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Immunogenicity of a live bivalent non-enterotoxigenic *Escherichia coli* (non-ETEC) vaccine and dietary clinoptilolite efficacy against postweaning diarrheal disease of pigs due to F4$^+$ and F18$^+$ ETEC strains

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ABSTRACT

No safe and effective vaccine exists against porcine enterotoxigenic *Escherichia coli* (ETEC) strains, which are the etiological agents of post-weaning diarrhoea (PWD), economically one of the most significant diseases of swine, which encountered for major productive losses in swine industry worldwide. The current study was designed to evaluate: (1) efficacy of an oral bivalent F4ac$^+$/F18ac$^+$ non-ETEC live vaccine candidate (VACCINE) in stimulating systemic and intestinal cellular immunity in 4-week-old weaned pigs, (2) the onset and duration of protective immunity of weaned pigs against naturally occurring PWD during the period of 6 weeks following weaning, and (3) the dietary supplement potential of zeolite clinoptilolite (CPL), an antimicrobial mineral and/or immunomodulator/ vaccine adjuvant (VACCINE + CPL). The pigs immunized either with the VACCINE or its combination with dietary CPL had significantly increased body weight gain from Day 7 to Day 42 ($P<0.05$) of the experiment, as compared to the control

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pigs (CONTROLS). Conversely, the pigs that were only supplemented with CPL had mostly significantly lower ($P<0.05$) body weight. The pigs that received VACCINE + CPL were neither diarrheic nor were there any mortalities during the entire period of the experiment. On Day 42 the total bacterial load in the jejunum was much lower in the pigs from all three principal groups than in the CONTROLS (30×10^8 *vs.* 18×10^7 *vs.* 14×10^7 *vs.* 13×10^7 CFU/mL, respectively). Regarding $CD4^+$ and $CD8^+$ T cells, specific immunization with either VACCINE or with VACCINE + CPL stimulated a significantly higher proportion of these cells ($P<0.05$) from Day 7 to Day 42 of the experiment. Quite similar findings were obtained for $CD21^+$ B cells, as their proportion was significantly elevated in the pigs treated with either VACCINE or VACCINE + CPL ($P<0.05$ and $P<0.05$, respectively). The pigs from the VACCINE + CPL group had a significantly higher proportion of $CD45^+$ lymphoid cells than the pigs from the CONTROL group ($P<0.05$). Quite sparsely distributed $CD45RA^+$ naïve T lymphocytes were observed in the jejunal *lamina propria* of the intestinal villi, within the Lieberkühn crypts (LC) and in the submucosa of the CONTROLS and CPL supplemented pigs. The treatment with the VACCINE induced moderate recruitment of CD45 RA^+ cells within both the IFA and FA of the jejunal PP of the pigs 6 weeks following the vaccination. In the CONTROLS, CD45 RC^+ memory T cells were not abundant and were mostly dispersed in the middle of the villous *lamina propria,* but only rarely adjacent to the basal membrane of the intestinal enterocytes. These cells were more frequent within the *lamina propria* of the villi in the CPL group of pigs than in the CONTROLS. Even more numerous CD45 RC^+ memory T cells were observed in the villous *lamina propria* of the jejunum of the pigs from the VACCINE group. These cells were predominantly found in the villous *lamina propria* and in the IFA, but were less frequent in the FA of the jejunal PP of the pigs that received the VACCINE and CPL. Due to the nature of the disease, challenges in PWD vaccine development will continue to exist. Although our research approach was at least partially successful, developing a safe and immunogenically effective live oral vaccine against PWD that will provide solid protection and sustained cellular and humoral immune responses remains a significant challenge

Key words: immunogenicity; bivalent; live vaccine; non-enterotoxigenic Escherichia coli; clinoptilolite; post-weaning diarrhoea; pigs

Introduction

Postweaning colibacillosis, associated with enterotoxigenic Escherichia coli (ETEC) strains affects pigs in all swine-rearing countries and has been recognized as an epidemiologically and economically significant diarrheal disease of pigs for over 50 years. It usually occurs about 1-2 weeks following weaning or after abrupt changes in the environment and nutrition. Antimicrobial intervention has often been practised by using various antibiotics as performance enhancers in food producing animals for almost 35 years, but antimicrobial resistance has led to the prohibition of this strategy over the long term (SUN and KIM, 2017). The total prohibition of antibiotic growth promoters (AGP) in the EU and the USA since 2006 and 2017, respectively, and their use in swine production has had a serious impact on both the health and productivity of pigs, underlining the need to develop alternative strategies to prevent postweaning colibacillosis. Since then, a great amount of research has been focused on alternatives to the use of AGP against enteric diseases in order to prevent postweaning diarrheal disease (PWD) in pigs, and many excellent reviews have been published on this issue (GALLOIS et al., 2009; LALLES et al., 2009; VONDRUSKOVA et al., 2010; ZHANG, 2014; LUPPI, 2017; SUN and KIM, 2017; LUISE et al., 2019; DUAN et al., 2020). Generally, seven strategies should be considered when establishing control measures for PWD-affected swine farms. First, management interventions that include minimizing microenvironmental stressors (particularly in farrowing and nursery facilities) and using an all in/all out system, with thorough sanitation, are inevitable. Second, nutritional strategies that include creep, restricted and/or multiple feedings after weaning, adding functional feedstuffs (such as those high in fibre and low in protein, blood plasma and egg yolk antibodies) acidifiers (organic or inorganic acids), antimicrobial minerals (zinc oxide, and copper sulphate), and direct feeding with selected microbiotics, phytobiotics or feed additives (such as nucleotides, feed enzymes,

prebiotic oligosaccharides β-glucan and mannan, clay and zeolite minerals) may enhance intestinal health and thus indirectly but effectively prevent PWD. Further, antimicrobial strategies have been often used various antibiotics and were formerly the most effective way to prevent PWD, however, with increased bacterial resistance to antibiotics, alternatives to antibiotics are urgently needed.

Thus, immunoprophylaxis may be successful through both passive immunity (after oral ingestion of plasma immunoglobulins containing allogeneic antibodies which is, however, only applicable very shortly after farrowing) and active immunity (following production of autologous antibodies against specific adhesins and/or toxins), which can be induced by oral vaccination with live or attenuated ETEC strains that produce F4 and F18 fimbriae but lack toxin genes. This has been shown to be the most promising strategy against PWD. Moreover, another nutritional intervention, known as a competitive exclusion dietary regime, whereby enterocyte receptors for adherence of ETEC strains are occupied by nonvirulent agents/ nontoxic molecules, such as probiotics or their structural components prebiotics, may have potential for broadening protective efficacy against PWD in combination with non-ETEC vaccine candidates. Finally, there are two more approaches, one of which is based on eradication of PWD in herds negative for F4+ and F18+ ETEC strains by careful scrutiny of disease history in the breeding stock, and by using depopulation and disinfection. The other is based on natural resistance to PWD in pigs genetically lacking intestinal receptors for F18 and/or F4 fimbriae, which may help to develop breeding for genetically related resistance to some forms of colibacillosis. In swine, passive lactogenic protection by maternal antibodies is rapidly lost after weaning and active mucosal immunity is needed to protect against PWD. Thus, active intestinal mucosal immunization with oral vaccines that is able to stimulate the mucosal immune system, particularly production of antigen-specific secretory IgA responses, and to establish protective immunity against F4+ and F18+ ETEC strains on the intestinal mucosal surfaces, is the logical and the most rational approach to preventing and controlling PWD (NADEAU et al., 2017). Besides, a high prevalence of both F4 and F18-ETEC in PWD cases in European countries was recently reported (LUPPI et al., 2016). Hence, oral vaccination of weaned pigs against both F4+ and F18+ ETEC represents a sustainable, practical, and effective goal in this situation. Administration of live attenuated or live wild-type non-ETEC strains carrying fimbrial adhesins to immunized pigs is performed by the oral route using either a syringe or intragstrical sonde, or via drinking water to nursery pigs at weaning or at 4 weeks of age. This can provide intestinal colonization by the vaccinal strain(s) and stimulation of intestinal immunity, resulting in an increase in secretory IgA antibodies on the mucosal surfaces, and finally blocks the adherence of ETEC strains producing F4 and F18 fimbrial antigens A wide range of on-farm studies have shown reduced mortality and reduced use of antibiotics after vaccination with a live attenuated or live F4+ non-ETEC strain in pigs immediately following weaning (RUAN and ZHANG, 2013; FAIRBROTHER et al., 2016). The immunogenicity and protective potential of these strains were already tested/evaluated as live monovalent vaccine candidates in *in vivo* studies on weaned pigs, as reported by numerous researchers (VALPOTIĆ et al., 1994; BOŽIĆ et al., 2002; VIJTIUK et al., 2005; BOŽIĆ et al., 2006; KOVŠCA-JANJATOVIĆ et al., 2009; KOVŠCA-JANJATOVIĆ et al., 2010; FAIRBROTHER et al., 2016), but data on avirulent bivalent vaccines against porcine coli diarrhoea and/or coli enterotoxaemia are still scarce (NADEAU et al., 2017). Although these vaccines, prepared as either live attenuated or wild type avirulent non-ETEC vaccines, are a promising category of vaccines against PWD, further research is needed to improve the stability and efficacy of such vaccines, and consequently to facilitate the development of a combined vaccine against both F4 and F18 infections (MELKEBEEK et al., 2013). Furthermore, an alternative strategy to AGP might be via parenteral immunization, although these vaccines generally stimulate systemic rather than gut mucosal immune responses.

However, RUAN et al. (2011) reported that the use of a tripartite FaeG-FedF-LT192 A2: B fusion

antigen, constructed using epitopes from adhesive subunits of predominant fimbriae (F4 and F18), which resulted in the induction of IgA antibodies in serum, faeces and intestinal washes that neutralized cholera toxin (CT), inhibited adherence of F4 and F18 and protected pigs against clinical signs of PWD after F4+ ETEC infection. The use of a fusion protein/antigen might be an interesting approach towards the development of a multivalent vaccine against multi virulence factors produced by ETEC strains that induce PWD. Recent results show the potential of such a vaccine candidate, targeting all 5 fimbriae of ETEC strains, which indicates promising broad protection against PWD (DUAN et al., 2020). Development of broadly protective vaccines against PWD should remain a top priority for veterinary science and practice, and future work will contribute to optimizing the vaccine format to elicit both systemic and gut mucosal immune responses, to verify the protective efficacy induced against one of the most important swine diseases, for which there are currently no prevention strategies to effectively protect against it (ZHANG, 2014). With the still increasing incidence of E. coli–associated PWD, and the resistance of E. coli strains to antibiotics, it is important and urgent to introduce naturally-originated and efficient alternatives to the use of conventional AGPs, which are now forbidden as feed additives in food animals. The current study was designed to evaluate/investigate: (1) the efficacy of an oral bivalent F4ac+/F18ac+ non-ETEC vaccine candidate in stimulating systemic and intestinal cellular immunity in 4-week-old weaned pigs, (2) the onset and duration of the protective immunity of weaned pigs against naturally occurring PWD during the critical period of 6 weeks following weaning, and (3) the dietary supplement potential of zeolite clinoptilolite (CPL) as an antimicrobial mineral and/or immunomodulator as well as the vaccine adjuvant.

Materials and methods

Bivalent vaccine candidate non-ETEC 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 (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^{0}C until used.

Pigs and treatments. Fifty-six crossbred pigs (Swedish Landrace x Yorkshire x Pietrain) were used, of both sexes and an average body weight of 7.05 kg, as the progeny of five litters (from 3rd parity sows) from a large-scale swine farm in eastern Croatia. The pigs were weaned at 26 days of age, housed, managed and fed with a standard weaner diet, according to the rearing technology of the farm. 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). Two days after weaning, at 28 days of age, the pigs were randomly divided into four groups comprising 14 animals each, ear-tagged with numbers 1-14 and kept in the same rearing facility of the farm in separate pens, as detailed previously (VALPOTIĆ et al. 2014). The experimental diets were corn- and soybean meal-based, and formulated as standard weaner Phase 1 (from Day 0 to Day 21) and Phase 2 (from Day 22 to Day 42 of the study) diets to meet their nutrient requirements (VALPOTIĆ et al., 2016). At 28 days of age (or Day 0 of the study) the pigs were treated as follows: the control group (1) received 60 mL of Trypticase soya broth (TSB) intragastrically (i. g.) as a placebo. The diet for the pigs from the principal group (2) that were vaccinated i. g. with 1010 colony forming units (CFU) mL of F4ac+ F18ac+ non- ETEC vaccine candidate in 60 mL of TSB, was also not supplemented with CPL, whereas the diets for both the remaining groups of principal pigs were either supplemented with 0.5% of zeolite CPL (Vetamin®, Panaceo, Austria) only (3) or treated i. g. with the bivalent vaccine candidate in combination with the in-feed CPL supplement (4). The experiment was conducted for

a period of 42 days or until 70 days of life. The pigs were monitored daily and weighed/sampled (for peripheral blood samples and rectal swabs) at seven-day intervals starting at Day 0 before the treatments.

Growth performance. The pigs were weighed at weekly intervals during the experiment, and changes in their body mass were recorded. The changes in body mass within the experimental group 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, 21, 28, 35 and 42 of the experiment, in comparison to the average body weight of the pigs from the non-treated group.

Diarrhoea evaluation. The pigs were monitored daily for diarrhoea and/or other clinical signs of gut disorders (such as anorexia and weight loss) and the incidence/severity of diarrhoea 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 42 days. In addition to morbidity due to diarrheal disease, mortality was also monitored, and dead pigs were necropsied and examined for gross pathology changes.

Bacteriological analysis of intestinal microbiota. In order to monitor gut health, we also determined the bacterial species/serovars isolated from the rectal swabs and jejunal content. Rectal swabs (r. s.) were taken from all the pigs per group on Days 0, 21, and 42 of the experiment, for isolation and serotyping of the enteric bacteria. On Day 0 and Day 42 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 mid jejunum with digestive content was first ligated and taken for counting of intraluminal bacteria. For determination of the total number of E. coli cells in 1 mL of the jejunal content (CFU/mL), the samples were diluted in serial dilutions up to 1010 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 to two Petri dishes, into which Tryptone-bile glucuronic medium (TBX; contains bile salts No. 3 and 5-bromo-4-chloro-3-indolil β-d-glucuronic acid (BCIG)) was poured. 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 were calculated as detailed earlier (VALPOTIĆ et al., 2014). For identification and serotyping, one loop (0.1 mL) of the jejunal 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 the specific commercial antisera (Denka Seiken, Japan). The haemolytic isolates of E. coli were identified by plating the jejunal content onto TSB with 5% of defibrinated sheep blood with esculine. The identification of 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 jejunal content (VALPOTIĆ et al., 2014).

Analysis of peripheral blood lymphoid cell subsets by the flow cytometry (FCM). At the same weekly intervals blood samples (1 mL) from 7 of the 14 pigs from each group (ear-tagged with numbers 1-7) were taken from the vv. cava cranialis into glass tubes (Beckton Dickinson Plymouth, UK) with ethylenediaminetetraacetic acid (EDTA) as the anticoagulant (Sigma, St. Louis, USA) for FCM analysis. The monoclonal antibodies (mAbs) reactive with swine leukocyte surface molecules, i.e. the clusters 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 been described previously (VALPOTIĆ et al., 2014)). Single cell suspensions (100 µL) were prepared in triplicate (comprising 10 000 cells each), incubated

with mAbs (50 µL) and processed as described previously (BOZIC 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). Isotype-matched mouse immunoglobulins were used to detect nonspecific fluorescence in the control cell suspensions.

Immunohistological analysis of intestinal naïve and memory immune cells. Immediately following euthanasia on either Day 0 or Day 42 of the experiment, five specimens of the mid jejunum from each of two pigs per group (either 5-6 cm from 4-week-old pigs or 8-9 cm from 10-week-old pigs, respectively) were taken and fixed in 10% neutral-buffered formalin (pH 7.0i7.6) for 24 h until used for immunohistology analysis. After fixation, the specimens of jejunum 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 jejunal lymphoid cell subsets, with naïve and memory 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). The 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 porcine CD45RA+ naive or CD45RC+ memory T cells, respectively, and secondary polyclonal Abs conjugated with horseradish peroxidase were used to study the in situ identification and distribution patterns of these cells residing the jejunal 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 tested cell subsets were photographed by digital camera (DMX1200, Nikon, Tokyo, Japan).

Statistical analyses. Statistical analyses of the 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 animal effect 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 the statistical model included 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

Growth kinetics. The pigs immunized either with bivalent live F4ac+ F18ac+ non-ETEC vaccine (with an exception at Day 35) or its combination with dietary CPL had significantly increased body weight gain from Day 7 to Day 42 ($P<0.05$) of the experiment, respectively, compared to the control pigs (Table 1).

Table 1. Comparative changes in the body weight (BW) of weaned pigs supplemented with dietary CPL, perorally immunized with either a live F4ac$^+$ F18ac$^+$ non-ETEC vaccine (VACCINE) candidate against porcine colibacillosis or with their combination (VACCINE + CPL) at Day 0 (or 4 weeks of age) during 6 weeks of the experiment

Treatment of pigs*		Day of the experiment (0 = 28 days of age)							Pooled SEM values
		0	7	14	21	28	35	42	
CONTROL**	MEAN BW (kg) ***	7.34^{A}	8.75^{A}	10.29^{A}	11.43^{A}	14.19^{A}	19.16^{A}	23.66^{A}	0.141
VACCINE		7.19^{AB}	9.02^{C}	10.78^{B}	12.17^{C}	15.03^{B}	18.59^{C}	24.24^{C}	0.133
CPL		7.08^{B}	7.50^{B}	10.11^{A}	11.72^{B}	14.25^{A}	17.93^{B}	20.34^{B}	0.132
VACCINE + CPL		6.57^{C}	9.97^{D}	10.80^{B}	12.54^{D}	14.98^{B}	19.84^{D}	24.93^{D}	0.151

*Groups comprised 14 pigs each; **Control pigs received *p. o.* 60 mL of TSB as a placebo, and in addition to this group of pigs the VACCINE group of pigs was also not supplemented with CPL during the 42 days of the experiment; ***Values with different superscripts in the same column differ significantly between the groups at P<0.05 or lower.

Also, these pigs had significantly higher (P<0.05) average body weight (24.9 kg or 24.2 kg vs. 23.7 kg, respectively) on Day 42 of the experiment. Conversely, the pigs that were only supplemented with CPL had mostly significantly lower (P<0.05) body weight during experimental period of 6 weeks, with the exception of Day 21 when the control pigs had slightly but significantly lower body weight (P<0.05).

Morbidity and mortality rates due to postweaning diarrhoea. The pigs that received a single dose of bivalent live F4ac+ F18ac+ non-ETEC vaccine candidate and were fed with CPL supplement were neither diarrheic nor were there any mortalities during the entire period of the experiment (Table 2). However, the pigs that were only either vaccinated or just fed with the CPL supplemented diet had a much lower incidence of diarrhoea (by 59.98% and 39.99%, respectively) than the control pigs.

Table 2. Incidence and severity of postweaning diarrhoea, and mortality of diarrheic pigs in-feed treated on Day 0 (or 4 weeks of age) with either CPL or *p. o.* with a live F4ac$^+$ F18ac$^+$ non-ETEC vaccine (VACCINE) candidate against porcine colibacillosis, or with their combination (VACCINE + CPL) during the 6 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	5/14 (35.71)	12	/	0.29	/	3/14 (21.43)
VACCINE	2/14 (14.29)	4	- 66.67	0.10	- 65.52	1/14 (7.14)
CPL	3/14 (21.43)	4	- 66.67	0.10	- 65,52	1/14 (7.14)
VACCINE + CPL	0/14 (0.00)	0	- 100.00	0.00	- 100,00	0/14 (0.00)

*Control pigs received *p. o.* 60 mL of TSB as a placebo, and in addition to this group of pigs the VACCINE group of pigs was not supplemented with the CPL during the 42 days of the experiment; **Groups comprised 14 pigs each; ***diarrhoea severity score (DSS): 0 = normal faeces, 1 = soft faeces, 2 = fluid faeces or 3 = projectile diarrhoea as summarized during 42 days of the experiment; ****Sum of DSS/42 days.

Also, the pigs from these groups had a considerably lower mortality rate (66.68% lower) compared to the controls. The control pigs had three times higher total DSS than the principal pigs (12 vs. 4) from both the vaccine treated and the CPL supplemented group. Thus, the total DSS observed in both these groups of principal pigs was 66.67% lower than in the controls. Also, the control pigs had an almost three times higher ADS ratio than the principal pigs (0.29 vs. 0.10). Hence, the pigs from these two principal groups had a 65.52% lower ADS ratio after 42 days of the experiment compared to the controls.

*Intestinal bacteriological parameters.*The haemolytic/nonhaemolytic *E. coli* isolate counts were higher in the controls than in the principals (4/2 *vs.* 1/0, 2/2 or 2/2) on Day 0 before the treatments regardless of the group of pigs (Table 3).

Table 3. Bacterial isolates from rectal swabs (r. s.) and the number of bacteria (CFU mL) in the jejunal contents (j.c.) from weaned pigs supplemented with dietary CPL (from Day 0 to 42 of the study, or Day 28 to Day 70 of life), intragastrically immunized (on Day 0) with either the F4ac$^+$ F18ac$^+$ non-ETEC vaccine candidate (VACCINE) against porcine postweaning colidiarrhoea or with their combination (VACCINE + CPL) during the 6 weeks of the experiment

Day of the study	Bacterial isolates* (r. .s.)/ No. of bacteria (j. c.)	Treatment of pigs ****			
		CONTROL ***	VACCINE	CPL	VACCINE + CPL
0	*E. coli* O157:K119:F18ac	+	+		
	E. coli (nHly) + *Enterococcus* spp.	+		+	+
	E. coli (nHly)	+		+	+
	Negative findings (NF)		+	+	+
	E. coli O149:K91:F4ac (Hly) + *Enterococcus* spp.	+	+	+	++
	Enterococcus spp.		+++	+	+
	Enterococcus faecium			+	+
	E. coli O149:K91:F4ac (Hly)	+ +		+	
	E. coli O8:K87:F4ac (Hly)	+			
	CFU/mL	27 x 10^8	31 x 10^8	29 x 10^8	28 x 10^8
42	*E. coli* (nHly)		+++		
	Enterococcus faecium	+	+	++	++
	E. coli (nHly) + *Enterococcus* spp.	+		++	+
	E. coli O149:K91:F4 (Hly)	+			
	E. coli O157:K119:F18ac (Hly) + *Enterococcus* spp.	+	+		
	E. coli O149:K91:F4 (Hly) + *Enterococcus* spp.	+			
	E. coli O8:K87:F4ac (Hly) + *E. faecium*	+			
	*E. coli (*nHly) + *E. faecium*		++		+
	E. coli O157:K119:F18ac	+		+	
	NF		+	++	+++
	CFU/mL	30 x 10^8	18 x 10^7	14 x 10^7	13 x 10^7

*Species/serovar of the isolates = no. of positive (+) or negative (-) findings (NF) per pig/group; Hly = haemolytic or nHly = nonhemolytic; **r. s. and j. c. were taken either from all pigs per group on Days 0, 21 and 42, or from 2 euthanatized pigs per group on Days 0 and 42 of the study, respectively; ***Standard weaner diet only (nontreated CONTROL and VACCINE-treated pigs) or the diet supplemented with 0.5% CPL; ****Groups comprised 14 pigs each on Day 0 of the study.

After the treatments and the period of 6 weeks of the experiment, on Day 42 the control pigs had very similar findings (4/1) to those recorded on Day 0 (4/2) with regard to haemolytic/nonhaemolytic isolates. In contrast, the principal groups of pigs had either lower numbers of haemolytic isolates, (0/3 - CPL group and 0/2 - VACCINE + CPL group), or the same as recorded on Day 0 (1/5) in the pigs from the VACCINE group. On Day 42 the total bacterial load in the jejunum was much lower in the pigs in all three principal groups (VACCINE, CPL and VACCINE + CPL) than in the control pigs (30×10^8 *vs.* 18×10^7 *vs.* 14×10^7 *vs.* 13×10^7 CFU/mL, respectively) at the end of the study (Table 3).

Systemic circulating immune cell responses. Changes in the proportion of 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 during the 42 days of the experiment (Figs. 1 to 4). Regarding $CD4^+$ T cells, specific immunization with either $F4ac^+$ $F18ac^+$ non-ETEC vaccine candidate (VACCINE) or with the bivalent vaccine in combination with CPL supplementation (VACCINE + CPL) stimulated a significantly higher proportion of these cells ($P<0.05$) from Day 7 to Day 42 of the experiment compared to the values obtain in the control pigs (Fig. 1).

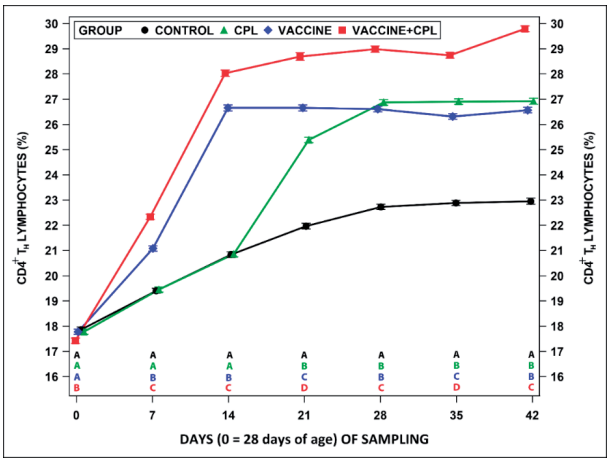

Fig. 1. Changes in $CD4^+$ T cell proportions in the peripheral blood of weaned pigs supplemented on day 0 with either dietary clinoptilolite (CPL) or perorally immunized with the $F4ac^+$ $F18ac^+$ non-ETEC vaccine (VACCINE) candidate against porcine colibacillosis, and with their combination (VACCINE + CPL) during the 6 weeks of the experiment. Values with different letters on the same day differ significantly at $P<0.05$ or lower

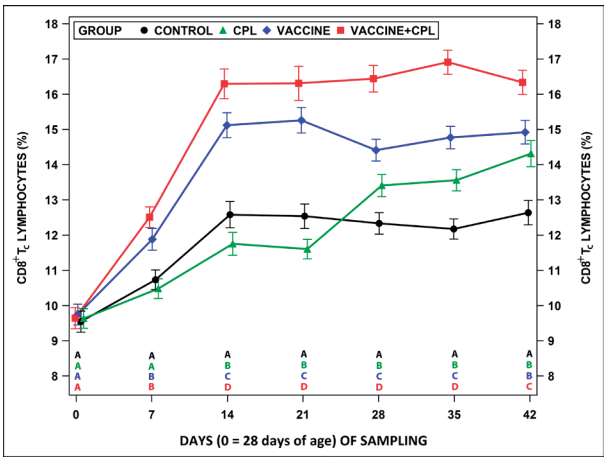

Fig. 2. Changes in $CD8^+$ T cell proportions in the peripheral blood of weaned pigs supplemented on day 0 with either dietary clinoptilolite (CPL) or perorally immunized with the $F4ac^+$ $F18ac^+$ non-ETEC vaccine (VACCINE) candidate against porcine colibacillosis, and with their combination (VACCINE + CPL) during the 6 weeks of the experiment. Values with different letters on the same day differ significantly at $P<0.05$ or lower

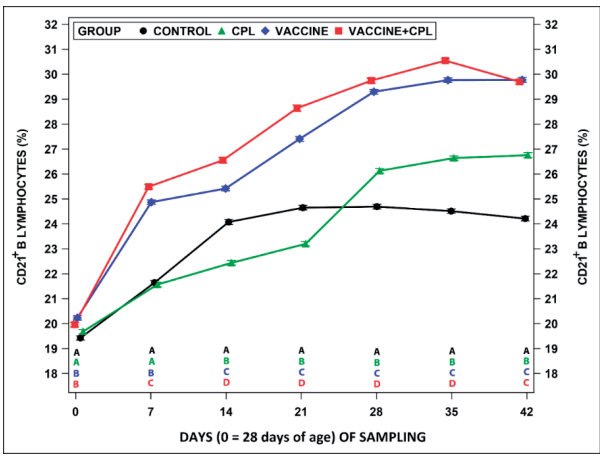

Fig. 3. Changes in $CD21^+$ B cell proportions in the peripheral blood of weaned pigs supplemented on day 0 with either dietary clinoptilolite (CPL) or perorally immunized with the $F4ac^+$ $F18ac^+$ non-ETEC vaccine (VACCINE) candidate against porcine colibacillosis, and with their combination (VACCINE + CPL) during the 6 weeks of the experiment. Values with different letters on the same day differ significantly at $P<0.05$ or lower

The pigs only fed CPL supplement also had a significantly higher proportion of $CD4^+$ T cells ($P<0.05$) than the controls, but in the period from Day 21 to the end of the experiment. The proportion of $CD8^+$ T cells was much higher in the pigs that

were treated with either VACCINE or VACCINE + CPL (P<0.05 and P<0.05, respectively) than in the control pigs over the entire course of the post treatment period, *i. e.* from Day 7 to Day 42 (Fig. 2). Interestingly, the pigs treated with only CPL had either a significantly lower proportion of this T cell subset (P<0.05) from Day 14 to Day 21 or it increased from Day 28 to Day 42 (P<0.05) of the experiment.

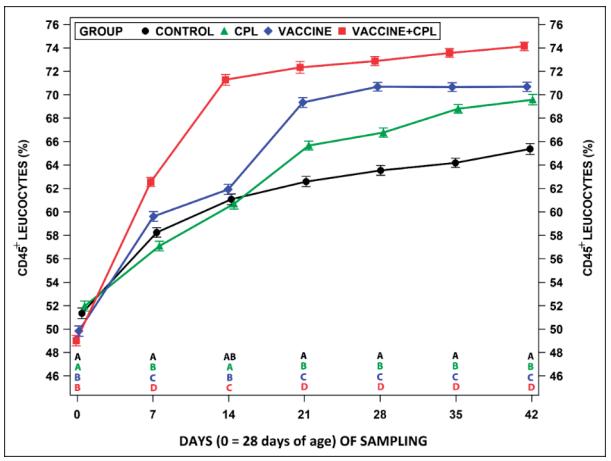

Fig. 4. Changes in CD45$^+$ lymphoid cell proportions in the peripheral blood of weaned pigs supplemented on day 0 with either dietary clinoptilolite (CPL) or perorally immunized with the F4ac$^+$ F18ac$^+$ non-ETEC vaccine (VACCINE) candidate against porcine colibacillosis, and with their combination (VACCINE + CPL) during the 6 weeks of the experiment. Values with different letters on the same day differ significantly at *P*<0.05 or lower

Quite similar findings were obtained for CD21$^+$ B cells as their proportion rose significantly in the pigs treated with either VACCINE or VACCINE + CPL (P<0.05 and P<0.05, respectively) during the same period of the study (Fig. 3). Also, the pigs supplemented with CPL had either much lower values for this cell subset (P<0.05) from Day 14 to Day 21 or much higher values (P<0.05) from Day 28 to Day 42 of the study.

The pigs from the principal groups had a significantly higher proportion of CD45$^+$ lymphoid cells than the pigs from the control group (P<0.05) during the period from Day 21 to Day 42 (Fig. 4). A significantly higher proportion of these cells was also recorded on Day 14 of the experiment, but only for the pigs treated with VACCINE + CPL, whereas the two other treatments did not induce significant differences between the experimental groups of pigs. However, on Day 7 of the study the pigs treated with either VACCINE or VACCINE + CPL had significantly higher proportions of CD45$^+$ lymphoid cells (P<0.05), while the pigs fed the CPL supplement had significantly lower levels of these cells (P<0.05).

Identification and distribution patterns of gut CD45RA$^+$ and CD45RC$^+$ T cells. The *in situ* immunohistological identification of either small intestinal CD45RA$^+$ naïve or CD45 RC$^+$ memory T cells and their localization/distribution patterns within the mucosal sites of the jejunum in the 10-week-old control and principal pigs, following 6 weeks of the experiment, are shown in Figures 5 and 6, respectively. Rather sparselydistributed CD45RA$^+$ naïve T lymphocytes were observed in the jejunal *lamina propria* of the intestinal villi, within the Lieberkühn crypts (LC), and in the submucosa of the control pigs (Fig. 5a). These cells were very rarely visible adjacent to the basal membrane of enterocytes, or within the epithelial layer. The CD45RA$^+$ cells from the pigs fed a diet supplemented with CPL were similarly distributed within the villous *lamina propria* as was observed in the control pigs (Fig. 5b).

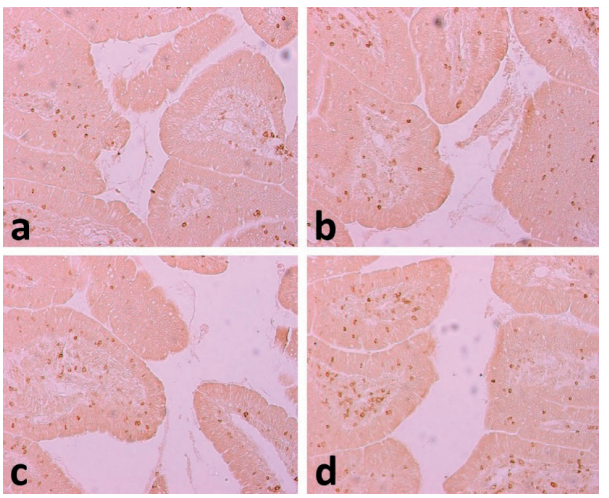

Fig. 5. Identification and localization of CD45 RA$^+$ naive T cells in the jejunal villous *lamina propria* of a10-week-old pig from the control group (a) that received on Day 0 (28 days of age) 60 mL of TSB *p. o.* as a placebo, and the treated groups of pigs that received either dietary immunomodulator CPL (b), *p. o.* F4ac$^+$ F18ac$^+$ non-ETEC vaccine candidate (c) or their combination (d) after the 6 weeks of the experiment, as demonstrated by an indirect IP method using ABC staining; x 200

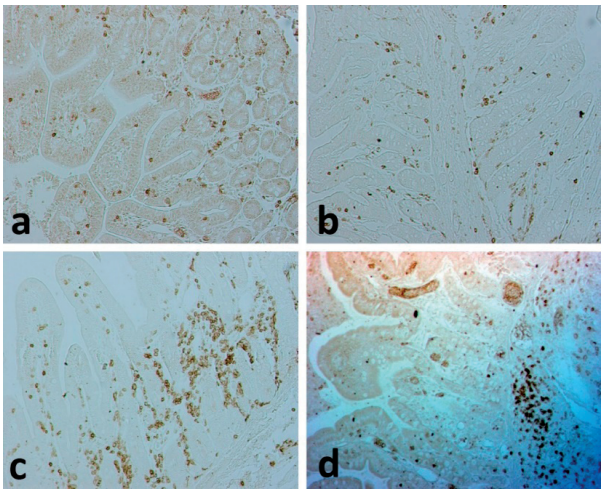

Fig. 6. Identification and localization of CD45 RC$^+$ memory T cells in the jejunal villous *lamina propria* and LC of a 10-week-old pig from the control group (a) that received at Day 0 (28 days of age) 60 mL of TSB *p. o.* as a placebo, and the treated groups of pigs that received either dietary immunomodulator CPL (b), *p. o.* F4ac$^+$ F18ac$^+$ non-ETEC vaccine candidate (c) or their combination (d) after the 6 weeks of the experiment, as demonstrated by an indirect IP method using ABC staining; x 200

However, it was noticed that there were slightly more dense patterns of these cells in the interfollicular areas (IFA) and more strongly in the follicular areas (FA) of the jejunal Peyer's patches (PP) of the CPL-supplemented pigs in comparison to those observed in the control pigs. As observed by microscopic examination, it seems that the treatment with the VACCINE induced moderate recruitment of CD45 RA$^+$ cells within both the IFA and FA of the jejunal PP of the pigs, 6 weeks after the vaccination (Fig. 5c). Namely, numerous CD45RA$^+$ naïve T cells were visible in the *lamina propria* of the intestinal villi, within the LC, and particularly close to the basal membrane of the intestinal epithelial cells. Also, these cells were rarely observed within the jejunal enterocytes. A greater frequency of CD45RA$^+$ cells was observed in the middle of the villous *lamina propria* and in the IFA and FA of the pigs that received the VACCINE and were fed a diet supplemented with CPL as compared to the nonvaccinated/ non-supplemented control pigs (Fig. 5d). Solitary cells of naive immunophenotype were visible within the epithelial cells at the tip of the villi.

The distribution patterns of the CD45 RC$^+$ memory T lymphocytes within the jejunal mucosa/ submucosa from the control pigs after 6 weeks of the experiment are shown in Fig. 6a. In the control pigs the CD45 RC$^+$ cells were not abundant and were mostly dispersed in the middle of the villous *lamina propria*, but only rarely adjacent to the basal membrane of the intestinal enterocytes. These cells were more frequent within the *lamina propria* of the villi in the CPL-supplemented pigs than in the controls (Fig. 6b). Even more numerous CD45 RC$^+$ memory T cells were observed in the villous *lamina propria* of the jejunum of the pigs that had been immunized with the F4ac$^+$ F18ac$^+$ non-ETEC vaccine candidate, 6 weeks after the vaccination (Fig. 6c). These cells were predominantly found in the villous *lamina propria* and in the IFA, but were less frequent in the FA of the jejunal PP of the pigs that received the VACCINE and CPL on day 0 of the experiment (Fig. 6d). The localization and frequency of the naive CD45 RA$^+$ and memory CD45 RC$^+$ T lymphocytes indicated their different distribution patterns within particular tissue structures (the villi, LC, epithelium and *lamina propria*) and areas (IFA, FA, PP) of the jejunal mucosa and submucosa, which may indicate their different functions in the intestinal immune response to intraluminal foreign organisms/ substances such as microbes and their antigens, vaccinal immunogens and/or immunomodulators/ adjuvants.

Discussion

Our study confirmed the efficacy of a live oral bivalent F4/F18 non-ETEC vaccine candidate consisting of avirulent *E. coli* strains, either the recombinant F4ac$^+$ strain 2407 (without plasmid for STb enterotoxin) or an attenuated F18ac$^+$ strain 2143 (that lost toxicity by a special co-culturing procedure, as describedabove) by showing the immunogenicity of both systemic and intestinal cellular immunity, and protective potential for pigs against PWD. Similarly, the live bivalent *E. coli* vaccine against oral challenges with F4$^+$ and F18$^+$ ETEC stimulated systemic production of specific anti-F4 and anti-F18 antibodies of IgM and IgA class, and induced clinical protection of weaned

pigs immunized against PWD (NADEAU et al., 2017). These two studies are fairly complementary with regard to the study design, and comparable regarding the results obtained in relation to the most important question of the onset and duration of protective cellular and humoral immunity, when the immunogenicity of bivalent F4/F18 non-ETEC vaccines is evaluated.

According to our experience and that of other researchers, the promptness and intensity of the local intestinal mucosal immune responses, including both cellular and humoral (particularly secretory IgA production) components is crucial for effective and substantial protection against PWD (VALPOTIĆ et al., 1994; BOŽIĆ et al., 2002; VERDONCK et al., 2004; VIJTIUK et al., 2005; SNOECK et al., 2006: BOŽIĆ et al., 2006; KOVŠCA-JANJATOVIĆ et al., 2009; KOVŠCA-JANJATOVIĆ et al., 2010; DELISLE et al., 2012; FAIRBROTHER et al., 2016).

Porcine gut-associated lymphoid tissues (GALT) within the intestinal mucosa/submucosa sites, their organization and functions are fundamentally important for homeostasis on the mucosal surfaces of the digestive tract. One of their basic immunological functions is that the epithelium of the intestinal mucosa and the GALT constitute an essential physical and immunological barrier against porcine enteric pathogens (BAILEY et al., 2005), such as F4/F18 ETEC strains, which are the most common causative agents of PWD. The intestinal mucosal surfaces represent an entry route for a wide range of pathogenic microbes, including *E. coli,* but also comprise the immune system defences organized as the GALT, which may protect against porcine PWD, and also induce maximal immunity after prospective live attenuated oral F4/F18 vaccines reach the susceptible/ inductive sites, such as the PP (VIJTIUK et al., 2002; BOŽIĆ et al., 2006; SNOECK et al., 2006; KOVŠCA-JANJATOVIĆ et al., 2009; KOVŠCA-JANJATOVIĆ et al., 2010).

The success of vaccination against PWD in our model system depends mainly upon the qualitative and quantitative parameters of intestinal cellular immunity, but is also based on data regarding morbidity (the incidence/severity of diarrhoea) and mortality due to PWD, growth performance and the shedding of virulent strains of ETEC. Hence, we consider that live bivalent (F4/F189 non-ETEC) vaccines against PWD are promising as they target inductive sites of the GALT, such as the PP, to induce and develop intestinal cell-mediated and humoral immunity by producing locally secretory IgA and systemic IgM and IgA antibodies. This approach is still very rarely seen. Growth kinetics were much better in the pigs that were either orally vaccinated with a bivalent F4/F18 non-ETEC vaccine or in addition to the vaccination fed with CPL supplement, in relation to the body weight gain of the controls over the 6 weeks of the study, At the end of the study the principal pigs from these groups also had significantly higher average body weight compared to the controls. However, the pigs fed a diet only supplemented with 0.5% CPL generally had a lower body weight during the experimental period of 6 weeks.

There are controversial reports on the influence of CPL on growth performance in swine. Namely, some authors have suggested that dietary CPL improves body weight gain and decreases the feed conversion rate in growing (from 25 to 70 days of age), but not in finishing pigs (from 71 to 161 days of age) by adding 2% of CPL to the diet (SUBRAMANIAM and KIM, 2015). With the same inclusion rate of 2% of CPL in feed from weaning to slaughter, ALEXOPOULOS et al. (2007) reported the enhanced performance of growing and fattening pigs. Others did not observe any body weight differences in either suckling (7 days of life) or growing pigs (1-45 days of life) fed a diet with either 2% or 0.5% of CPL supplement (SARDI et al., 2002; PRVULOVIĆ et al., 2007), respectively. As our approach in the current study was primarily testing CPL as a dietary supplement with its already reported immunomodulatory properties, (VALPOTIĆ et al., 2016) and thus with putative adjuvanticity for the mucosal non-ETEC vaccine candidate against porcine PWD, rather than as a growth enhancer, the data obtained are not comparable with those in the aforementioned studies. None of the vaccinated and CPL supplemented pigs developed diarrhoea or died during the observation period of the experiment.

Since there are no similar model systems in the available literature, our finding could only be related to the data reported by NADEAU et al. (2017) regarding the protective efficacy of the live oral bivalent vaccine F4/F18 non-ETEC against PWD due to $F4^+$ and $F18^+$ ETEC challenge strains.

These authors also did not record mortality cases among the vaccinated and challenged pigs, but three nonvaccinated control pigs died after 7 days post challenge. However, the identical incidence of mortality, with 3 cases among the controls in our experiment, was recorded on days 17, 20 and 22 after weaning (Day 28 of age and Day 0 of the study), and could be attributed, as found at necropsy, to naturally occurring PWD. Namely, the number of diarrheic pigs in the control group was much higher: 5/14 *vs* 0/14 (35.71 % higher) than in the pigs that received vaccine and were fed with the CPL supplement. The pigs that were either vaccinated or fed a diet supplemented with CPL had fewer diarrheic cases: 2/14 *vs* 5/14 (21.42 % fewer) or 3/14 *vs* 5/14 (14.28 % fewer), respectively, compared with the controls.

Consequently, the mortality rates in the control group of pigs and both the principal groups (either vaccine-treated or fed CPL supplement) also differed (3/14 *vs* 1/14 and 1/14), and were rather lower (by 14.29 % and 14.29%) in the principal pigs. The pigs were monitored daily for general health (body condition, appetite, behaviour, faecal consistency score and dehydration) which was evaluated as detailed earlier (FAIRBROTHER et al., 2016). Our scoring system for diarrhoea was slightly different comprising only 4 levels of diarrhoea severity, expressed as the faeces consistency (0 = normal, 1= soft, 2 = fluid and 3 = profuse/projectile) over the 42 days of the experiment, as mentioned above. Accordingly, the control pigs had a 3 to 12 times higher total DSS, which means that the principal pigs (in the vaccinated or CPL fed group) had either 66.67 % or 100 % lower DSS (vaccinated and fed CPL supplement, respectively). Generally, our results are comparable to those obtained by NADEAU et al. (2017) due to the fact that data of these authors are quite similar regarding mortality/morbidity, and in particular the fact that they also applied F4/F18 bivalent vaccine and successfully protected pigs against PWD. Faecal shedding analyses of faecal samples from the rectal swabs confirmed the presence of the vaccinal $F18ac^+$ non-ETEC strain (O157: K119: F18ac) in the pigs from the control group and the VACCINE group, and the absence of the $F4ac^+$ non-ETEC strain (O9: K36: H19: F4ac, LT^- STb) before vaccination on Day 0. The latter strain was not identified in the rectal swab samples from any pigs regardless of the group during the experiment. Interestingly, the $F18ac^+$ non-ETEC strain was isolated at the end of the experiment (Day 42) from 2 samples from pigs, one from the CONTROL and one from the CPL group, but again not from the VACCINE group.

However, quantitative analyses of CFU per mL of jejunal contents from 2 pigs per group euthanized on Days 0 and 42 of the study showed the immunogenic potential of applied treatments. The values for CFU/mL on Day 0 of the study ranged between the groups of pigs from 27 x 10^8 (CONTROLS) to 31 x 10^8 (VACCINE), but after 6 weeks following the treatments these values were significantly lower, as recorded on Day 42 of the study, ranging from 13 x 10^7 (VACCINE + CPL) to 18 x 10^7 (VACCINE). The value obtained for the control pigs was slightly increased on Day 42 (30 x 10^8 *vs* 27 x 10^8 CFU/mL) in relation to Day 0 of the study, but it is certain that non-treated control pigs were not protected against potential development of PWD like the treated groups of pigs, according to the clinical and bacteriological examinations and parameters obtained. Thus, it is very likely that the pigs that either received F4/F18 non-ETEC vaccine candidate alone or combined with dietary CPL, or that were fed with CPL supplement induced active protective systemic and intestinal immunity against naturally occurring ETEC infection and the development of PWD. Similar protection has been reported from a monovalent F4 vaccine (FAIRBROTHER et al., 2016) and bivalent F4/F18 vaccine NADEAU et al., 2017) in either an F4-ETEC or an 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).

However, in our study we evaluated the immunogenicity of the oral bivalent F4/F18 vaccine candidate for recruitment of both systemic and local intestinal cellular immunity, which were obviously at protective levels as seen in the reduced onset and duration of PWD clinical signs and faecal shedding of F4- ETEC and F18-ETEC. Immunity against each of the fimbriae F4 and F18 has been previously reported in relation to serological antibody kinetics and levels, for monovalent vaccines (MELKEBEEK et al., 2013; FAIRBROTHER et al., 2016). However, we also tested this immunity by immunohistological identification/localization and histomorphometric quantification of cellular proliferation/distribution patterns, in order to assess its significance and the protective potential of either $F4^+$ or $F18^+$ non-ETEC vaccine candidate strains (BOŽIĆ et al., 2006; KOVŠCA-JANJATOVIĆ et al., 2009; KOVŠCA-JANJATOVIĆ et al., 2010; KOVŠCA-JANJATOVIĆ et al., 2011), or $F4ac^+$ pathogenic ETEC strains (VALPOTIĆ et al., 1994; LACKOVIĆ et al., 1997).

Namely, we based our approach on the fact that intestinal mucosal surfaces represent the entry route for harmless dietary antigens, but also for harmful viral and bacterial pathogens, including porcine ETEC strains, the etiological agents of PWD, which may alter or awaken the GALT defences. Hence, it is important for prospective vaccines to stimulate maximal immunity at these susceptible sites, and they should include live avirulent non-ETEC strains carrying F4 and/or F18 adhesins (FAIRBROTHER et al., 2005). More recent studies have shown that live monovalent and bivalent vaccines against PWD, when applied orally, stimulated both systemic and intestinal humoral immunity by serum or mucosal IgM and IgA antibodies, and that there were no indicative differences between the kinetics and intensity of these responses (NADEAU et al., 2017; FAIRBROTHER et al., 2016). Our data on the systemic cellular immunity that developed following oral immunization with F4/F18 non-ETEC are not comparable with those from these authors, with the exception that we observed slightly earlier maximum responses (from Day7 to 14) of $CD4^+$, $CD8^+$ T cells and $CD21^+$ B cells, but not of $CD45^+$ lymphoid cells (from Day 21 to 42) than those they recorded for serum IgM and IgG antibodies (NADEAU et al., 2017). However, this is logical since mucosal immune T and B cells, after contact with foreign antigens or, in our case, with vaccinal immunogens of the bivalent non-ETEC vaccine, immediately migrate from the intestinal mucosa to the systemic circulation to disseminate information to other immune cells residing in other mucosal sites. We also examined the distribution and quantitative patterns of jejunal T cell subsets CD45RA and CD45RC that are of great relevance for the establishment and duration of protective immunity, following either on-farm naturally-occurring ETEC infection or experimentally induced peroral immunization with $F4ac^+$ and $F18ac^+$ non-ETEC vaccine candidate strains. The functional phenotype of these subsets is expressed as either immunologically naïve in $CD45RA^+$ or immunologically memory in $CD45RC^+$ T cells (BOŽIĆ et al., 2002).

The localization of naïve $CD45RA^+$ and memory $CD45RC^+$ T cells showed their different distribution patterns within particular areas of the mucosa and submucosa, indicating their different functions in intestinal immune responses (KOVŠCA-JANJATOVIĆ et al., 2009; KOVŠCA-JANJATOVIĆ et al., 2010). These authors noticed that $CD45RC^+$ cells were mostly found in the villous *lamina propria* and in the IFA, but quite rarely in the PP, which is agreement with our findings. In their study, however, they also reported the presence of microfold (M) cells within the intestinal epithelial layer, particularly covering the PP, and since their role is the intake and transport of intraluminal pathogens or their products to the immune cells within the *lamina propria* their number was higher in the pigs that had been immunized with a bivalent F$4F18 non-ETEC vaccine (KOVŠCA JANJATOVIĆ et al., 2011). Visually, the localization/distribution patterns of jejunal naïve CD45RA and memory CD45RC cells are in accordance with those demonstrated in our previous studies (BOŽIĆ et al., 2006; SNOECK et

al., 2006; KOVŠCA-JANJATOVIĆ et al., 2009; KOVŠCA-JANJATOVIĆ et al., 2010; KOVŠCA-JANJATOVIĆ et al., 2011).

However, the present study did not include quantitative histomorphometric data, which is the only difference between its study design and those of our earlier studies on this issue cited above. Although progress has been made in the past few decades in developing effective vaccines against PWD, challenges still exist due to the disease's complexity and the immunological heterogeneity among ETEC strains. However, recent progress in using a multiple fusion antigen approach to developing multivalent vaccines means that broad protection against ETEC-associated PWD should be attained in the near future.

Conclusion

Due to the nature of the disease, challenges in PWD vaccine development will continue to exist. Although our research approach was at least partially successful, developing a safe and immunogenically effective live oral vaccine against PWD that will provide solid protection and sustained cellular and humoral immune responses remains a remarkable challenge.

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SAŽETAK

Dijareja nakon odbića (DNO) prasadi uzrokuje glavninu proizvodnih gubitaka u svinjogojskoj industriji širom svijeta. Ne postoji sigurno i učinkovito cjepivo protiv svinjskih enterotoksigenih sojeva bakterije *Escherichia coli* (ETEC) koji se smatraju etiološkim uzročnicima DNO. Ovo je istraživanje planirano radi vrednovanja: (1) učinkovitosti oralnog bivalentnog F4ac$^+$/F18ac$^+$ ne-ETEC pokusnog živog cjepiva (CJEPIVO) u stimuliranju sistemske i crijevne stanične imunosti 4 tjedna stare odbijene prasadi, (2) pojave i trajanja zaštitne imunosti odbijene prasadi protiv prirodnog zaražavanja uzročnicima DNO tijekom razdoblja od 6 tjedana nakon odbića i (3) potencijala dodatka hrani zeolita klinoptilolita (KPL), antimikrobnog minerala i/ili imunomodulatora/adjuvansa cjepiva (CJEPIVO + KPL). Prasad imunizirana ili cjepivom ili njegovom kombinacijom s dodatkom hrani KPL-om imala je znakovito povećan prirast tjelesne mase od 7. do 42. dana (P<0,05) pokusa u usporedbi s kontrolnom skupinom prasadi (KONTROLA). Suprotno tome, prasad koja su primila samo dodatak hrani KPL, imala su pretežito znakovito nižu (P<0,05) tjelesnu masu. Prasad koja je primila CJEPIVO + KPL nije imala ni dijareju, niti je bilo uginuća tijekom razdoblja trajanja pokusa. Ukupni broj izdvojenih bakterija iz jejunuma bio je mnogo manji u prasadi iz sve tri pokusne skupine od onoga u kontrolne prasadi (30 x 10^8 *vs.* 18 x 10^7 *vs.* 14 x 10^7 *vs.* 13 x 10^7 CFU/mL). Specifična imunizacija s CJEPIVOM ili CJEPIVOM + KPL stimulirala je znakovit porast udjela CD4$^+$ i CD8$^+$ T stanica (P<0,05) od 7. do 42. dana pokusa. Vrlo su slični nalazi dobiveni za CD21$^+$ B stanice, s obzirom da je njihov udjel bio znakovito povišen u prasadi tretirane ili s CJEPIVOM ili s CJEPIVOM + KPL (P<0,05 ili P<0,05). Prasad iz skupine koja je primila CJEPIVO + KPL, imala je značajno viši udjel CD45$^+$ lifoidnih stanica od prasadi iz KONTROLA skupine (P<0,05). Rijetko raspodjeljeni CD45RA$^+$ naivni T limfociti opaženi su u lamini propriji crijevnih resica jejunuma, u Lieberkühnovim kriptama te u submukozi prasadi KONTROLE skupine i skupine hranjene s dodatkom KPL. Tretman CJEPIVOM izazvao je umjerenu proliferaciju CD45RA$^+$ u interfolikularnim (IFP) i folikularnim područjima (FP) Peyerovih ploča (PP) jejunuma u prasadi 6 tjedana nakon cijepljenja. U KONTROLNE prasadi, CD45RC$^+$ memorijske T stanice nisu bile brojne i uglavnom su bile dispergirane u sredini lamine proprije resica, a samo rijetko u blizini bazalne membrane crijevnih enterocita. Te su stanice brojnije u lamini propriji resica u prasadi iz KPL skupine u odnosu na KONTROLNU prasad. No, CD45RC$^+$ memorijske T stanice još su brojnije, što je primijećeno u lamini propriji resica jejunuma prasadi koja je primila CJEPIVO. Te su stanice pretežito nađene u lamini propriji resica i u IFP-u, ali su manje brojne u FP-u jejunalnih Peyerovih ploča u prasadi koja je primila CJEPIVO i KPL. Zbog naravi bolesti, izazovi vezani za razvoj cjepiva protiv DPO će i dalje postojati. Premda je naš istraživački pristup bio samo djelomično uspješan, razvoj sigurnog i imunogeno učinkovitog živog oralnog cjepiva protiv DPO, koje će pružati solidnu zaštitu i održive stanične i humoralne imunosne odgovore, i nadalje je izvanredan izazov.

Ključne riječi: imunogenost; bivalentno živo cjepivo; ne-enterotoksigena *Escherichia coli*; klinoptilolit; dijareja nakon odbića; prasad