned protective mechanism based on humoral immunity which may be induced by fimbrial and/or toxin antigens of ETEC strains, the current study evaluated

the immunogenicity of the oral bivalent F4/F18 non-ETEC vaccine candidate for recruitment of both systemic and intestinal cellular immunity. Obviously, the immunization of pigs with this vaccine stimulated cellular immune responses to reach protective levels, since the onset and severity/duration of clinical signs of PWD, as well as growth retardation were reduced. In this regard, the kinetics of changes in the proportions of the selected peripheral blood total $CD45^+$ lymphoid cells and the most important T and B lymphocyte subsets, either $CD4^+$ and $CD8^+$ or $CD21^+$, respectively, were phenotyped and quantified on a weekly basis for the 5 weeks of the experiment, starting on Day 0 before the treatments were applied. Regardless of the treatment applied, the pigs from the principal groups had significantly increased percentages of the tested lymphoid cells, as determined between Day 28 and Day 35. However, in the VACCINE+MOS group of pigs, all four lymphoid cell subsets tested were significantly increased in comparison to the CONTROLS, as determined on all the days of sampling following the treatment on Day 0 of the experiment. Further, the pigs treated with either VACCINE had significantly higher $CD45^+$ cells from Day 7 to Day 35 of the experiment. The immunization of the pigs with the VACCINE+MOS supplementation stimulated significantly increased percentages of $CD4^+$ T cells from Day 7 to Day 35 of the experiment, as compared to the values obtained in the CONTROLS.

There is a significant body of literature on evaluating vaccinal immunity against each of the fimbrial F4 and/or F18 antigens, as well as against the multi-epitope fusion antigen construct, by serological antibody response kinetics and levels for monovalent (MELKEBEEK et al., 2013; FAIRBROTHER et al., 2017), bivalent (NADEAU et al., 2017; LUISE et al, 2019) and multivalent vaccines (DUAN et al., 2020). However, data on the impact of specific immunizations on either systemic or intestinal mucosal cellular immunity are rather scarce. Actually, no closely related or at least comparable references on the latter issue were available to us, and thus, we must discuss these issues by objectively analysing our earlier data. Moreover, we also tested mucosal cellular and humoral immunity, in particular secretory IgA^+ (sIgA) cells, by histomorphometric identification and quantification of their proliferation patterns, in order to assess their protective potential following immunization with either $F4^+$ and/or $F18^+$ non-ETEC vaccine candidate strains (VIJTIUK et al., 2005; BOŽIĆ et al., 2006; KOVŠCA JANJATOVIĆ et al., 2009; KOVŠCA JANJATOVIĆ et al., 2010; KOVŠCA JANJATOVIĆ et al., 2011). Also, we examined the immunogenic/antigenic potential of pathogenic $F4ac^+$ ETEC strains (VALPOTIĆ et al., 1994; LACKOVIĆ et al., 1997).

Our approach was based on the well-known fact that intestinal mucosal surfaces are the entry route for harmless dietary antigens, but also for harmful microbial pathogens, including porcine ETEC strains that are etiological agents of PWD, which may alter or excite the GALT defences. ETEC can adhere to the brush borders of enterocytes, and damage the cell tight junctions (TJ), leading to an impaired intestinal barrier which allows bacteria and their toxins to enter the mucosa, inducing intestinal inflammation and diarrhoea (DUBREUIL, 2017). The gut mucosal immune system contains specialised lymphoid tissues, *i. e*. the GALT, where environmental antigens (including those of ETEC) are shown to induce T and B cell responses. These responses are regulated by T cells and cytokines, and they lead to B cell differentiation into IgA^+ plasma cells secreting s IgA onto the intestinal mucosal surfaces (STOKES et al., 1994). After contact with foreign antigens or, as in our case, with the vaccinal immunogens of a bivalent non-ETEC vaccine, these immune cells immediately migrate from the intestinal mucosa to systemic circulation to disseminate information to other immune cells residing at other mucosal sites. Hence, it is important that prospective vaccines stimulate maximal immunity at these susceptible mucosal sites, particularly TJ, and they should include live avirulent non-ETEC strains expressing F4 and/or F18 fimbriae (FAIRBROTHER et al., 2005; DUBREUIL, 2017). More recent studies have shown that live monovalent and bivalent vaccines against PWD, when applied orally, stimulate both systemic and intestinal humoral immunity by serum or mucosal IgM and IgA/sIgA antibodies,

and that there are no indicative differences between the kinetics and intensity of these responses (FAIRBROTHER et al., 2017; NADEAU et al., 2017). Our data on the systemic cellular immunity that developed following oral immunization with F4/F18 non-ETEC are not comparable with those from the latter authors, because they applied a challenge infection with a bivalent ETEC strain expressing the same fimbriae, and evaluated the immune response to oral immunization by determining serum kinetics and the levels of IgM and IgG antibodies. However, the data obtained in both studies regarding immune responsiveness to either ETEC challenge or potentially occurring PWD due to ETEC infection, could be at least considered as complementary. In order to gain an insight into the potential activation of the ileal mucosa residing T cell subsets of naïve CD45RA and memory CD45RC phenotypes following the treatments applied, we examined their distribution and frequency patterns *in situ*. Namely, these cells are of great relevance for the establishment and duration of protective immunity following either on-farm naturally occurring ETEC infection or peroral immunization with F4ac$^+$ and F18ac$^+$ non-ETEC vaccine candidate strains. (BOŽIĆ et al., 2002). The *in situ* findings of naïve CD45RA$^+$ and memory CD45RC$^+$ T cells demonstrated their different distribution patterns within particular areas of the mucosa, indicating their different functions in intestinal immune responses (KOVŠCA JANJATOVIĆ et al., 2009; KOVŠCA JANJATOVIĆ et al., 2010). Naive CD45RA$^+$ cells were more abundant in the LC area whereas memory CD45RC$^+$ cells were mostly found in the villi and IFA, and predominantly in the PP and the extrafollicular areas. Memory CD45RC$^+$ cells were abundant in the mucosal LP and in the IFA, but quite rare in the PP. These findings were in the agreement with our recent findings in this study. Again, no other similar or related resources with data have been found as yet. In our earlier study we reported the presence of microfold (M) cells within the intestinal epithelial layer, particularly covering the PP, the major inductive sites for the antigen-specific response (SNOECK et al., 2006). Since the role of M cells is the intake and transport of intraluminal pathogens, or their products, to the immune cells within the LP their number increased in the pigs that were immunized with bivalent F4$^+$ F18$^+$ non-ETEC vaccine (KOVŠCA JANJATOVIĆ et al., 2011). Visually, the localization/distribution patterns of ileal naïve CD45RA$^+$ and memory CD45RC$^+$ cells are in accordance with those demonstrated in our previous studies (BOŽIĆ et al., 2006; KOVŠCA JANJATOVIĆ et al., 2009; KOVŠCA JANJATOVIĆ et al., 2010; KOVŠCA JANJATOVIĆ et al., 2011).

In the present study, we evaluated the immunogenicity of a live oral non-ETEC vaccine carrying the most common F4ac$^+$ and F18ac$^+$ fimbrial antigens characteristic for PWD in pigs, and investigated the efficacy of dietary MOS supplementation (AGAZZI et al., 2020). This was primarily seen in blocking the adherence of pathogenic ETEC strains expressing F4/F18 fimbriae to the specific enterocyte receptors, thus supporting gut health, promoting growth, ameliorating protective immune response (as an immunomodulator or as an adjuvant for non-ETEC vaccine against PWD), and modulating gut morphology and residing microbiota. Nutritional application of only MOS to protect newly weaned pigs against on-farm occurring PWD, associated with ETEC strains expressing F4 and F18 fimbriae, may provide protection of weaned pigs on the basis of its multi beneficial properties, especially by competitive exclusion mechanism (HARVEY et al., 2005).

Conclusions

Administration of a single oral dose of the live bivalent F4/F18 non-ETEC vaccine protected pigs over 5 weeks following the specific immunization at weaning or 4 weeks of age against on-farm occurring PWD due to ETEC strains. Protection was demonstrated by a significant decrease in the number of pigs with moderate to high frequency and severity of diarrhoea, by reduced faecal shedding of haemolytic F4$^+$ and F18$^+$ ETEC strains, reduced ileal bacterial load (CFU/mL), and by a reduction in mortality, and BW loss. The vaccine induced optimal systemic and intestinal cellular immunity by significantly increasing all 4 lymphoid cell subsets, and conferred protection against naturally occurring

on-farm PWD due to infection with $F4^+$ and $F18^+$ ETEC strains. However, only the pigs that received MOS-adjuvanted vaccine had early protection from Day 7 to Day 35 (or the end of the experiment). The pigs that were immunized with the vaccine had a similar trend of increased proportions of peripheral blood lymphoid cells, with the exception of significantly decreased $CD8^+$ T cells on Day 7. The group of the pigs that were fed with MOS supplement had significantly increased proportions of all 4 lymphoid subsets tested, but solely from Day 28 to Day 35 of the experiment, showing their immunomodulatory potential. Both intestinal and systemic cellular immune responses regularly increased in the ileal mucosal compartments (such as the PP, FA, IFA, villous LP and the LC), as anticipated and observed for naïve $CD45RA^+$ and memory $CD45RC^+$ T cells, or significantly increased in the peripheral blood as determined for $CD4^+$ and $CD8^+$ T lymphocytes, $CD21^+$ B lymphocytes and $CD45^+$ lymphoid cells in the pigs immunized with either the vaccine candidate or with the dietary MOS-adjuvanted vaccine candidate. To conclude, active mucosal immunity is urgently needed to protect pigs against PWD. Hence, oral vaccination of pigs against both F4 and F18 ETEC represents a sustainable, practical and effective approach in the search for such a bivalent, highly immunogenic and safe vaccine. In this regard, our research approach was at least partially successful and innovative for the development of a safe and effective live oral vaccine against PWD that will provide solid protection and sustained cellular and humoral immune responses to recall antigens, such as F4- and F18-ETEC adhesins. Currently, there are no preventive approaches available to protect pigs effectively against PWD. Due to the disease's complexity and the immunological heterogeneity among ETEC strains, challenges in vaccine development against PWD still exist. However, recent research on the MEFA approach to develop multivalent vaccines for broad protection against ETEC-associated PWD, should obtain promising results in the very near future.

References

AGAZZI, A., V. PERRICONE, F. OMODEI ZORINI, S. SANDRINI, E. MARIANI, X-R. JIANG, A. FERRARI, M. CRESTANI, T. X. NGUYEN, V. BONTEMPO, C. DOMENEGHINI, G. SAVOINI (2020): Dietary mannan oligosaccharides modulate gut inflammatory response and improve duodenal villi height in post-weaning piglets improving feed efficiency. Animals 10, 1283.

DOI: 10.3390/ani10081283

ANDRIŠIĆ, M., I. ŽARKOVIĆ, K. ŠANDOR, A. VUJNOVIĆ, E. PERAK JUNAKOVIĆ, H. VALPOTIĆ, K. BENDELJA, Ž. CVETIĆ, F. BOŽIĆ, S. TERZIĆ (2020): Phenotype analysis of CD3+CD16+ lymphocytes in the peripheral blood of pigs. Vet. stn. 51, 117-128.

DOI: 10.46419/vs.51.2.2

BAILEY, M., K. HAVERSON, C. INMAN, C. HARRIS, P. JONES, G. CORFIELD, B. G. MILLER, C. R. STOKES (2005): The development of the mucosal immune system pre- and post-weaning: balancing regulatory and effector function. Proc. Nutr. Soc. 64, 451-457.

DOI: 10.1079/PNS2005452

BAILEY, M. (2009): The mucosal immune system: recent developments and future directions in the pig. Dev. Comp. Immunol. 33, 375-383.

DOI: 10.1016/j.dci.2008.07.003

BECKER, S. L., Q. LI, E. R. BURROUGH, D. KENNE, O. SAHIN, S. A. GOULD, J. F. PATIENCE (2020): Effects of an F18 entertoxigenic *Escherichia coli* challenge on growth performance, immunological status, and gastrointestinal structure of weaned pigs and the potential protective effect of direct-fed microbial blends. J. Anim. Sci. 98, 1-10.

DOI: 10.1093/jas/skaa113

BOŽIĆ, F., V. BILIĆ, I. VALPOTIĆ (2002): Modulation by levamisole of CD45RA and CD45RC isoforms expression in the gut of weaned pigs vaccinated against colibacillosis. J. Vet. Pharmacol. Therap. 25, 69-72.

DOI: 10.1046/j.1365-2885.2002.00379.x

BOŽIĆ, F., G. LACKOVIĆ, A. KOVŠCA JANJATOVIĆ, O. SMOLEC, I. VALPOTIĆ (2006): Levamisole synergized experimental $F4ac^+$ *Escherichia coli* oral vaccine in stimulating ileal Peyer's patch T cells in weaned pigs. J. Vet. Pharmacol. Therap. 29, 199-204.

DOI: 10.1111/j.1365-2885.2006.00731.x

BURKEY, T. E., K. A. SKJOLAAS, J. E. MINTON (2009): Porcine mucosal immunity of the gastrointestinal tract. J. Anim. Sci. 87, 1493-1501.

DOI: 10.2527/jas.2008-1330

CASEY, T. A., and H. W. MOON (1990): Genetic characterization and virulence of enterotoxigenic *Escherichia coli* mutants which have lost virulence genes in vivo. Infect. Immun. 58, 4156-4158.

DOI: 10.1128/iai.58.12.4156-4158.1990

DAVIS, M. E., C. V. MAXWELL, D. C. BROWN, B. Z. DE RODAS, Z. B. JOHNSON, E. B. KEGLEY, D. H. HELLWIG, R. A. DVORAK (2002): Effect of dietary mannan oligosaccharides and(or) pharmacological additions of copper sulfate on growth performance and immunocompetence of weanling and growing/finishing pigs. J. Anim. Sci. 80, 2887-2894.

DOI: 10.2527/2002.80112887x

DUAN, Q., S. PANG, W. WU., B. JIANG, W. ZHANG, S. LIU, X. WANG, Z. PAN, G. ZHU (2020): A multivalent vaccine candidate targeting enterotoxigenic *Escherichia coli* fimbriae for broadly protecting against porcine post-weaning diarrhea. Vet. Res. 51, 93-105.

DOI: 10.1186/s13567-020-00818-5

DUBREUIL, J. D. (2017): Enterotoxigenic *Escherichia coli* and probiotics in swine: what the bleep do we know? Bioscience of Microbiota. Food Health 36, 75-90.

DOI: 10.12938/bmfh.16-030

FAIRBROTHER, J. M., E. NADEAU, C. L. GYLES (2005): *Escherichia coli* in postweaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. Anim. Health Res. Rev. 6, 17-39.

DOI: 10.1079/AHR2005105

FAIRBROTHER, J. M., E. NADEAU, L. BELANGER, C.- L. TREMBLAY, D. TREMBLAY, M. BRUNELLE, R. WOLF, K. HELLMANN, A. HIDALGO (2017): Immunogenicity and protective efficacy of a single-dose live non-pathogenic *Escherichia coli* oral vaccine against F4-positive enterotoxigenic *Escherichia coli* challenge in pigs. Vaccine 35, 353-360.

DOI: 10.1016/j.vaccine.2016.11.045

GORDON, V. M., S. C. WHIPP, H. W. MOON, A. D. O'BRIEN, J. E. SAMUEL (1992): An enzymatic mutant of Shiga-like toxin-II variant is a vaccine candidate for oedema disease of swine. Infect. Immun. 60, 485-490.

DOI: 10.1128/iai.60.2.485-490.1992

HALAS, V., I. NOCHTA (2012): Mannan oligosaccharides in nursery pig nutrition and their potential mode of action. Animals 2, 261-274.

DOI: 10.3390/ani2020261

HARVEY, R. B., R. C. ANDERSON, K. J. GENOVESE, T. R. CALLAWAY, D. J. NISBET (2005): Use of competitive exclusion to control enterotoxigenic strains of Escherichia coli in weaned pigs. J. Anim. Sci. 83, 44-47.

HAVERSON, K., M. BAILEY, C. R. STOKES (1999): T-cell populations in the pig intestinal lamina propria: memory cells with unusual phenotypic characteristics. Immunology 96, 66-73.

DOI: 10.1046/j.1365-2567.1999.00658.x

HEO, J. M, F. O. OPAPEJU, J. R. PLUSKE, J. C. KIM, D. J. HAMPSON, C. M. NYACHOTI (2013): Gastrointestinal health and function in weaned piglets: a review of feeding strategies to control post-weaning diarrhoe without using in-feed antimicrobial compounds. J. Anim. Physiol. Anim. Nutr. (Berl). 97, 207-237.

DOI: 10.1111/j.1439-0396.2012.01284.x

KIM, J., Y. HYUN, K. SOHN, T. KIM, H. WOO, I. HAN (2000): Effects of mannanoligosaccharide and protein levels on growth performance and immune status in pigs weaned at 21 days of age. Korean J. Anim. Sci. 42, 489-498.

DOI: 10.22358/jafs/68051/2000

KOVŠCA-JANJATOVIĆ, A., G. LACKOVIĆ, F. BOŽIĆ, D. ŠPOLJARIĆ, M. POPOVIĆ, H. VALPOTIĆ, N. VIJTIUK, Ž. PAVIČIĆ, I. VALPOTIĆ (2009): Histomorphometric characteristics of immune cells in small intestine of pigs perorally immunized with vaccine candidate F18ac$^+$ nonenterotoxigenic *E. coli* strain. Eur. J. Histochem. 53, 189-198.

DOI: 10.4081/ejh.2009.189

KOVŠCA-JANJATOVIĆ, A., G. LACKOVIĆ, F. BOŽIĆ, D. KEZIĆ, M. POPOVIĆ, H. VALPOTIĆ, I. HARAPIN, Ž. PAVIČIĆ, B. NJARI, I. VALPOTIĆ (2010): Histomorphometric evaluation of intestinal cellular immune responses in pigs immunized with live oral F4ac$^+$ non-enterotoxigenic *E. coli* vaccine against postweaning colibacillosis. Eur. J. Histochem. 54, 18-24.

DOI: 10.4081/ejh.2010.e4

KOVŠCA JANJATOVIĆ, A., G. LACKOVIĆ, F. BOŽIĆ, H. VALPOTIĆ, M. TOMINAC-TRCIN, S. SLADOLJEV, B. ŠEOL, I. VALPOTIĆ, M. POPOVIĆ (2011): De novo differentiation of intestinal villous M cells in weaned pigs immunized with levamisole-adjuvanted vaccine candidate F4ac$^+$ or F18ac$^+$ non-enterotoxigenic *Escherichia coli* strains. Vet. arhiv 81, 77-90.

LACKOVIĆ, G., N. VIJTIUK, S. ĆURIĆ, E. A. DEAN-NYSTROM, T. A. CASEY, I. VALPOTIĆ (1997): Detection of wCD1, SWC1a, SWC2 and CD45 molecules by immunofluorescence or immunoperoxidase techniques in porcine gut-associated lymphoid tissues following experimentally induced colibacillosis. Period. biol. 99, 343-350.

LEMIEUX, F. M., L. L. SOUTHERN, T. D. BIDNER (2003): Effect of mannan oligosaccharides on growth performance of weanling pigs. J. Anim. Sci. 81, 2482-2487.

DOI: 10.2527/2003.81102482x

LUISE, D., C. LAURIDSEN, P. BOSI, P. TREVISI (2019): Methodology and application of *Escherichia coli* F4 and F18 encoding infection models in post-weaning pigs. J. Anim. Sci. Biotechnol. 10. 53.

DOI: 10.1186/s40104-019-0352-7

LUPPI, A. (2017): Swine enteric colibacillosis, therapy and antimicrobial resistance. Porc. Health Manag. 3, 16.

DOI: 10.1186/s40813-017-0063-4

MELKEBEEK, V., B. M. GODDEERIS, E. COX (2013): ETEC vaccination in pigs. Vet. Immunol. Immunopathol. 152, 37-42.

DOI: 10.1016/j.vetimm.2012.09.024

MIGUEL, J. C., S. L. RODRIGUEZ-ZAS, J. E. PETTIGREW (2004): Efficacy of a mannan oligosaccharide (Bio-Mos®) for improving nursery pig performance. J. Swine Health Prod. 12, 296-307.

NADEAU, E., J. M. FAIRBROTHER, J. ZENTEK, L. BELANGER, D. TREMBLAY, C.-L. TREMBLAY, I. RÖHE, W. VAHJEN, M. BRUNELLE, K. HELLMANN, D. CVEJIĆ, B. BRUNNER, C. SCHNEIDER, K. BAUER, R. WOLF, A. HIDALGO (2017): Efficacy of a single oral dose of a live bivalent *E. coli* vaccine against post-weaning diarrhea due to F4 and F18-positive enterotoxigenic *E. coli*. Vet. J. 226, 32-39.

DOI: 10.1016/j.tvjl.2017.07.004

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

DOI: 10.1016/j.ijmm.2005.07.003

NOCHTA, I., T. TUBOLY, V. HALAS, L. BABINSZKY (2009): Effect of different levels of mannan-oligosaccharide supplementation on some immunological variables in weaned piglets. J. Anim. Physiol. Anim. Nutr. 93, 496-504.

DOI: 10.1111/j.1439-0396.2008.00835.x

RUAN, X., W. ZHANG (2013): Oral immunization of a live attenuated *Escherichia coli* strain expressing a holotoxin-structured adhesin-toxoid fusion (I FaeG-FedF-LTA; SLIB) protected young pigs against enterotoxigenic *Escherichia coli* infection. Vaccine 31, 1458-1463.

DOI: 10.1016/j.vaccine.2013.01.030

RUAN, X., M. LIU, T. A. CASEY, W. ZHANG (2011): A tripartite fusion, EaeG-FedF-LT (192) A2: B of enterotoxigenic *Escherichia coli* (ETEC) elicits antibodies that neutralize cholera toxin, inhibit adherence of K88 (F4) and F18 fimbriae, and protect pigs against K88ac/heat-labile toxin infection. Clin. Vaccine Immunol. 18, 1593-1599.

DOI: 10.1128/CVI.05120-11

RUAN, X., D. E. KNUDSEN, K. M. WOLLENBERG, D. A. SACK, W. ZHANG (2014): Multiepitope fusion antigen induces broadly protective antibodies that prevent adherence of *Escherichia coli* strains expressing colonization factor antigen I (CFA/I), CFA/II, and CFA/IV. Clin. Vaccine Immunol. 21, 243-249.

DOI: 10.1128/CVI.00652-13

SMITH, M. G., D. JORDAN, T. A. CHAPMAN, J. J. CHIN, M. D. BARTONT, T. N. DO (2010): Antimicrobial resistance and virulence gene profiles in multi-drug resistant enterotoxigenic *Escherichia coli* isolated from pigs with post-weaning diarrhea. Vet. Microbiol. 145, 299-307.

DOI: 10.1016/j.vetmic.2010.04.004

SNOECK, V., T. VERFAILLE, F. VERDONCK, B. M. GODDEERIS, E. COX (2006): The jejunal Peyer's patches are the major inductive sites of the F4-specific immune response following intestinal immunization of pigs with F4 (K88) fimbriae. Vaccine 24, 3812-3820.

DOI: 10.1016/j.vaccine.2005.07.025

STOKES, C. R., M. BAILEY, K. HAVERSON, C. HARRIS, P. JONES, C. INMAN (2004): Postnatal development of intestinal immune system in piglets: implications for the process of weaning. Anim. Res. 53, 325-334.

DOI: 10.1051/animres:2004020

STOKES, C. R., M. BAILEY, A. D. WILSON (1994): Immunology of the porcine gastrointestinal tract. Vet. Immunol. Immunopathol. 43, 143-150.

DOI: 10.1016/0165-2427(94)90130-9

SUN, Y., S. W. KIM (2017): Intestinal challenge with enterotoxigenic *Escherichia coli* in pigs, and nutritional intervention to prevent postweaning diarrhea. Anim. Nutrition 3, 322-330.

DOI: 10.1016/j.aninu.2017.10.001

VALPOTIĆ, H., I. ŽURA ŽAJA, M. SAMARDŽIJA, B. HABRUN, M. OSTOVIĆ, D. ĐURIČIĆ, N. MAĆEŠIĆ, Ž. MIKULEC, P. KOČILA, P. SOBIECH, I. VALPOTIĆ, S. VINCE (2018): Dietary supplementation with mannan oligosaccharide and clinoptilolite modulates innate and adaptive immune parameters of weaned pigs. Polish J. Vet. Sci. 21, 83-93.

VALPOTIĆ, H., R. BARIĆ-RAFAJ, V. MRLJAK, Ž. GRABAREVIĆ, M. SAMARDŽIJA, M. ŠPERANDA, I. ŽURA ŽAJA, D. ĐURIČIĆ, A. BACH, I. HARAPIN, I, FOLNOŽIĆ, S. VINCE, I. VALPOTIĆ (2017): Influence of dietary mannan oligosaccharide and clinoptilolite on hematological, biochemical and gut histological parameters in weaned pigs. Period. biol. 119, 63-73.

VALPOTIĆ, H., M. SAMARDŽIJA, S. TERZIĆ, S. VINCE, M. ŠPERANDA, G. LACKOVIĆ, B. HABRUN, N. MAS, D. ĐURIČIĆ, P. KOČILA, F. MARKOVIĆ, I. VALPOTIĆ (2016): Effect of mannan oligosaccharide supplementation on blood and intestinal immune cells, bacteria numbers and performance in weaned pigs. Acta Vet. Brno 85, 267-276.

DOI: 10.2754/avb201685030267

VALPOTIĆ, H., M. ŠPERANDA, A. KOVŠCA-JANJATOVIĆ, M. ĐIDARA, G. LACKOVIĆ, F. BOŽIĆ, B. HABRUN, S. SREČEC, M. MATAUŠIĆ-PIŠL, I. VALPOTIĆ (2014): Levamisole stimulates proliferation of circulating and intestinal immune cell subsets, gut health and performance in weaned pigs. Can. J. Anim. Sci. 94, 43-53.

DOI: 10.4141/cjas2013-097

VALPOTIĆ, H., G. MRŠIĆ, B. GRŠKOVIĆ, D. ŠPOLJARIĆ, D. KEZIĆ, S. SREČEC, M. MATAUŠIĆ-PIŠL, G. LACKOVIĆ, D. CAPAK, D. MIHELIĆ, K. VLAHOVIĆ, I. VALPOTIĆ, A. PIRKIĆ, D. ANĐELINOVIĆ, M. POPOVIĆ (2013): Effect of polyoxyethylene and polyoxypropylene nonionic block copolymers on performance and recruitment of immune cell subsets in weaned pigs. Acta Vet. Scand. 55, 54-62.

DOI: 10.1186/1751-0147-55-54

VALPOTIĆ, I., N. VIJTIUK, K. TRUTIN-OSTOVIĆ, T. A. CASEY, E. DEAN-NYSTROM, G. LACKOVIC (1994): Identification and distribution of CD+ T cell subsets in porcine gut following experimental infection with F4ac$^+$ enterotoxigenic *Escherichia coli* (ETEC) or non-ETEC strains. Reg. Immunol. 6, 387-390.

VAN den BROECK, W., E. COX, B. M. GODDEERIS (1999): Induction of immune responses in pigs following oral administration of purified F4 fimbriae. Vaccine 17, 2020-2029.

DOI: 10.1016/S0264-410X(98)00406-X

VIJTIUK, N., L. ŠVER, G. LACKOVIĆ, M. POPOVIĆ, F. BOŽIĆ, I. VALPOTIĆ (2005): Intestinal immune response of weaned pigs experimentally vaccinated with F4ac$^+$ non-enterotoxigenic strains of *Escherichia coli*. Acta Vet. Brno 74, 595-601.

DOI: 10.2754/avb200574040595

WINN, W., S. ALLEN, W. JANDA, E. KONEMAN, G. PROCOP, P. SCHRECKENBERGER, G. WOODS (2006): Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Williams and Wilkins, Philadelphia, PA.

WHITE, L. A., M. C. NEWMAN, G. L. CROMWELL, M. D. LINDEMANN (2002): Brewers dried yeast as a source of mannan oligosaccharides for weanling pigs. J. Anim. Sci. 80, 2619-2628.

DOI: 10.2527/2002.80102619x

ZHAO, P.Y., J. H. JUNG, I. H. KIM (2012): Effect of mannan oligosaccharides and fructan on growth performance, nutrient digestibility, blood profile, and diarrhea score in weanling pigs. J. Anim. Sci. 90, 833-839.

DOI: 10.2527/jas.2011-3921

ZHANG, W. (2014): Progress and challenges in vaccine development against enterotoxigenic *Escherichia coli* (ETEC) - associated porcine post-weaning diarrhea (PWD). J. Vet. Med. Res. 1, 1006.

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VALPOTIĆ, H., D. SVOBODA, D. ŠPOLJARIĆ, D. LEINER, B. ŠPOLJARIĆ, N. VIJTIUK, B. HABRUN, H. CAPAK, Ž. VIDAS, S. VINCE, N. MAĆEŠIĆ, M. SAMARDŽIJA, M. POPOVIĆ, A. KOVŠCA JANJATOVIĆ, G. LACKOVIĆ, I. VALPOTIĆ, M. ĐURIĆ JARIĆ, F. MARKOVIĆ: Vrednovanje profilaktičkog potencijala imunizacije cjepivom neenterotoksigenog soja bakterije *Escherichia coli* (ne-ETEC) i pogodnosti kompetitivne ekskluzije dodatkom hrani manan-oligosaharida protiv infekcija izazvanih sojevima ETEC u odbijene prasadi. Vet. arhiv 92, 53-72, 2022.

SAŽETAK

Enterotoksigeni sojevi bakterije *Escherichia coli* (ETEC), koji proizvode F4 i F18 fimbrije, najuobičajeniji su uzročnici dijareje nakon odbića (DNO) u prasadi. Rastuće globalno ograničavanje uporabe antibiotika u farmskih životinja usmjeruje istraživanja prema razvijanju nutritivnih i prehrambenih strategija, kao i prema cijepljenju protiv DNO-a. Cilj ovog istraživanja bio je vrednovanje učinkovitosti živog, oralnog F4ac$^+$F18ac$^+$ ne-ETEC cjepiva-kandidata (VAK) u poticanju crijevne i sistemske stanične imunosti u prasadi u dobi od 4 tjedna, tijekom 5 tjedana nakon imunizacije. U pokusu su određivani početak i trajanje zaštitne imunosti od pojavnosti farmskog DNO-a, proizvodni rezultati, ocjenjivanje učestalosti i jačine dijareje te mortalitet i fenotipski udjeli imunosnih stanica. Uzimani su uzorci fecesa i ileuma za određivanje sastava mikrobiota ili za fenotipiziranje naivnih/memorijskih T-limfocita. Procijenjena je i učinkovitost prebiotika manan-oligosaharida (MOS), kao dodatka hrani, koji bi mogao uspostaviti kompetitivnu ekskluziju naseljavanja tankog crijeva sojevima ETEC, a mogao bi pokazati i adjuvantnost u testiranoj kombinaciji (VAK + MOS). Prasad hranjena dodatkom MOS-a u hrani, ili koja je primila VAK, imala je znakovito povećanu ($P < 0,05$) tjelesnu masu 14. dan pokusa, dok je prasad tretirana kombinacijom VAK-a i MOS-a imala znakovito nižu tjelesnu masu 35. dan pokusa. Tretman kombinacijom VAK-a i MOS-a rezultirao je znatno blažom kliničkom slikom DNO-a, napose u odnosu na pojavnost i jačinu dijareje te na mortalitet. Ukupno bakterijsko opterećenje u ileumu bilo je mnogo niže u prasadi iz sve tri pokusne skupine (MOS, VAK i VAK + MOS) od onog u kontrolnoj (KON) skupini (19×10^7, 17×10^7 i 12×10^7 prema 23×10^8 CFU/mL) 35. dan pokusa. Prasad iz pokusnih skupina imala je znakovito veće udjele testiranih imunosnih stanica ($P < 0,05$) 28. i 35. dan pokusa. Lokalizacija i učestalost naivnih CD45RA$^+$ te memorijskih CD45RC$^+$ T-limfocita pokazuju njihove različite obrasce smještanja u posebne tkivne strukture, kao što su crijevne resice, kripte, epitelij i *lamina propria* te područja u sluznici ileuma, što može specificirati njihove različite funkcije u crijevnim imunosnim odgovorima na intraluminalne mikrobe i njihove proizvode, vakcinalne imunogene i/ili imunomodulatore/adjuvanse. Zaključujemo da je radi zaštite prasadi od DNO-a nužno uspostaviti aktivnu mukoznu imunost. Stoga je oralno cijepljenje prasadi protiv F4 i F18 ETEC-a održiv, praktičan i učinkovit pristup u pronalaženju odgovarajućega bivalentnog, izrazito imunogenog i sigurnog cjepiva.

Ključne riječi: imunizacija; *Escherichia coli*; kompetitivna ekskluzija; manan-oligosaharid; prasad