Vaccine 37 (2019) 1340–1349

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Multi-antigenic recombinant subunit vaccine against *Lawsonia intracellularis*: The etiological agent of porcine proliferative enteropathy

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ARTICLE INFO

Article history:
Received 25 September 2018
Received in revised form 10 January 2019
Accepted 11 January 2019
Available online 30 January 2019

Keywords:
Lawsonia intracellularis
Recombinant vaccine
Proliferative enteropathy
Pig immune response

ABSTRACT

Proliferative enteropathy, caused by *Lawsonia intracellularis*, represents a threat for swine industry. Current vaccines are effective but difficult to obtain and scaled up, because of demanding bacterial culture conditions. In this work, a subunit vaccine candidate against *L. intracellularis* was developed and its efficacy was evaluated *in vivo*, alone or co-formulated with pig recombinant IFN-α.

The vaccine formulation contains three chimeric antigens: two outer membrane proteins and a secreted one, which were engineered by adding T epitopes using bioinformatics tools. After simultaneously expressing the three antigens in *E. coli*, its immunogenicity was tested in mice and pigs. Antigens co-formulated with porcine IFN-α were also assayed in the last species. Immune response was assessed by ELISA and qPCR, and histopathological studies of intestinal epithelial tissue were performed after challenge.

Mice and pigs showed an increased IgG response against chimeric antigens. Particularly, there were significant differences in the antibody response when porcine IFN-α was co-administrated with *L. intracellularis* antigens. Besides, mRNAs from *il12* and *cd4* marker were detected during the first week after immunization of pigs, suggesting a Th1-type cellular immune response. The significant enhancement of *oas2* gene expression indicates the effect exerted by porcine IFN-α. Post-mortem histopathological analysis post-challenge revealed damage only into epithelial cells of the gastrointestinal tract from animals of the negative control group. Injuries were related to atrophy of the intestinal villi, where a decrease of globet cells and a greater migration of lymphocytes were observed.

Overall, our results demonstrated that the vaccine candidate elicited significant humoral and cellular immune responses. Besides, histopathological analysis suggested that vaccinated animals were protected against experimental *L. intracellularis* infection. This research constitutes a step forward to the generation of the first recombinant chimeric vaccine against *L. intracellularis*, representing a faster, easier and cost effective approach to counteract the porcine proliferative enteropathy.

1. Introduction

The obligate intracellular bacteria *Lawsonia intracellularis* (*L. intracellularis*) generates significant economics losses in swine by reducing the feed conversion efficiency up to 50% and the average gain weight from 17% to 84% compared to unaffected pigs [1]. Taking into account the cost per infected pig is around $ 10 USD, this microorganism is considered a huge problem for the porcine industry worldwide [2,3]. The disease associated with *L. intracellularis* was first reported in pigs [4]. Also, it was described in horses and rabbits [5–7]. The bacteria use the active mitotically intestinal crypt cells for their propagation [8,9]. As a thickened intestinal mucosa is produced due to proliferation of undifferentiated

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https://doi.org/10.1016/j.vaccine.2019.01.029
0264-410X/

enterocytes, this intestinal infectious disease was called proliferative enteropathy. Anorexia, acute or chronic diarrhea, weight loss and occasionally death are classical sings of this disease.

Damage caused by *L. intracellularis* in pig-producing countries has motivated the development of new and more effective vaccines for counteracting this pathogen. Live attenuated or inactivated vaccines are available in the market (*Enterisol ileitis*, produced by Boehringer-ingelheim and *Porcilis ileitis* produced by Intervet, Merck [10]), which implies a large-scale production of infective bacteria in cell cultures. However, it is well documented that only few laboratories in the world successfully maintain *L. intracellularis* in culture due to the complexity of this culture system [11,12]. Thus, culture scale-up of *L. intracellularis* and the technology transfer to the veterinary pharmaceutical industry remain difficult to achieve.

Specifically, the live attenuate vaccine has additional limitations related to the environmental risk due to the probable pathogen dissemination into countries where the vaccine is being introduced [13]. Also, the simultaneous use of antimicrobial drugs is not allowed, which increases the occurrence of bacterial infections in weaned piglets.

The development of recombinant vaccines based on highly immunogenic proteins containing engineered T cell epitopes could be an effective approach and a potential solution for the pathogenicity produced by *L. intracellularis*. Advancements arising from the knowledge of the entire genome of *L. intracellularis* provide new tools to identify prominent sequences for designing chimeric immunogens. However, there are no available vaccines against *L. intracellularis* based on the identification and recombinant expression of specific antigens from this microorganism [14,15].

Here, we have developed a new multi-antigenic subunit vaccine candidate against *L. intracellularis*, containing two outer membrane proteins and a secreted one, from this pathogen. Epitopes for porcine T and B cells were also identified and incorporated into the design. The proteins contained in the subunit vaccine were produced in *E. coli* and their immunogenicity was demonstrated in mice and pigs. Normal intestinal tissue observed in vaccinated pigs after challenge suggested protection from *L. intracellularis* infection.

2. Materials and methods

2.1. Bacterial strains, restriction endonucleases and animals

SHuffle T7 *E. coli* strain (New England Biolabs, UK) was used for the expression of the recombinant antigens. The restriction endonucleases were purchased from New England Biolabs, UK. C57BL/6 female mice of six weeks old were acquired from Chilean Public Institute of Health to evaluate *in vivo* IgG immune response against the recombinant *L. intracellularis* antigens. Healthy Duroc/Yorkshire piglets of four weeks old were acquired from an intensive pig production of a Chilean farm to performing the immunization and challenge trials. Commercial Ileitis Antibody ELISA (SVANOVA, Sweden) was used to corroborate that pigs were free from *L. intracellularis*.

2.2. Bioinformatic prediction of potential antigens

The *L. intracellularis* entire genome (PHE/MN1-00, N343) was analyzed by Tblastn from the Blast 2.2 database. Open reading frame prediction of potential antigens was carried out by the finder tool NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) [16]. The sequences were selected considering the subcellular localization and the presence of B- and T-cell epitopes based on the Server for Biological Sequences Analysis, CBS. B-cell and T-cell epitopes were predicted by using ABCpred and BCEpred tools respectively, from the Microbial Technology Institute server and NetMHC tools [17–20]. T-cell epitopes with low coverage were replaced and additional T-cell epitopes were included in loops regions (Fig. 1). The prediction of antigenic structures was performed using Robertta-server Protocols (RSP) [21]. The stability and validity of the three models were confirmed by energy minimization analysis using the Swiss-PdbViewer, and the computation Ramachandran plot method using RAMPAGE tool, respectively [22].

2.3. Expression of recombinant antigens

The chimeric DNA sequences were synthetized and cloned in the bacterial expression vector pET-22b by Genescript (https://www.genscript.com/gene_synthesis.html). All sequences were inserted in the same vector, which comprised: T7 promoter/Lac Operator – chimeric gene –6 histidine residues – T7 terminator (Fig. 2). Antigens were expressed in the genetically transformed SHuffle® T7 *E. coli* strain which was grown in liquid LB-Ampicillin medium up to the optical density (OD) of 0.6 and induced with 0.5 mM isopropyl β-D1-thiogalactopyranoside (IPTG) (Santa Cruz Biotechnology, USA). Analytical expression was carried out in 300 mL of LB medium (Liofilchem, Italy) supplemented with 50 μg/mL of ampicillin (USBiological, USA) at 30 °C under constant stirring for 6 h of induction. Batch cultures of 5 L were further developed in a bioreactor (Winpact, USA). The growth parameters were: temperature of 30 °C, pH 7.0 and stirring of 200 rpm until reaching a DO of 0.6, measured at 600 nm. Expression was induced with IPTG 0.5 mM for 12 h. The cell pellet was resuspended in PBS

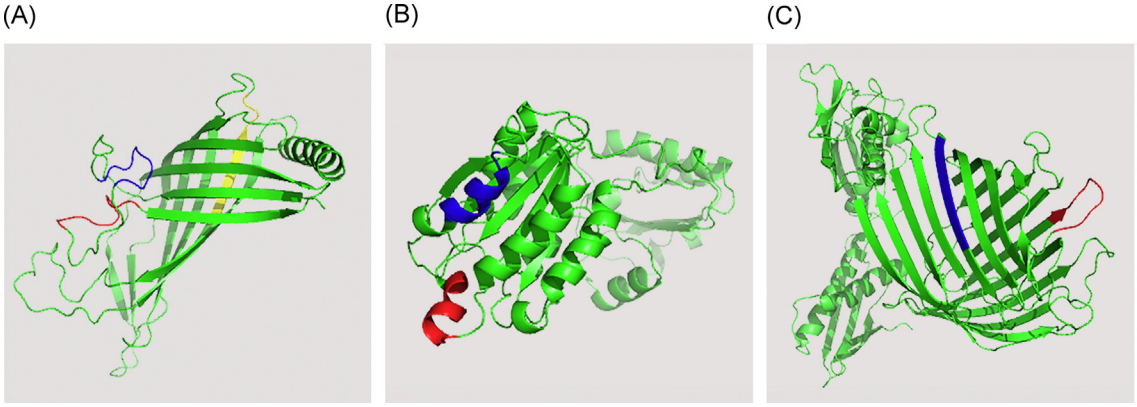

Fig. 1. Structures prediction for chimeric antigens from *L. intracellularis*. Modeling was performed by Robetta-server (http://robetta.bakerlab.org). (A) INVASc, (B) OMP2c and (C) OMP1c. The green color shows the protein backbone; blue (FSFPYWFTF), red (KQFNLNTLL) and yellow (FSYATDLSY) correspond to inserted T-cell epitopes.

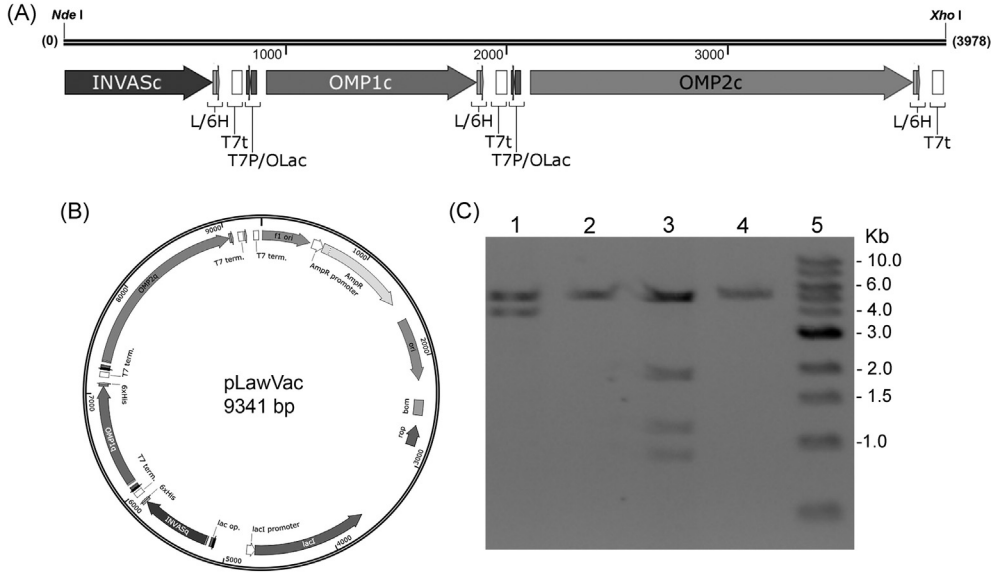

Fig. 2. Design and construction of the pLawVac vector. (A) Transcriptional units for expression of INVASc, OMP1c and OMP2c in *E. coli*. The three consecutive transcriptional units contain: T7 promoter/T7-lacO operator (T7P/OLac), chimeric gene with a spacer and a 6XHist tag (L/6H), and T7 transcriptional terminator (T7t). (B) Map of pLawVac expression vector containing the chimeric genes. (C) Restriction analysis for the pLawVac expression vector. Lanes 1 and 2: *Xho* I and *Bgl* II enzymatic digestion for pLawVac and pET22b vectors, respectively. Lanes 3 and 4: *Xho* I, *Bgl* II and Sal I digestion for pLawVac and pET22b vectors, respectively. Lane 5: 1 kb DNA ladder.

buffer (137 mM NaCl (Merck, Germany), 2.7 mM KCl (Merck, Germany), 10 mM Na_2HPO_4 (Merck, Germany) 1.8 mM KH_2PO_4 (Merck, Germany), pH 7.4) containing 0.1% Triton X-100) and disruption was performed using an ultrasonic homogenizer (QSonica, USA) at 95 Hz equipped with a titanium probe of 13 mm diameter. Cells were sonicated on ice at 10-second intervals with 10 s of rest. The total sonication time was 10 min.

2.4. Extraction of inclusion bodies

The extraction of inclusion bodies was performed according to procedures previously described [23]. Briefly, the biomass resuspended in PBS containing 0.1% Triton X-100 was lysed by mechanical cell disruption in a French press EmulsiFlex C-5, (Avestin, Canada). The pressure was adjusted to 1000 psi and the flow rate was 18–20 drops per minutes. The supernatant and pellet of cellular lysate were separated by centrifugation at 4342*g* for 20 min and the pellet was washed 2 times in 1 M NaCl plus 1% Triton X-100 and once in PBS. Finally, the inclusion bodies obtained in the insoluble fraction were resuspended in PBS and stored at −20 °C. Quantification was assessed using the Odyssey imaging system (LI-COR, USA), after applying into polyacrylamide gel electrophoresis using bovine serum albumin (Sigma, USA) as standard.

2.5. SDS-PAGE and western blotting

SDS-PAGE analysis was performed as described by Laemmli (1970) [24] in 12% polyacrylamide gels. Subsequently, proteins were transferred to nitrocellulose membranes (Schleicher and Schuell, Germany) using a semidry electroblotter (BioRad, USA). Monoclonal mouse anti-His (Clontech, USA) was used as the primary antibody, and goat Alexa fluor® 680 anti-mouse (Jackson ImmunoReseach, USA) was used as secondary antibody. The infrared signals were measured using the Odyssey imaging system.

2.6. Immunization and challenge experiments

Mouse and pigs studies were done in compliance with national guidelines and the authorization of the Ethical Committee from Universidad de Concepción.

2.6.1. Immunization assay in mice

The immunogenicity of recombinant antigens was evaluated in two experimental groups of five C57BL/6 female mice each. Antigens, as inclusion bodies, were emulsified in Montanide ISA 15A VG (Seppic, France) using an antigen: adjuvant ratio of 80:20. Dose of 50 μg per 100 μL of total volume were intramuscularly administered using 25 G needles. The same amount of protein from untransformed bacteria was used as negative control. Immunization scheme comprised one dose at day 0 and a booster at day 21. Blood samples were collected every week until week 6.

2.6.2. Immunization and challenge assay in pigs

The immune response of recombinant antigens was evaluated in healthy Duroc/Yorkshire piglets of four weeks old. Three experimental groups of eight pigs each were randomly gathered. Antigens were emulsified in Montanide ISA 15A VG using an antigen: adjuvant ratio of 80:20. Dose of 200 μg per 1 mL of total volume were intramuscularly administered using a 19 G needles. The first group (G1) was immunized with chimeric antigens and the second group (G2) was immunized with antigens co-formulated with 10^6 IU of recombinant porcine IFN-α (pIFN-α). The same amount of proteins from untransformed bacteria was administered to the third group (G3) as negative control.

Immunization scheme comprised one dose at day 0 and a booster at day 21. Blood samples were collected every week until week 6 and stored at −20 °C for ELISA assays. Challenge was performed in G2 and G3 experimental groups after 7 weeks of the primary immunization. Each animal was orally administered with 40 mL of ileum macerate diluted in physiological serum from a naturally infected animal with *L. intracellularis*. Pigs were monitored daily during the challenge looking for changes in behavior or the appearance of clinical signs. After 30 days of challenge, pigs were sacrificed and intestinal epithelial tissue was collected for histopathological analysis.

2.7. Immune response evaluation by ELISA assays

2.7.1. Mice and pigs sera evaluation by indirect ELISA

Flat-bottom 96 well ELISA plates (Nunc, USA) were coated with 100 ng per well of the chimeric antigens from *L. intracellularis*

solubilized in urea 8 M (Merck, Germany) during 16 h. Plates were washed three times with PBS plus 0,05% Tween 20 (PBST) and blocked with 3% of skimmed milk in PBS for one hour at 37 °C. After washing, diluted sera from mouse or pig were added (100 μL/well) for 2 h at 37 °C. Plates were washed and a secondary antibody conjugated to horseradish peroxidase (HRP) was added. For mice sera evaluation, goat anti-mouse IgG-HRP antibody (Abcam, USA) diluted 1/1000 was used. Sera of pigs were evaluated using 1/10000 diluted of goat anti-pig IgG-HRP polyclonal antibody (Abcam, USA). After one hour at 37 °C, plates were washed and revealed with a solution of o-phenylenediamine dihydrochloride (OPD) 0.4 mg/mL (Sigma, USA) diluted in citrate-buffer. Absorbance was measured using a SPECTROstar® *Nano* microplate reader (Labtech, Germany) at 492 nm.

2.7.2. Competitive ELISA assay

Flat-bottom 96 well ELISA plates were coated, washed and blocked as previously described. Pooled sera from naturally *L. intracellularis* infected pigs and uninfected pigs (positive and negative serum, respectively) were used. The competition was established with a pooled serum of mice immunized with chimeric antigens from *L. intracellularis*. After washing, the positive serum from pigs undiluted, diluted 1/10 and 1/100 were added. Plates were washed and the positive serum from mice diluted 1/100 was added. After washing, the secondary antibody goat anti-mouse IgG-HRP diluted 1/10,000 was added. The reaction revealing, stop and the absorbance measure were performed as described above.

2.8. RNA extraction and real time qPCR

Total RNA was isolated from lymphocytes purified from peripheral blood at days 0, 4 and 8 after the primary immunization, using a NucleoSpin®RNA kit (Macherey-Nagel, Germany). Analysis of relative gene expression of *il12*a, *cd4* and *oas*2 were done by real-time quantitative PCR using an AriaMx Real-Time PCR System (Agilent Technologies, USA) and a KAPA SYBR® FAST One-Step qRT-PCR kit (Kapa Biosystems, USA). The primers using in the experiment are listed:

il12a: forward (CTCAACCACCTGGACCATCT); reverse (TGGC CTTCTGAAGTGTGTTG)

cd4: forward (TCCCTGAAGGACAGGAAGGT); reverse (TACTGA AGCAAGGCCTGGAG)

oas2: forward (TCCAGCAACTCAAGAAACCCA); reversed (ACCCA TCCAGATTCTTGCAG)

gapdh: forward (CCACCCAGAAGACTGTGGAT); reversed (TTGA GCTCAGGGATGACCTT)

Cycling conditions were 3 min at 95 °C, followed by 40 repeats of 95 °C for 5 s, 58 °C for 30 s. Relative quantification of mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method [25]. The comparative threshold cycles values were normalized using gapdh mRNA [26].

2.9. Histopathological analysis

After challenge, animals were sacrificed and histopathological analysis was performed following the protocols describe by Luna (1968) and Guedes et al. (2002) [27,28]. The intestinal epithelial tissue slides were fixed in 10% formalin for 24–48 h. A Shandon Citadel 1000 tissue processor with a vacuum pump and an inclusion center Microm A280 (Thermo Fisher Scientific, USA) was used. The cross sections were observed in a photomicroscope Axiokop 40 (Carl Zeiss, Germany) equipped with a 10-megapixel digital camera (Canon, Japan) and an image processor analysis Axiovision Rel. 4.6 (Carl Zeiss, Germany).

2.10. Statistical analysis

Statistical analyses were performed using GraphPad Prism Software version 5.0 (GraphPad, San Diego, CA, USA). The competitive ELISA was compared by the Kruskal-Wallis test and the Dunn post-test of multiple comparisons. The humoral immune responses in mice and pigs, as well as the cellular immune response in pigs, were compared by ANOVA test and a Tukeýs post-test of multiple comparison. Significance was considered for $p < 0.05$.

3. Results

3.1. Identification of potential antigens from the genome of L. intracellularis

The analysis of the *L. intracellularis* complete genome (PHE/MN1-00, N343) allowed the identification of 1340 potential gene sequences encoding proteins. From this information, 33 sequences corresponded to secretory proteins and 306 sequences to membrane proteins, including 123 sequences with a single spanning region (type 1). After a B- and T-cell epitopes prediction analysis, the proteins with at least five B-cell epitopes and cut-off values over 0.9 were selected (Supplementary materials, Annex 1). The presence of T-cell epitopes with coverage over 50% for all alleles evaluated was taken into consideration for antigens selection (Supplementary materials, Annex 2), as well as the molecular size (less than 70 kDa) and the antigenic potential described before [29]. Three antigens were selected as potential components of the subunit vaccine: a 65 kDa outer membrane protein 1 (OMP1c), a 35 kDa outer membrane protein 2 (OMP2c) and a 25 kDa secretion protein classified as invasin (INVASc). In addition, T-cell epitopes with 100% coverage were added for each MHC isotype. They were designed to be mainly localized in loops regions without disturbing protein stability [30]. In some cases T-cell epitopes low coverage were replaced (Table 1). Sequences modeling corroborated the predicted designs for chimeric protein structures (Fig. 1).

3.2. Expression and characterization of chimeric antigens

The *E. coli* expression vector pLawVac containing the antigenic sequences (Fig. 2A–B) was submitted to a restriction analysis for corroborating its correct assembly. The digestion of pLawVac with the endonucleases *Xho* I/*Bgl* II endonucleases showed the expected two bands of 5259 bp and 4076 bp, corresponding to the three repeated transcriptional units in tandem and the rest of pET-22b vector, respectively (Fig. 2 C). A triple digestion with the endonucleases *Xho* I/*Bgl* II/*Sal* I resulted in DNA bands of 909 bp, 1195 bp and 1972 bp, corresponding to INVASc, OMP1c and OMP2c recombinant genes, respectively. SDS-PAGE and Western blot analysis of the E. coli strain K12 (Shuffle T7) transformed with the pLawVac expression vector showed the presence of three bands of proteins at molecular weight of around 65 kDa, 35 kDa and 25 kDa, which were observed mainly in the pellet of cellular lysate at 6 and 12 h of expression induction. The molecular weight of these bands matches with those predicted for chimeric antigens OMP1c, OMP2c and INVASc, respectively (Fig. 3A–B). The protein expres-

Table 1
T-cell epitopes added to the protein sequences.

Protein ID	Start position	Sequence
WP_011526745.1_694	201	FSYATDLSY
WP_011526133.1_53	357	FSFPYWFTF
WP_011526136	427	KQFNLNTLL

sion levels did not show significant changes in the experiment conducted at different induction time (6 or 12 h).

The antigens production was scaled up into bioreactor. Quantification of the chimeric antigens was estimated to be approximately 60% of the total proteins, according to SDS-PAGE (Fig. 3C–D). This material was used for immunization assays.

3.3. Chimeric antigens from L. intracellularis are highly immunogenic in mice and preserve epitopes from native proteins

C57BL/6 mice immunized with chimeric antigens displayed low IgG levels in the first two weeks after the initial immunization. The mean optical density (mOD) was around 0.2 and there was no significant difference between the control group and the group immunized with chimeric antigens. At week 3, there was a significant variation in the mOD of the vaccinated group compared with the control group ($p < 0.01$). After the booster, the IgG levels of the vaccinated group reached mOD above 0.75, which were significantly different from the control group ($p < 0.001$) (Fig. 4).

Moreover, a competitive ELISA was performed to evaluate the capacity of positive and negative sera from pigs to compete with a serum of mice immunized with chimeric antigens from *L. intracellularis*. The mOD significantly decreased from 0.6 to 0.4 when undiluted positive serum from pigs was added ($p < 0.01$). However, the decrease in the mOD when the positive serum from pigs was diluted 1/10 and 1/100 was not significantly different from the negative control (Fig. 5A). The negative serum from pigs did not significantly alter the mOD of any experimental group (Fig. 5 B). These assays demonstrated the presence of common immunogenic epitopes in the chimeric antigens and in the native proteins from *L. intracellularis*.

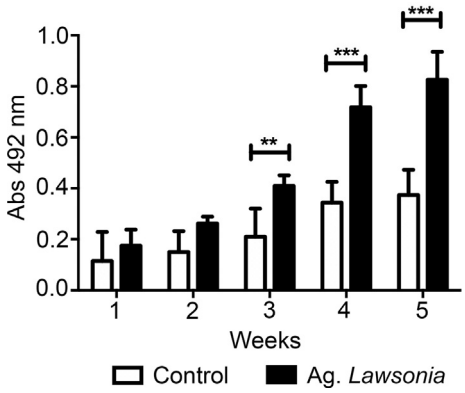

Fig. 4. Time-course of IgG response against *L. intracellularis* antigens in mice. Sera were evaluated by indirect ELISA. Bars represent the standard deviation of IgG values. Results were statistical analyzed by a Kruskal-Wallis test and the Dunn post-test of multiple comparisons (***$p \leq 0.001$, **$p \leq 0.01$).

3.4. Immune responses against chimeric antigens from L. intracellularis in piglets

The humoral immune response against chimeric antigens in the first 3 weeks was low, showing an average mOD below 0.4. There was not significant difference among the experimental groups. The induction of IgG antibodies against *L. intracellularis* antigens after the booster exhibited a significant increment from week 4 in the experimental groups vaccinated with chimeric antigens (mOD above 0.7), compared to the control group ($p < 0.001$) (Fig. 6). Likewise, a significant difference between the group vaccinated with the chimeric antigens and the group vaccinated with

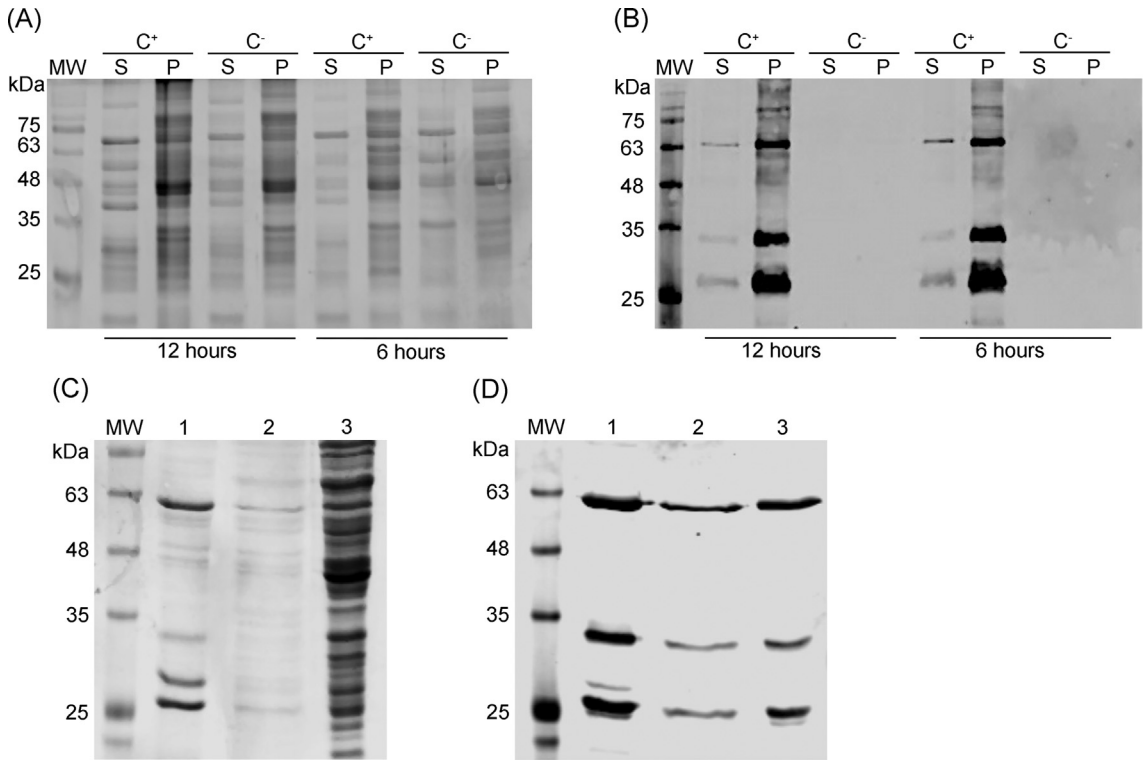

Fig. 3. Expression of chimeric antigens OMP1c, OMP2c and INVASc in the *E. coli* strain K12 (SHuffle® T7) transformed with the pLawVac plasmid. (A) SDS-PAGE and (B) Western blot analysis of *E. coli* transformed (C+) and non-transformed (C−) at 6 and 12 h after induction with IPTG. The chimeric proteins OMP1c, OMP2c and INVASc were observed at 65, 35 and 25 KDa, respectively. Lane S and lane P: supernatant and pellet of cellular lysate, respectively. (C) SDS-PAGE and (D) Western blot analysis, of proteins recovered from cellular lysate. Lane 1: pellet of cellular lysate; lane 2: supernatant after washed pellet; lane 3: supernatant of cellular lysate. Mouse anti-Hist and goat anti-mouse Alexa fluor 680 were used as primary and secondary antibodies, respectively. Immune detection was measured as infrared signal using the Odyssey imaging system.

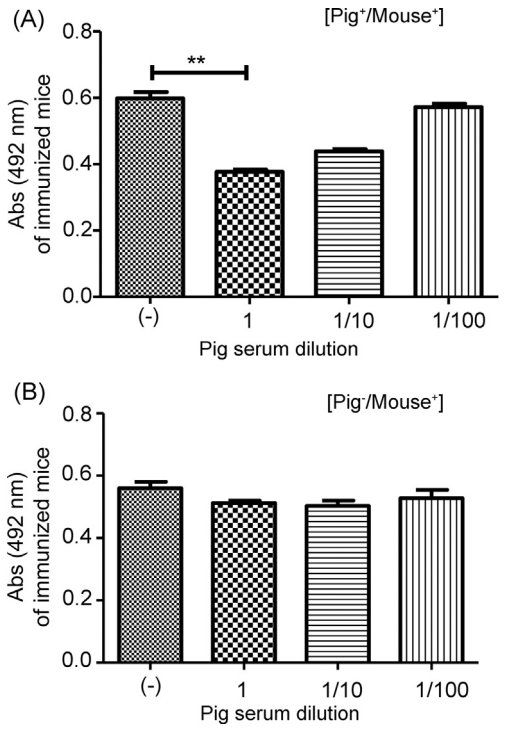

Fig. 5. Competitive ELISA using positive (A) and negative (B) sera from pigs. The absorbance was measured at 492 nm. Bars represent the standard deviation. The data were statistical analyzed by the Kruskal-Wallis test and the Dunn post-test of multiple comparisons (**$p \leq 0.01$).

the same antigens co-formulated with pIFN-α, was observed since week 5 post-vaccination ($p < 0.05$). Results showed a significant enhancement of the humoral immune response when pIFN-α was combined with *L. intracellularis* antigens.

The cellular immune response was assessed by the measurement of the relative quantification of mRNA from *il12* and *cd4* marker. The *il12* transcripts were detected from day 4 after the first immunization. By day 8, the mRNA level of this cytokine significantly increased compared to the control group ($p < 0.05$). There was not significant difference between the groups vaccinated with the chimeric antigens (Fig. 7A). Furthermore, proliferation of $CD4^+$ T lymphocyte cells showed a tendency to increase in the last day evaluated (Fig. 7B). The related expression of the gen *oas*2 at day 4 post-immunization showed significant difference in the experimental group immunized with the *L. intracellularis* antigens compared to control group ($p < 0.05$). As expected, the *oas*2 mRNA levels were significantly higher at days 4 and 8 post-immunization in the group immunized with the formulation containing pIFN-α compared to the other two experimental groups ($p < 0.05$) (Fig. 7C).

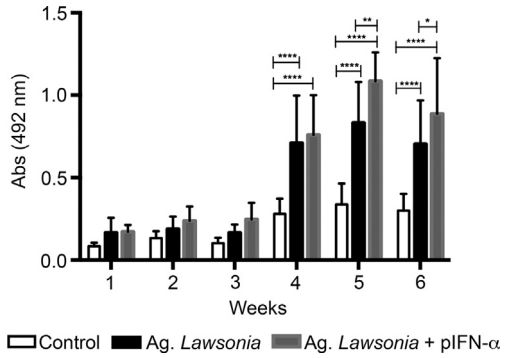

Fig. 6. Humoral immune response against *L. intracellularis* antigens assayed in pigs. The IgG antibody response was measured by indirect ELISA. Bars represent the standard deviation. The data statistical analysis was performed by a two way ANOVA and a Tukey's post-test of multiple comparisons (****$p \leq 0.0001$, **$p \leq 0.01$, *$p \leq 0.05$).

3.5. Histopatological analysis of intestinal epithelial tissue

Epithelial tissue from gastrointestinal tract was evaluated after challenge in the experimental group immunized with the chimeric antigens co-formulated with pIFN-α and in the negative control. The necropsy of animals from the control group showed a thickened mucosa in the gastrointestinal tract. Such lesion was not observed in the group immunized with *Lawsonia* antigens

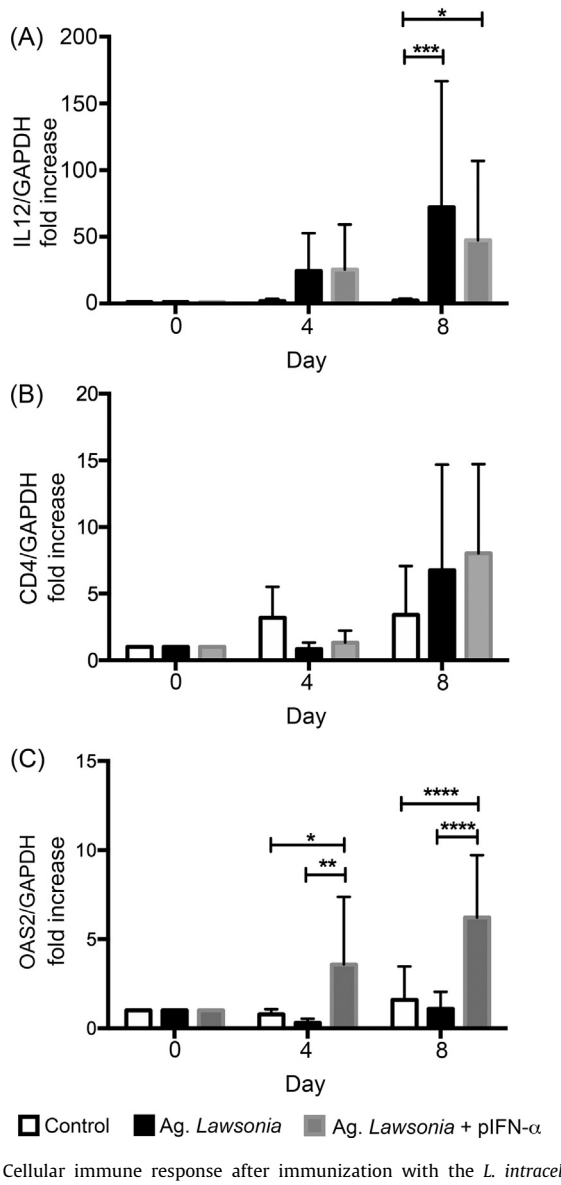

Fig. 7. Cellular immune response after immunization with the *L. intracellularis* antigens in pigs. (A) Fold increase of proinflammatory cytokine IL12; (B) proliferation of $CD4^+$ T lymphocytes and (C) *oas*2 gen were determined by real-time qPCR using mRNA isolated from lymphocytes of peripheral blood. Bars represent the standard deviation. Statistical analysis was performed by a one way two way ANOVA with Tukey's post test of multiple comparisons (****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$).

co-formulated with pIFN-α, (Table 2, Fig. 8). Samples of the last experimental group showed a preserved structure of intestinal villi and crypts. Also, size and number of goblet cells were maintained according to the species (Fig. 8A–D). The presence of cryptosporidium on the surface of villi denotes conserved epithelium morphology. Meanwhile, in the control group the ileum mucous showed altered morphology, characterized by hyperplasic and branched intestinal crypts and a diminished number of goblet cells. Also, there was an evident intestinal villi atrophy, exfoliation of superficial enterocytes and lymphocyte migration to the lamina propria of damaged epithelium (Fig. 8E–H). During the experiment, weight loss of animals was not evident in neither of the experimental groups evaluated. Changes in the behavior or fever symptoms were not observed. No animal died.

4. Discussion

Lawsonia intracellularis, the etiological agent of proliferative enteropathy in piglets, is described as a gram negative and intracellular obligated bacterium. This disease has globally expanded to more than 90% of herds [9,31]. The infection begins at eight weeks of age and remains active during fattening and breeding stages, which lead to a delayed animal growth and a reduced food conversion rate. These issues cause huge losses to veterinary industry [3,32].

Vaccines have been a successful method in the control and eradication of infection diseases worldwide [33,34]. The vaccine research field has positively impacted by genome sequencing platforms, gathering vast and updated databases with sequences of several pathogens [35]. This technology has allowed the identification of antigens through bioinformatics, enabling the generation of effective subunit vaccines [33,36]. The generation of new vaccines against *L. intracellularis* is imperative due to the intrinsic drawbacks associated to the production of conventional vaccines against this pathogen.

In this work, bioinformatics tools were used for designing a novel chimeric recombinant vaccine for controlling proliferative enteropathy. The ELISA assays carried out with sera of mice and pigs vaccinated with the chimeric antigens from *L. intracellularis* showed high IgG levels after the booster, demonstrating the immunogenicity of vaccine candidate. High antibody levels have also been obtain with a single dose of inactivated vaccine against *L. intracellularis* when it was intramuscularly administered [10]. However, no measurable levels of IgG have been detected after oral vaccination with attenuated vaccine [37]. This controversial results using conventional vaccine could be due to nature of the vaccine, differences in the experimental design, among other features.

Noticeably, sera from naturally infected pigs recognized chimeric antigens from *L. intracellularis* produced in *E. coli* in a competitive ELISA, indicating that these chimeric antigens, produced as inclusion bodies, preserved epitopes from native proteins of the pathogen.

The humoral response displayed by animals vaccinates with chimeric antigens co-formulated with pIFN-α was significantly higher compared with those vaccinates without pIFN-α. This finding corroborates the increasing of antibody levels by this cytokine. It could be related to the fact that pIFN-α increases the humoral antibody response inducing all IgG subclasses with extended half-life [38]. The use of pIFN-α have been used to potentiate the immune response of vaccinate candidates [39].

Relative mRNA expression analysis for several genes associated to cellular immune response (*il12*, *cd4* and *oas2*) was performed in vaccinated and control pigs. Significant increase in the relative mRNA expression of the immune-modulatory cytokine IL12 was observed in vaccinated groups compared to the control. Also, the population of CD4 cells showed a tendency to increase, according to *cd4* mRNA expression. These results suggested the development of an innate and adaptive immune response in the vaccinated pigs. Among other biological functions described for IL12, this cytokine induces proliferation and differentiation of naive $CD4^+$ T cells into Th1 cells, and plays an important function in their trafficking and migration [40].

On the other hand, activity of pIFN-α was confirmed by the significant increase observed in the expression of *oas*2 gene in vaccinated pigs. Upon IFN-α secretion, cells activate protective mechanisms against virus. One of those mechanisms involve *oas*2 gene, which encode 2′,5′ oligoadenylate synthetase 2. The role of this enzyme is crucial in viral RNA degradation taking place after the activation of 2′,5′ oligoadenylate, which in turn interact with ribonuclease L triggering the effector function [41].

Finally, expected results were observed after challenge, where vaccinated pigs did not show epithelial damages at macroscopic level. However, lesions characteristic of a pathogenic infection were present in the control group. A thickening of the normal bowel structure was observed, where necrotic cells were accumulated. At microscopic level, immature, elongated and branched

Table 2
Epithelial tissue damages observed in the vaccinated *vs* control pigs.

Animal number	Epithelium damage	Reduction in globet cells	Adhered bacteria	Lymphocytes migration
Vaccinated group				
9	0.5	0	0.5	0.5
10	0	0	0	0
11	0	0	0	0
12	0	0	0	0
13	0	0	0	0
14	0	0	1	0
15	1	0	0	1
16	0	0	0	0
Damage accumulation	1.5	0	1.5	1.5
Control group				
17	2	1	0	0
18	1	1	1	0.5
19	1	0	0	0
20	0	1	1	1
21	0	0	0	0
22	0	0	0	0
23	1	0	0	1
24	2	2	0	0.5
Damage accumulation	7	5	2	3

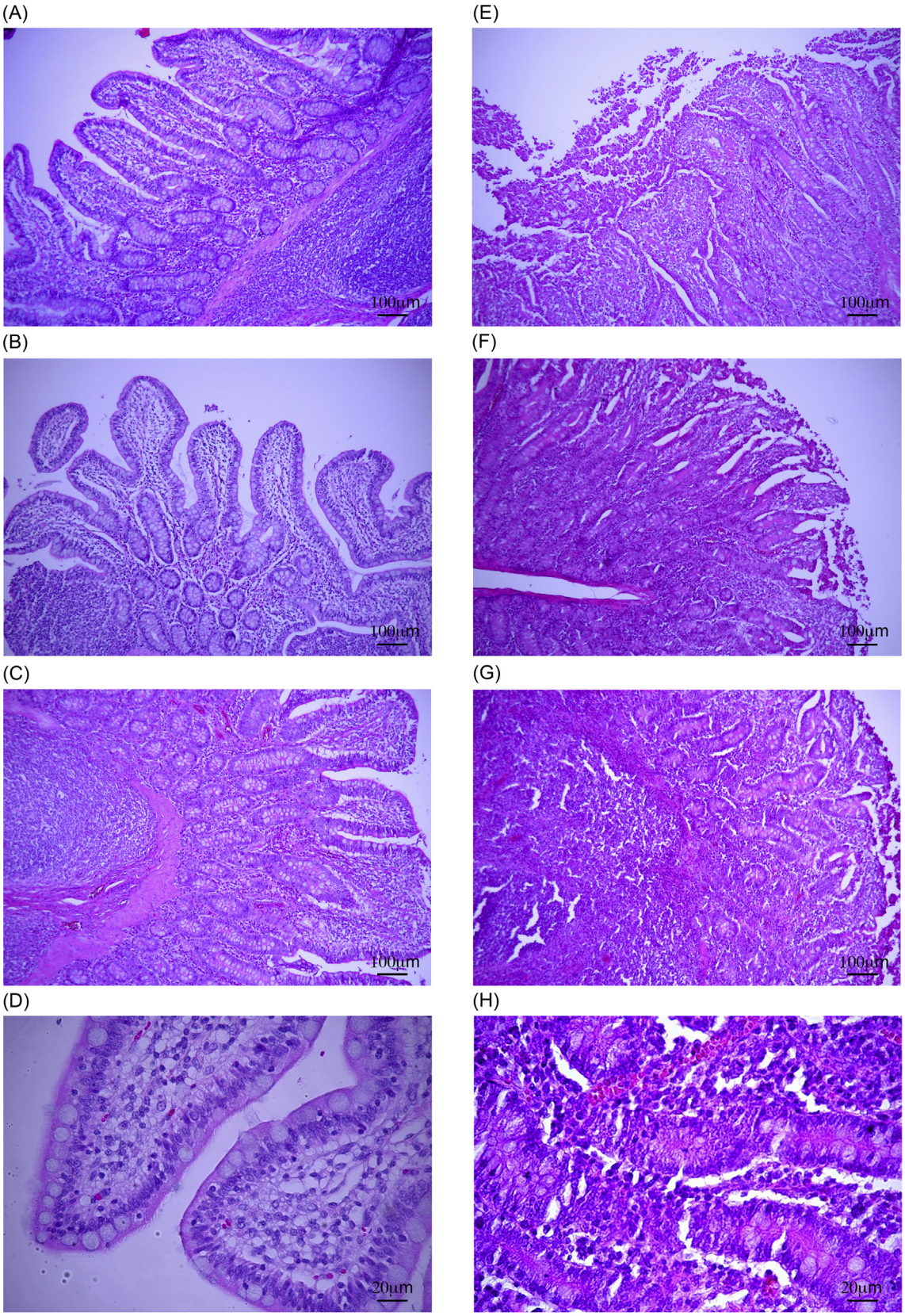

Fig. 8. Histopathological analysis after challenge. Slides of intestinal epithelial tissue of vaccinated (A–D) and unvaccinated (E–H) pigs.

cells were arrange in a stratified epithelium structure, leading to intestinal cell hyperplasia in the cryptic area of the ileum. The absence of goblet cells in the crypt zone was also observed. These epithelial damages were almost absent in the vaccinated animals. Replication of *L. intracellularis* occurs in the cytoplasm after its internalization. Protein interactions between cell membrane proteins of the pathogen and the host could mediate the invasion [42,43]. Enterocytes maturation is impaired by the infection and consequently, absorptive capacity is reduced. Intestinal hypertrophy and inflammatory lesions characterize this injury, where mononuclear leukocytes and macrophages infiltration is observed [44].

The novel vaccine candidate induced effective humoral and cellular immune response. Histopathological analysis after challenge suggested protection of vaccinated pigs. Further experiment must be performed to evaluate the *cd4* marker later in time. Moreover, several pIFN-α concentrations must be tested for selecting an adequate dose inducing the desired immune response and the contribution of the *Lawsonia* antigens by themselves to the vaccine efficacy must be evaluated.

Summarizing, bioinformatics tools were used to select three proteins from the bacterium *L. intracellularis* for designing a subunit vaccine candidate against this pathogen. The antigens co-expressed in *E. coli* were highly immunogenic in mice and pigs, and preserved native epitopes from the pathogen. Specifically, a co-formulation of the chimeric antigens and pIFN-α significantly improved the humoral immune response in pigs. Moreover, immunization and challenge trials allowed evaluating the efficacy of the vaccine candidate. This study establishes the initial bases for the generation of subunit vaccine, which induce an efective protection against porcine proliferative enteropathy.

Acknowledgments

The authors greatly appreciate the financial support provided by the Chilean Government through Fondef Grant: ID14i10335 and INNBIO Grant: VET007F.

Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.01.029.

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