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Establishment of a production process for a novel vaccine candidate against *Lawsonia intracellularis* ☆

S. Salazar [a], N. Gutiérrez [b], O. Sánchez [c], E. Ramos [b], A. González [c], J. Acosta [b], T. Ramos [a], C. Altamirano [d], J.R. Toledo [b], R. Montesino [b,*]

[a] Department of Live Sciences and Agriculture, Universidad de las Fuerzas Armadas ESPE, Quito, Ecuador
[b] Biotechnology and Biopharmaceuticals Laboratory, Pathophysiology Department, School of Biological Sciences, Universidad de Concepción, Victor Lamas 1290, P.O. Box 160C, Concepción, Chile
[c] Recombinant Biopharmaceuticals Laboratory, Pharmacology Department, School of Biological Sciences, Universidad de Concepción, Victor Lamas 1290, P.O. Box 160C, Concepción, Chile
[d] School of Biochemical Engineering, Pontificia Universidad Católica de Valparaíso, Chile

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ABSTRACT

Background: *Lawsonia intracellularis* remains a problem for the swine industry worldwide. Previously, we designed and obtained a vaccine candidate against this pathogen based on the chimeric proteins: OMP1c, OMP2c, and INVASc. These proteins formed inclusion bodies when expressed in *E. coli*, which induced humoral and cellular immune responses in vaccinated pigs. Also, protection was demonstrated after the challenge. In this study, we established a production process to increase the yields of the three antigens as a vaccine candidate.

Results: Batch and fed-batch fermentations were evaluated in different culture conditions using a 2 L bioreactor. A fed-batch culture with a modified Terrific broth medium containing glucose instead of glycerol, and induced with 0.75 mM IPTG at 8 h of culture (11 g/L of biomass) raised the volumetric yield to 627.1 mg/L. Under these culture conditions, plasmid-bearing cells increased by 10% at the induction time. High efficiency in cell disruption was obtained at passage six using a high-pressure homogenizer and a bead mill. The total antigen recovery was 64% (400 mg/L), with a purity degree of 70%. The antigens retained their immunogenicity in pigs, inducing high antibody titers.

Conclusions: Considering that the antigen production process allowed an increment of more than 70-fold, this methodology constitutes a crucial step in the production of this vaccine candidate against *L. intracellularis*.

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1. Introduction

The porcine proliferative enteropathy is caused by the obligate intracellular bacteria *Lawsonia intracellularis*, which has provoked massive damages to the swine industry. These harms include sub-optimal weight gain in animals during the fattening stage, a low feed conversion rate, and a decrease in the number of live-born pigs [1,2]. Previous studies showed economic losses between 3 and 25 USD per pig due to the increase in feeding costs, time to achieve slaughter weight, and costs of medications [3].

Vaccination is highly recommended for counteracting this disease [4]. However, current vaccines are difficult to obtain because of the high production costs associated with the specific culture conditions of this intracellular pathogen [5,6]. This scene could be reverted by using recombinant vaccines instead of vaccines based on the attenuated or inactivated microorganism. Recently, our group designed and produced the first subunit recombinant vaccine candidate against *L. intracellularis* composed by three proteins of the pathogen: 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) [7].

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* Corresponding author.
E-mail address: rmontesino@udec.cl (R. Montesino).

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Nomenclature

μ	Specific growth rate
CFU	Colony forming unit
DCW	Dry cell weight
INVASc	Chimeric secretion protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria Bertani broth
LBA	Luria Bertani broth with ampicillin added
MTB	Modified Terrific broth
OD_{600}	Optical density measured at 600 nm
OMP1c	Chimeric outer membrane protein 1
OMP2c	Chimeric outer membrane protein 2
OPD	O-phenylenediamine dihydrochloride

Moreover, the coding sequences of these proteins were engineered for improving their immunogenicity. The efficacy of this vaccine candidate, produced in *E. coli* as inclusion bodies, was demonstrated in pigs. We obtained a significant increase in humoral and cellular responses in vaccinated animals, which did not show any damages in the intestinal epithelial tissue after a challenge assay [7].

E. coli is a widely used microorganism for the expression of recombinant proteins in small and large scales [8,9,10]. The volumetric yield of this bacterial culture has thoroughly been improved by the fed-batch culture. The feeding on the fed-batch method prevents the excessive accumulation of toxic substances, which allow proper cell growth. Moreover, the fed-batch strategy provides the means for the accurate control of the dissolved oxygen and the specific growth rate. Transformed *E. coli* also required a suitable culture medium, the control of pH, temperature, induction time, and inductor concentration for increasing the productivity of the desired recombinant protein [11,12,13].

Most of the recombinant proteins produced in *E. coli* remain inside the cell as inclusion bodies. Hence, it is necessary to release the intracellular components by disrupting the wall of microbial cells using mechanical, chemical, or enzymatic procedures. Mechanical methods are efficient for microbial cell disruption, and they are the most used for large-scale production, compared to enzymatic techniques. The cell wall-lysis based on enzymatic treatments is expensive and needs an additional step to remove the enzyme from the final product [14]. High-pressure homogenizer and bead mill stands out within mechanical methods, and their effectiveness depends on the microbial strain, cell concentration, and the age of culture, among other features [15].

This work aimed to improve the expression of *L. intracellularis* antigens, which constitute the biologically active components of the vaccine candidate. For this purpose, we studied the culture and downstream stages of the production process. We performed a fed-batch culture in a 2 L bioreactor with a modified TB medium. Additionally, we evaluated the induction time, the inductor concentration, and the plasmid stability. The efficacy of mechanical disruption methods was assessed by measuring the recovery of *Lawsonia* antigens. Finally, the antigen immunogenicity was confirmed in vaccinated pigs.

2. Materials and methods

2.1. Pre-inoculum and inoculum preparation

The recombinant antigens (OMP1c, OMP2c, and INVASc), previously engineered for improving their immunogenicity, were expressed as Montesino and coworkers in 2019 [7]. Pre-inoculum was prepared in a propylene tube of 50 mL with 10 mL of Luria Bertani broth (Lioilchem, Italy) and ampicillin at 100 μg/mL (USBiological, USA) (LBA). A glycerol stock of Shuffle® T7 transformed with the plasmid pLawVac (100 μL) was added to the culture medium. The pre-inoculum was incubated at 37°C for 10 h under constant stirring at 150 rpm. Two inoculums were prepared in Erlenmeyer flasks with 100 mL of LBA. The flasks were inoculated with 1 mL of the pre-inoculum and incubated at 37°C for 10 h under constant stirring at 150 rpm.

2.2. Production of the recombinant antigens

The antigens were produced in a fermenter Winpact FS-06 (Major Science, USA). The inoculum of 200 mL at 1.2 g/L was added to complete a total volume of 2 L of LB, Terrific broth (TB) [16] or modified TB (MTB). All media were supplemented with ampicillin 100 μg/mL. MTB had the same composition of TB, but the glycerol concentration (3% instead of 0.2%). The culture conditions in the fermenter were the following: temperature 37°C, pH 7.0, dissolved oxygen over 20% by varying the culture stirring. The pH was adjusted by the addition of 25% (v/v) NH_3OH or 20% (v/v) H_3PO_4.

The fed-batch started after 6 h of culture. Yeast extract (176 g/L) and glycerol (221 mL/L) were added at a constant feed rate of 5 mL/min for 2 h. The fed-batch culture was also assayed by substituting the 3% of glycerol in MTB by 2% of glucose. The expression induction of the recombinant antigens was carried out with 0.75 mM or 1.5 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) (Santa Cruz Biotechnology, USA). The growth rate and duplication time were calculated as described by Weddel in 2007 [17], and the optical density was measured at 600 nm (OD_{600}) in a spectrophotometer UV-2505 (Labomed, Inc., USA). Dry cell weight (DCW) was determined at 90°C in a moisture analyzer (Radwag, Poland). One optical density unit corresponded to 0.49 g DCW/L.

2.3. Plasmid stability

The plasmid segregation was assessed on LB plates with and without ampicillin (100 μg/mL). The plasmid stability was determined as the percentage of plasmid-bearing cells compared to the total viable cells.

2.4. Cell viability

Samples for determining cell viability were collected every 2 h. They were diluted in a solution of 0.85% NaCl (Merck, Germany) and seeded in LB medium for 24 h at 37°C. The colony forming unit (CFU) was calculated by the formula: CFU/mL = (colony number × dilution factor)/ seeding volume (mL).

2.5. Obtaining of the inclusion bodies

Cell cultures were centrifuged at 4400 × g for 20 min at 4°C. The biomass was resuspended in PBS buffer (137 mM NaCl, 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 at 20 g DCW/L. Inclusion bodies were released using two devices of mechanical disruption. The EmulsiFlex-C5 pressure homogenizer (Avestin, Canada) was used at 12,000 psi with a flow rate of 40 mL/min. The Dyno-mill ML bead mill (Wab, Switzerland),

with a grinding chamber of 210 mL containing glass beads of 0.3 mm, was used at a flow rate of 260 mL/min with a residence time of 0.8 min/passage. The biomass suspensions were passed ten times by each equipment, which was collected and diluted in the same disruption volume to obtain a final concentration of 10 g DCW/L. After spinning the biomass at 4342 × g for 20 min, the pellet of the cellular lysate was washed twice in 1 M NaCl plus 1% Triton X-100 and once in 0.05 M Na_2HPO_4 pH 12.0. Finally, the inclusion bodies were resuspended in PBS and stored at −20°C.

2.6. Antigen analysis

The antigens were analyzed as previously described [7]. Briefly, SDS-PAGE analysis was done in 12% polyacrylamide gels. For the western blot assay, the proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell, Germany) using a semidry electroblotter (BioRad, USA). Monoclonal mouse anti-His (Clontech, USA) and goat anti-mouse Alexa fluor®680 (Jackson ImmunoReseach, USA) were used as primary and secondary antibodies, respectively. The purity and concentration of the antigens were determined by densitometry in SDS-PAGE stained with Coomassie Blue R250 (Sigma, USA). The purity was estimated according to the antigen band's intensity compared to the whole protein content. The concentration was determined using bovine serum albumin (Sigma, USA) as standard [7]. Western blot signals and densitometric assays were analyzed by the Odyssey imaging system (LI-COR, Biosciences, USA).

2.7. Immunization assay in pigs

The studies in pigs were done in compliance with national guidelines and the authorization of the Ethics Committee of the Universidad de Concepción, Chile. Healthy Duroc/Yorkshire piglets of three weeks old were acquired from an intensive pig production of a Chilean farm.

The animals were gathered into two groups of fourteen animals each and immunized following the scheme previously described [7]. *Lawsonia* antigens were emulsified in Montanide ISA 50V2 (Seppic, France) using an antigen:adjuvant ratio of 40:60. Doses of 1 mL, containing 200 μg of antigens, were intramuscularly administered. One group was immunized with chimeric antigens and the other group was immunized with the same amount of proteins from untransformed bacteria (negative control). Blood samples were collected every ten days until the day 30 and stored at 20°C for humoral immune response detection.

2.8. ELISA assay for immune response evaluation

The immune response evaluation was carried out according to Montesino et al. (2019) [7]. Briefly, flat-bottom 96 well ELISA plates (Nunc, USA) were coated with 100 ng per well of *Lawsonia* antigens solubilized in urea 8 M (Merck, Germany). Sera diluted 1/100 were added (100 μL/well) for 2 h at 37°C. As a secondary antibody, goat anti-pig IgG-HRP polyclonal antibody (Abcam, USA) diluted 1/10,000 was used. The signal was visualized with a solution of o-phenylenediamine dihydrochloride (OPD) at 0.4 mg/mL (Sigma, USA) diluted in citrate-buffer. Absorbance was measured at 492 nm using a SPECTROstar®Nano microplate reader (Labtech, Germany).

2.9. Statistical analysis

Statistical analyses were performed using the GraphPad Prism Software version 5.0 (GraphPad, San Diego, CA, USA). Humoral immune responses were compared by ANOVA test and a Tukey's post-test of multiple comparison. The antibody titration was compared by the Mann Whitney test. Significance was considered for $p < 0.05$.

3. Results

In a previous work, *Lawsonia* antigens (OMP1c, OMP2c, and INVASc) were produced as inclusion bodies in a batch culture using LB medium by inducing with 0.5 mM IPTG for 6 h in a fermenter of 2 L at low cell density [7]. In this study, we compared the *Lawsonia* antigen production in batch culture using LB and TB media. Subsequently, we evaluated fed-batch culture in the modified TB medium inducing the antigen expression at different times and distinct IPTG concentration. Low- and high-density culture conditions were also assayed. After mechanical disruption by bead mill and high-pressure homogenizer, we evaluated the yield recovery and the immunogenicity of *Lawsonia* antigens.

3.1. Production of Lawsonia antigens in batch culture

Preceding conditions for the production of *Lawsonia* antigens included the growth of transformed bacteria in batch culture with LB medium and 0.5 mM of IPTG. Under these conditions, 5.6 mg/L of the desired antigens were obtained at 8 h of culture (2 h post induction). The specific production was 1.75 g of antigens per grams of biomass and the productivity was 0.74 mg/L/h. Considering the low antigen yield in the previous assay, we used TB medium to improve the cell biomass and the antigen expression. The batch culture with this medium induced at 0.75 IPTG increased six-fold the antigen production (37.7 g/L). Specific production and productivity were also improved (Table 1).

3.2. Establishment of the cell culture conditions without induction

Taking into account that fed-batch cultivation improves cellular density and the yield of recombinant proteins, we performed a whole procedure for studying the production of *Lawsonia* antigens under fed-batch conditions. The first step was the establishment of optimal pre-inoculum and inoculum. Both kept a liquid/flask volume ratio of 0.25 to ensure a proper culture aeration [18]. The pre-inoculum lag phase lasted 4 h, followed by 6 h of exponential phase with a specific growth rate (μ) of 0.609 h^{-1} until 10 h of culture. In the last 2 h the μ decreased, indicating the beginning of the stationary phase. At 10 h the culture reached the highest cellular density without nutrient depletion. These conditions guaranteed that the inoculum started in the exponential phase. After seeding the inoculum, no lag phase was observed, and the exponential phase was maintained until 6 h with a μ of 0.760 h^{-1}. The specific growth of this phase was 0.115 h^{-1} (Fig. 1).

Since one of our goals was to improve the production of recombinant antigens by inducing the gene expression at higher cell densities, we evaluated the growth kinetics of the transformed *E. coli*

Table 1
Fermentation parameters of batch cultures.

Parameters	LB medium	TB medium
Cell concentration at induction (g/L)	0.3	0.4
IPTG concentration (mM)	0.5	0.75
Induction time (h)	6	8
Harvest (h)	7.5	10
Y P*/V (mg/L)	5.6	37.7
Productivity (mg/L/h)	0.74	3.77
Y P*/X (mg/g)	1.75	5.24

Y, yield; P, product (*Lawsonia* antigens); X, biomass; V, volume.
* These values were measured after cell disruption with the high-pressure homogenizer.

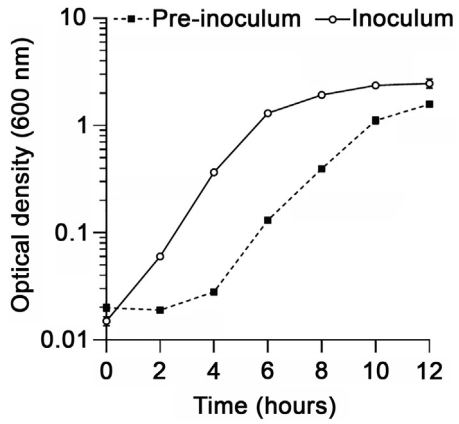


Fig. 1. Kinetic cell growth of the pre-inoculum and inoculum in LBA medium. The results represent the mean of three replicates. Bars represent the standard deviation.

strain with MTB medium. These experiments were done in a fermenter of 2 L under non-induced conditions (Fig. 2). The fermenter was seeded with 200 mL of the inoculum at 6 h of growing. Although the time of the inoculum at the moment of seeding the fermenter coincided with the end of the exponential phase, it ensured the absence of a lag phase and the required cell density in the fermenter. When 3% instead of 0.2% glycerol was used as a

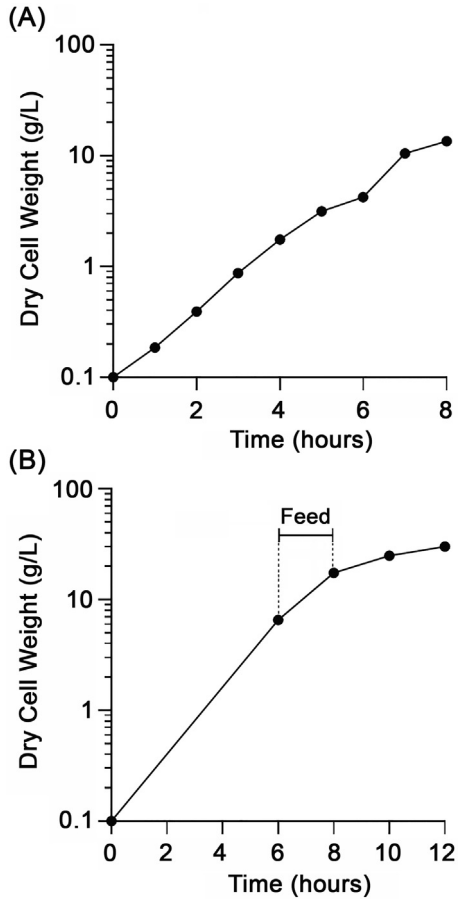


Fig. 2. Kinetic cell growth without induction in a 2 L fermenter with TB-medium (A), TB-medium plus 3% glycerol (B), and TB-medium plus 3% glycerol supplemented with yeast extract and glycerol (C). One optical density unit corresponded to 0.49 g DCW/L.

primary carbon source, an increase in the cell biomass (13.52 g/L) was observed at 8 h of culture compared to the batch culture in TB medium (7.2 g/L). Under these conditions, the exponential phase remained until 7 h of culture with a μ 0.657 h^{-1} (Fig. 2A).

Next, a fed-batch culture was performed using MTB (3% glycerol), and the feeding comprised glycerol and yeast extract. The performance of this fed-batch culture increased the biomass (17.40 g/L) with a μ 0.678 h^{-1} at 8 h of culture. The constant feeding allowed the culture extension to 12 h with a higher biomass concentration (30.04 g/L) (Fig. 2B). These results demonstrated the need to provide an additional feeding to increase cell density.

3.3. Antigen expression upon induction

The above fed-batch conditions were used to evaluate the *Lawsonia* antigen expression by inducing with 0.75 mM and 1.5 mM IPTG. The induction with 0.75 mM IPTG was done at 6 h, when the culture had 5.02 g/L of biomass. The total yield of this culture was 536 mg/L of recombinant antigens, with a biomass of 18.72 g/L. Nevertheless, the culture induced at a similar biomass concentration (5.23 g/L) with 1.5 mM IPTG, caused a decrease of the antigen expression levels (292 mg/L) (Fig. 3A). Regardless of the IPTG concentration used for the induction, a reduction of around 40% in the biomass yield was observed compared to the non-induced cultures. As the highest recombinant protein yields were obtained by inducing with 0.75 mM IPTG, this inductor concentration was selected for further experiments.

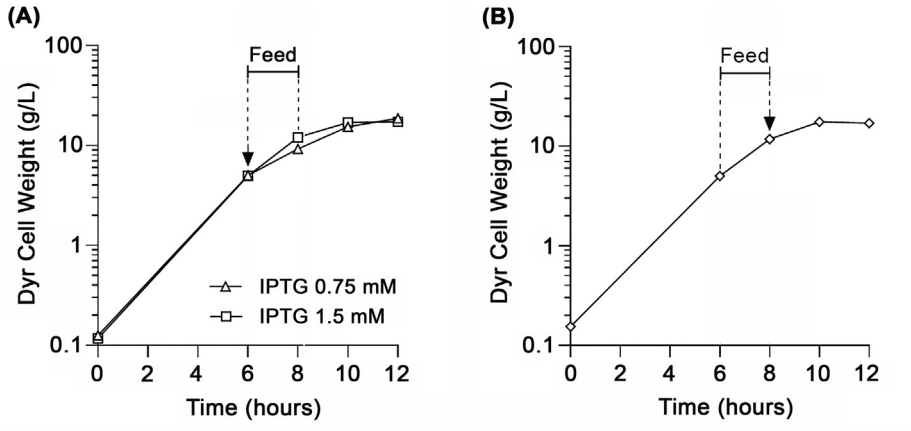
Afterwards, we evaluated the fed-batch culture induced with 0.75 mM IPTG at higher biomass (11.76 mg/mL). Under these experimental conditions, an increment of the *Lawsonia* antigen yield was obtained (602.50 mg/L), with no noticeable variation in the final biomass concentration compared to the previous experiment (17.08 g/L and 18.72 g/L, respectively) (Fig. 3B).

3.4. Plasmid segregation during the antigen expression

The level of protein expression not only depends on the cellular biomass produced in the fermenter, but also on the plasmid retention by the host. The plasmid stability was studied using the same culture condition as above and a different carbon source in MTB (2% glucose). Our results showed that the culture containing 3% of glycerol as a primary carbon source, had 62% of plasmid-bearing cells before the induction. However, this value decreased to 11% at the end of the culture. These parameters were improved when glucose was used as a primary carbon source. They were 75% and 19%, respectively (Fig. 4). Despite the decreasing of the plasmid-bearing cells, the last culture conditions guaranteed the highest yield of recombinant antigens.

3.5. Analysis of fed-batch cultures

The production of fed-batch cultures is presented in Table 2. The highest values of antigen production were observed when glucose was used as a carbon source and the induction was done with IPTG at 0.75 mM. Therefore, the final fed-batch culture conditions for the *Lawsonia* antigen production were: (i) MTB containing 2% glucose; (ii) feeding with yeast extract (176 g/L) and glycerol (221 mL/L) at 6 h; (iii) induction with 0.75 mM IPTG at 8 h with a biomass of 11 g/L, approximately; (iv) harvest at 12 h. The achievement of three successful fermentations as above corroborated the reproducibility of the culture system. The behavior of cell viability was similar to that of the cell biomass. The average antigen yield was 627.1 mg/L and the viable cells reached 75%, demonstrating the robustness of the production process (Fig. 5A). SDS-PAGE and western blot showed that the culture with glucose properly produced the *Lawsonia* antigens (Fig. 5B).



IPTG (mM)	Biomass (DCW) at induction time (g/L)	Biomass (DCW) at the end of the culture (g/L)	Antigens yield (mg/L)*
0.75	5.02	18.72	536.06
1.5	5.23	17.20	292.45

IPTG (mM)	Biomass (DCW) at induction time (g/L)	Biomass (DCW) at the end of the culture (g/L)	Antigens yield (mg/L)*
0.75	11.76	17.08	602.50

Fig. 3. Expression of *Lawsonia* antigens in a 2 L fermenter. (A) Culture induced at approximately 5 g/L with 0.75 and 1.5 mM IPTG. (B) Culture induced at 11.76 g/L with IPTG at 0.75 mM. The arrows indicate the induction time. The antigen yield corresponds to the three recombinant proteins (OMP1c, OMP2c, INVASc). * These values were measured after cell disruption with the high-pressure homogenizer.

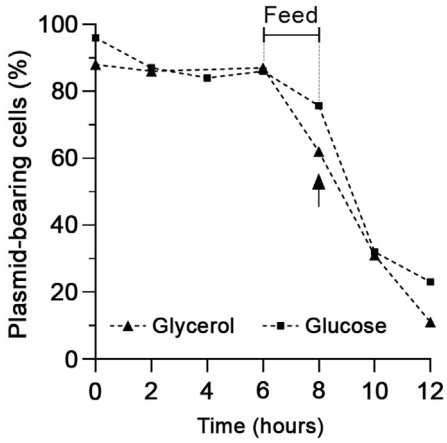


Fig. 4. Plasmid stability during the antigen expression in a 2 L fermenter. Fed-batch fermentations using glycerol and glucose as carbon sources during the exponential phase of the culture. The arrow indicates the induction with 0.75 mM IPTG.

3.6. Mechanical cell disruption

The efficacy of cell disruption was assessed by evaluating two mechanical devices: solid shear (bead mill) and liquid shear (high-pressure homogenizer). Our results showed no differences between both methods. The percentage of cell viability was around 5% in the second passage, which dropped to 1% at passage six with more than 99% of cell disruption (Fig. 6A). Hence, six passages were enough to obtain a proper cell disruption in each method. SDS-PAGE and western blot showed that the relative composition of recombinant antigens in the cell lysate was similar after the cell disruption for both devices, corresponding to 40% of total proteins, approximately. The protein INVASc showed the highest expression levels and the protein OMP2c the smallest one. This feature was maintained when comparing the three antigens with each other or with total proteins, showing the different expression levels of *Lawsonia* antigens. The ratio of the three antigens was maintained during the downstream process. After washing steps, the purity of inclusion bodies increased around 65–70%, and the final antigen yield was about 400 mg/L (Fig. 6B-D). Considering the previous results, we obtained a recovery percent of 64%. Additionally, western blot assays showed neither cleavage nor degradation of the three antigens at 4°C and −20°C for at least two months (Fig. S1).

3.7. Humoral immune response of Lawsonia antigens

The humoral response after the immunization of pigs with *Lawsonia* antigens was detected at day twenty, where the antibody levels were significantly higher than those of the control group. This behavior was also observed at day thirty (Fig. 7A). Sera with the highest absorbance values were selected for a titration experiment, displaying titers higher than 1/12,000 (Fig. 7B).

4. Discussion

The proper expression of recombinant proteins requires the establishment of robust production processes. They ensure high yields and reproducible batches of the active biopharmaceutical ingredient. These characteristics can be achieved by improving the microbial metabolism through standardized growing cycles under controlled conditions, which contribute to the high-quality

Table 2
Fermentation parameter of fed-batch cultures.

Fed-batch cultures	Y P*/S (mg/g)	Y P*/X (mg/g)	Y P*/V (mg/L)	Productivity (mg/L/h)
Carbon source: 3% glycerol. Induction with 0.75 mM of IPTG at 5.02 g/L of biomass	7.09	28.63	536.06	44.67
Carbon source: 3% glycerol. Induction with 1.5 mM of IPTG at 5.23 g/L of biomass	3.88	17.04	292.45	24.42
Carbon source: 3% glycerol. Induction with 0.75 mM of IPTG at 11.76 g/L of biomass	7.98	35.31	602.50	50.25
Carbon source: 2% glucose. Induction with 0.75 mM of IPTG at 10.24 g/L of biomass	10.85	35.07	627.1	52.25

Y, yield; P, product (*Lawsonia* antigen); S, carbon source; X, biomass; V, volume.
* These values were measured after cell disruption with the high-pressure homogenizer.

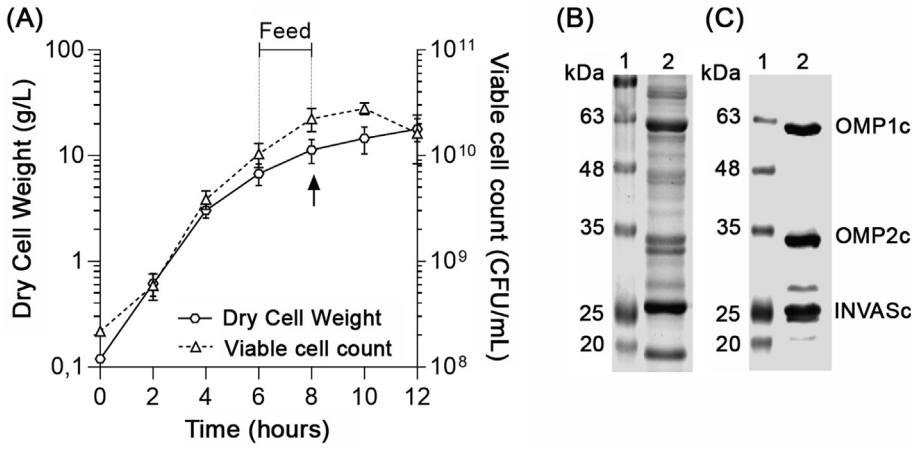
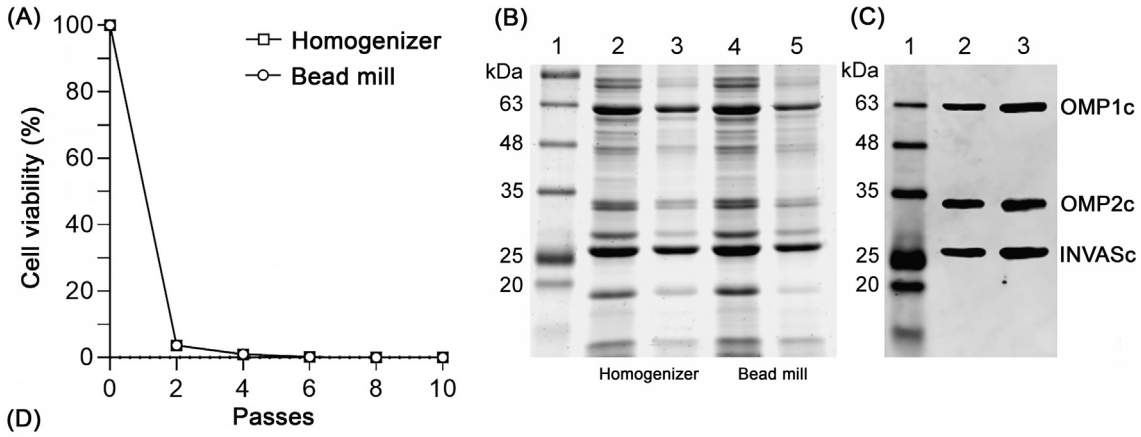


Fig. 5. Evaluation of *Lawsonia* antigen production using the optimal conditions. (A) The curves correspond to the mean of three batches of fermentation, showing the dry cell weight and cell viability. Bars represent the standard deviation. The arrow indicates the induction with 0.75 mM IPTG. SDS-PAGE (B) and western blot (C) of *Lawsonia* antigens. Lane 1: AccuRuler RGB PLUS prestained protein ladder (Maestrogen, Taiwan), lane 2: Sample of *Lawsonia* antigens. Immunodetection was carried out using the monoclonal mouse anti-His and goat anti-mouse Alexa fluor®680.



(D)

Lawsonia antigens	Homogenizer				Bead mill			
	Cell lysate		Washed pellet		Cell lysate		Washed pellet	
	Antigen percent (each other)	Antigen percent (total proteins)	Antigen percent (each other)	Antigen percent (total proteins)	Antigen percent (each other)	Antigen percent (total proteins)	Antigen percent (each other)	Antigen percent (total proteins)
OMP1c	40.46	17.65	35.09	24.53	41.17	16.63	32.33	21.64
OMP2c	11.54	5.03	12.97	9.07	12.43	5.02	14.01	9.38
INVASc	48.00	20.94	51.94	36.31	46.39	18.74	53.66	35.93
		43.62		**69.92**		**40.40**		**66.96**

Fig. 6. Cell disruption using two mechanical devices. (A) Percent of cell viability after the cell disruption with the high-pressure homogenizer EmulsiFlex-C5 or the bead mill Dyno-mill ML. (B) SDS-PAGE of cell lysates using the homogenizer and the bead mill. Lane 1: AccuRuler RGB PLUS prestained protein ladder (Maestrogen, Taiwan). Lanes 2, 4: Cell lysates after the mechanical disruption with the homogenizer and the bead mill, respectively. Lanes 3, 5: Washed pellets from the homogenizer and the bead mill, respectively. (C) Western blot of washed pellets from the homogenizer (lane 2) and the bead mill (lane 3). Protein detection was assessed as above. (D) Relative quantification of recombinant antigens in cell lysates and washed pellets.

of the desired product. In an earlier study, we designed and produced, for the first time, an effective recombinant vaccine candidate against *L. intracellularis* based on three recombinant proteins. The aim of the present research was to establish a consistent production process for this vaccine candidate.

It is known that cell densities obtained in TB medium could be ten times higher than in LB medium [11,19]. The culture medium must supply enough energy and carbon source for the growing and propagation of microbial cells [20]. Here, we evaluated three different culture conditions to increase the cell biomass production before inducing the expression of *Lawsonia* antigens: (1) batch culture with TB medium; (2) batch culture with MTB medium (3% glycerol) and (3) fed-batch culture using glycerol and yeast extract. The culture curve in TB medium showed a severe decrease in the growth rate after 6 h. The sudden entrance to the stationary phase could suggest the nutrient depletion. Then, we decided to modify the TB medium composition by increasing the carbon source (glycerol), which maintained a constant growth rate until the end of the culture and increased the biomass yield. The uncertain fate of the culture after 8 h and the need for higher cell density prompted us to use fed-batch cultivation.

The feeding method is frequently used to increase the biomass concentration and the recombinant product yield [21,22]. High biomass concentration (125 g DCW/L) has been described in non-transformed *E. coli*. However, the expression of a specific recombinant product in transformed *E. coli* is characterized by lower yields of biomass concentration (6–77 gDCW/L) [23,24]. It depends on the composition of the culture medium, the feeding strategy, the desired recombinant protein, among others. Here, a constant feeding rate for 2 h allowed to prolong the culture and to increase the

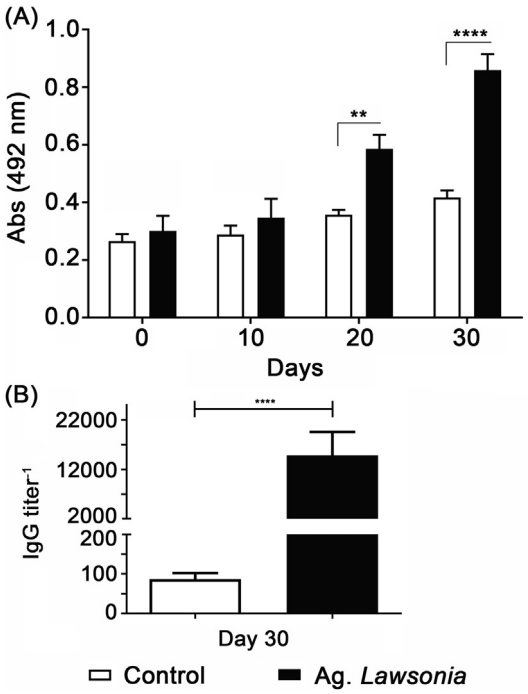


Fig. 7. Humoral immune response against *Lawsonia* antigens in pigs. (A) Absorbance values of sera from pigs immunized with *Lawsonia* antigens. (B) Titration of sera from day 30 after immunization. ELISA plates were coated with 100 ng per well of *Lawsonia* antigens (OMP1c, OMP2c, INVASc) solubilized in urea 8 M. Bars represent the standard deviation. Statistical analyses were performed by a two-way ANOVA and a Tukey post-test of multiple comparisons. The antibody titration was compared by the Mann Whitney test. ****$p < 0.0001$, **$p < 0.01$.

biomass. It was two-fold higher than biomass of the batch culture. Similar results of biomass were already obtained in a non-induced fed-batch culture [25].

After the fed-batch conditions were established, we did induction experiments using IPTG at 0.75 and 1.5 mM, based on the low yields of *Lawsonia* antigens obtained during the induction with 0.5 mM IPTG in a previous study [7]. The induction with 0.75 mM IPTG doubled the productivity of the culture compared to that obtained with 1.5 mM IPTG. The reduction of the antigen production after inducing with 1.5 mM IPTG could be provoked by the toxic effect of the IPTG and/or the metabolic burden imposed on the cells due to the heterologous gene expression [26,27]. The induction at higher biomass concentration should lead to an increase in the production of recombinant proteins since large amounts of transformed cells express the gene of interest [28]. Therefore, the fed-batch culture was induced at higher biomass concentration using 0.75 mM IPTG. As expected, we obtained an increment of *Lawsonia* antigen yields, which corroborated the dependence of the induction time and the expression of recombinant proteins [29,30].

The plasmid stability during the culture is an essential aspect regarding the efficiency of recombinant protein production. A decrease in plasmid-bearing cells during the culture is documented [31,32]. The plasmid imposes metabolic stress to the host cells, conducting to decrease the plasmid copy number. This metabolic stress not only is induced by the expression of the recombinant protein coded into the transcriptional unit, but also by the plasmid maintenance and its replication [33]. Moreover, the effect of the plasmid on the host cell physiology depends on the unique characteristics of each plasmid, which differ in the replication, the promoter system and the host-vector interaction [34].

According to our results, the substitution of glycerol by glucose as primary carbon source allowed higher plasmid stability. This effect could be related to the strong repression exerted by glucose to the transcriptional units under the control of T7 polymerase promoter, which could diminish the basal expression. It prevents the production of recombinant proteins before the induction [35].

The selection marker also determines the quantity of plasmid-bearing cells. In our study, ampicillin was used as selection marker to limit the growth of plasmid-free cells. However, the oversecretion of β-lactamase frequently leads to rapid degradation of ampicillin and a decrease in the amount of plasmid-bearing cells [36]. The use of other selection markers should be considered for increasing the plasmid-bearing cells and the specific product yield.

Bead mill and high-pressure homogenizer were used to evaluate cell disruption, which have proven effective in the scale-up production processes [37,38]. The efficiency of the cell disruption is often related to the number of passes, the selected pressure for the homogenizer, and the size and quantity of the beads for the bead mill. Moreover, two passes through the homogenizer are enough for the release of soluble proteins, and the inclusion body recovery requires at least four passes. Also, cell disruption by mechanical methods ensures a large quantity of inclusion bodies due to their mechanical stability [39]. According to our results, six passages were enough to release the inclusion bodies, which constitute the active component of the vaccine candidate. Although the desired proteins contained in the washed pellet were not obtained with high purity, several studies described the efficacy of veterinary vaccine candidates using the cell lysate of *E. coli* or soluble antigens containing bacterial debris [40,41]. The *Lawsonia* antigens produced by the methodology described in this study induced and effective immune response. It was superior to that obtained previously [7], because significant differences were detected in the antibody levels before the second immunization, and antibody titers increased four-fold (from 1/3000 to 1/12,000). Also, these antibody titers were higher than those obtained with conventional vaccines [42]. The increase in the immune response could be related to the use of Montanide ISA 50V2 instead of Montanide ISA 15A VG used in the preceding research. This result demonstrated that *Lawsonia* antigens produced under the established conditions retained their immunogenic characteristics.

5. Concluding remarks

The established production process for the obtaining of a novel vaccine candidate against *L. intracellularis* is a feasible process that allowed a 70-fold antigen increase and preserved its immunogenicity. The downstream process involves only the mechanical disruption of cells and the washing of the cell lysate. Taking into account that one dose contains 200 μg of antigens, one liter of fermentation could provide around 2000 doses of the future vaccine.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary data

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