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Identification of Leukotoxin and other vaccine candidate proteins in a *Mannheimia haemolytica* commercial antigen

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Abstract

Bovine Respiratory Disease is the most costly disease that affects beef and dairy cattle industry. Its etiology is multifactorial, arising from predisposing environmental stress conditions as well as the action of several different respiratory pathogens. This situation has hindered the development of effective control strategies. Although different type of vaccines are available, many currently marketed vaccines are based on inactivated cultures of the main viral and bacterial agents involved in this pathology. The molecular composition of commercial veterinary vaccines is a critical issue. The present work aims to define at the proteomic level the most relevant valence of a line of commercial respiratory vaccines widely used in Central and South America. Since *Mannheimia haemolytica* is responsible for most of the disease associated morbid-mortality, we focused on the main proteins secreted by this pathogen, in particular Leukotoxin A, its main virulence factor. By Western blot analysis and mass spectrometry, Leukotoxin A was identified as a major component of

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M. haemolytica culture supernatants. We also identified other ten *M. haemolytica* proteins, including outer membrane proteins, periplasmic transmembrane solute transporters and iron binding proteins, which are relevant to achieve protective immunity against the pathogen. This work allowed a detailed molecular characterization of this vaccine component, providing evidence of its quality and efficacy. Furthermore, our results contributed to the identification of several proteins of interest as subunit vaccine candidates.

Keywords: Biological Sciences, Microbiology, Immunology

1. Introduction

Bovine Respiratory Disease (BRD) is one of the leading causes of death and economic loss for the beef and dairy cattle industry. It has an estimated annual cost to the worldwide cattle industry of over $3 billion (Potter, 2015). This multifactorial syndrome can be caused either by viral or bacterial pathogens. Viral agents include bovine respiratory syncytial virus (BRSV) (Gershwin et al., 2000), parainfluenza III virus (PI-3) (Fulton et al., 2000), bovine viral diarrhea virus (BVDV) (Ridpath, 2010) and bovine corona virus (BCV) (Autio et al., 2007). These respiratory viruses along with some mycoplasmal agents are generally the primary infectious agents involved in BRD (Potter, 2015). This initial infection establishes a respiratory environment predisposing to the colonization and replication of pathogenic bacteria, including *Mannheimia haemolytica* (Al-Ghamdi et al., 2000), *Pasteurella multocida* (Dabo et al., 2007) and *Histophilus somni* (Corbeil, 2007), the former being responsible for up to 50% of the disease associated mortality (Narayanan et al., 2002; Potter, 2015). *M. haemolytica* is a Gram negative opportunistic bacterium. Although it is a common bacterium of the upper respiratory tract and nasopharynx of healthy ruminants, it can also act as an opportunistic pathogen infecting the lower respiratory tract under stressful situations such as shipping, adverse environmental conditions or previous respiratory viral infection (Youssef et al., 2004; Orouji et al., 2012).

Being BRD a multifactorial disease, the development of effective control strategies has been difficult to accomplish. Vaccination against viral and bacterial pathogens involved in BRD is a useful tool to reduce the risk of this disease. Many commercial respiratory vaccines are composed by inactivated cultures of the most relevant pathogens (Edwards, 2010; Rodrigues et al., 2015). These multivalent formulations are designed to reduce the impact of viral infections and to neutralize the severe associated bacterial infections. Therefore, to guarantee the efficacy of BRD vaccines, it is important to achieve a complete characterization of the antigens included in the vaccine and to verify the presence of those molecules critical for establishing an efficient immunological protection.

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In this work we attempt to characterize the main proteins of *M. haemolytica* in a polyvalent inactivated BRD line of vaccines currently commercialized in Central and South America (Neumosan, Virbac Uruguay S.A. (former Santa Elena), http://www.santaelena.com.uy). Among them we emphasize the study of Leukotoxin A (LKT), its main virulence factor, which has been shown to play an important role in the development of a protective immunity against the pathogen (Confer et al., 2009).

LKT is a potent cytolytic toxin, with an apparent molecular weight (MW) of 105 kDa, actively secreted by all serotypes of the bacterium during the logarithmic phase of in vitro growth (Shewen and Wilkie, 1985; Chang et al., 1987). It belongs to the family of the RTX (repeat in toxin) pore-forming cytolysins, which have been linked with the virulence of other bacterial species (Lo et al., 1987; Lainson et al., 1996). It specifically targets ruminant leukocytes and plays a major role in pathogenesis of BRD by impairing the primary lung defense mechanism and by inducing inflammation as a consequence of leukocyte lysis (Conlon et al., 1991). This phenomenon has been correlated with its ability to bind and interact with the ruminant beta2-integrin Lymphocyte Function-associated Antigen 1 (LFA-1) (CD11a/CD18) (Zecchinon et al., 2005; Singh et al., 2011). Neutralizing antibodies generation against the cytolytic toxin is relevant for achieving a high level of protection (Mosier et al., 1989). Immunity against *M. haemolytica* also requires antibodies against bacterial cell surface antigens (Shewen and Wilkie, 1988) and iron-regulated outer membrane proteins (Potter et al., 1999).

Our aim is to bring more insight into the molecular composition of a *M. haemolytica* inactivated antigen used in bovine commercial vaccines, focusing on the identification of relevant secreted or surface-exposed proteins. This knowledge will support the development of specific quality control techniques to assist the optimization and standardization of the industrial vaccine production process.

2. Materials and methods

2.1. Laboratory scale *M. haemolytica* culture

A seed culture was prepared by inoculation of an aliquot of master seed bank (MSB) of *M. haemolytica* serotype A1 (production strain code B059) in TSB-BHI (Tryptic Soy Broth, Brain Heart Infusion, Porcine) and incubation at 37.0 ± 1.0 °C, for 12 h with orbital agitation (220 rpm). Inoculum was prepared by a 1:100 dilution of the seed culture in TSB-BHI and further incubation at 37.0 ± 1.0 °C with orbital agitation until an optical density at 600 nm (OD_{600nm}) of 0.2 to 0.3 units was reached. A benchtop fermentor (Biostat A, New Brunswick) with 2 L of fermentation media (TSB-BHI supplemented with 0.4% glucose) was inoculated with 20 mL of the inoculum culture (1:100 dilution). The fermentation culture was grown at 37.0 ± 1.0 °C with dissolved oxygen set above 40% and variable

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agitation. During fermentation, samples were taken every one hour to measure OD_{600nm}, dissolved oxygen (%), glucose concentration (mg/dL) and pH. The culture was harvested when it reached stationary growth phase. Samples and the final culture were centrifuged at 8000×g for 30 min at 4 °C and culture supernatants were stored at −20 °C.

2.2. Industrial *M. haemolytica* antigen preparation

M. haemolytica culture for vaccine antigen preparation was performed following the industrial standardized protocol. Culture media used was developed by Virbac Uruguay S.A. and is based on protein hydrolysates, yeast extract, dextrose, and sodium and potassium salts. The inoculum was prepared as previously described using 1 vial of production seed of *M. haemolytica* serotype A1 (production strain code B059). The production fermentor was inoculated with a 1:100 dilution of the inoculum and the culture was performed at 37 °C with automatic agitation and aeration until the end of the logarithmic growth phase. After harvesting, culture was inactivated by incubation at 37 °C with formaldehyde 0.5% (v/v) during 24 h. To obtain culture supernatant the inactivated culture was centrifuged at 8000×*g* for 30 min at 4 °C. Inactivated culture supernatant was stored at −20 °C.

2.3. Recombinant LKT production

Specific primers were designed to amplify a portion of LKT which contains the C-terminal domain of the protein. An *in silico* prediction of the location of linear B-cell epitopes was performed using Bepipred algorithm (Larsen et al., 2006). As previously reported (Lee et al., 2001), the portion of LktA with more immunogenic epitopes was the C-terminal domain. Therefore, a sequence including the main predicted epítopes (amino acid 713 to 873) was selected. *M. haemolytica* (production strain code B059) was cultured as the seed culture described above. Culture was centrifuged at 10000×*g* and the cell pellet was stored at −70 °C. *M. haemolytica* genomic DNA was extracted using a standard protocol (Ausubel et al., 1999) and partial LKT sequence was amplified by PCR. For LKT expression, restriction sites were included in primers for a directional cloning of LKT sequence in pET28a(+) vector (Novagen). *E. coli* BL21 DE3 (Novagen) competent cells were transformed with the construct and stored at −70 °C in 15% glycerol. Plasmid DNA was isolated from *E. coli* using a commercial kit (QiaPrep Spin Miniprep, Qiagen). Plasmid sequence was confirmed by DNA sequencing at Institut Pasteur Montevideo (Uruguay). For LKT expression, a culture was performed in ZYM-5052 auto induction medium (Studier, 2005) for 18–20 h at 37.0 ± 1.0 °C. As expected, by SDS-PAGE a band of approximately 40 kDa corresponding to the recombinant protein was observed. The protein was purified by IMAC chromatography (HisTrap HP, GE Healthcare) in an ÄKTA Purifier 100 (GE Healthcare). The

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purified protein was dialyzed against PBS 1X pH 7.4 and quantified by BCA (Pierce BCA Protein Assay Kit, Thermo Scientific).

2.4. Rabbit anti-LKT serum production and evaluation

Two New Zealand white rabbits (2.5 Kg body weight) were maintained in the experimental animal facility (Instituto de Higiene, Universidad de la República, Uruguay) in accordance with animal procedures approved by the Honorary Commission of Animal Experimentation (CHEA protocol No 071140-000771-11). Both rabbits were immunized subcutaneously with 50 ug of recombinant LKT, on days 0 and 21 of the immunization protocol. Blood samples were taken on days 21, 45 and 115, and sera were prepared and stored at −20 °C. Serum titers were determined by ELISA using 0.1 μg/well of recombinant LKT for coating, serum dilutions of 1:100 to 1:100.000, and conjugate dilution of 1:10.000 (Goat anti-rabbit IgG (whole molecule)—Peroxidase, Sigma). High titer sera were pooled and purified by protein A chromatography (HiTraprProtein A, GE Healthcare). Purified IgG reactivity against native and recombinant LKT was evaluated by Western blot as described below.

2.5. SDS-PAGE and Western blot analysis

Culture supernatants were analyzed by 12% SDS-PAGE and coomassie blue staining (Ausubel et al., 1999). Molecular weight markers used were PageRuler Prestained Protein Ladder (Thermo Scientific) and Prestained Protein Ladder Broad Range (NEB).

For Western blot analysis, proteins were transferred onto a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences) for one hour at 400 mA. Rabbit antibodies against recombinant LKT, produced and purified by our work group, were used to identify LKT in culture supernatants. Briefly, membranes were incubated for 1 h at room temperature with anti-LKT antibodies at final concentration of 17.5 μg/mL in PBS 1X pH 7.4, 5% low fat milk, with gentle agitation. For antigen-antibody detection membranes were incubated for 1 h at room temperature with a 1:5000 dilution of anti-rabbit IgG (whole molecule)—alkaline phosphatase antibody produced in goat (Sigma) in PBS 1X pH 7.4, 5% low fat milk. The intensity of resulting bands was quantified using a Molecular Imaging Software (Carestream Health Inc).

For immune response evaluation bovine sera obtained from a calf infected with *M. haemolytica* and from a healthy calf were gently provided by Virbac Uruguay. Inactivated and non inactivated culture supernatant samples analyzed by SDS-PAGE were transferred onto membranes, as previously described. Membranes were incubated for 1 h at room temperature with a 1:500 dilution of each serum and afterwards for 1 h at room temperature with a 1:5000 dilution of anti-bovine

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IgG–HRP (Novex), all in PBS 1X pH 7.4, 5% low fat milk. Detection was performed using TMB liquid substrate system for membranes (Sigma).

2.6. Mass spectrometry (MS)

Inactivated culture supernatant obtained from the industrial process was concentrated 20X by ultrafiltration (Vivaspin 500, 5 kDa, GE Healthcare) and analyzed by SDS-PAGE and coomassie blue staining. The most defined and intense bands were identified by peptide mass fingerprinting at Institut Pasteur Montevideo (Uruguay) using a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems). Peptides from each sample were obtained by trypsin treatment (Sequencing Grade, Promega) overnight at 37 °C. Peptide mass spectra were acquired as previously described (Lima et al., 2011) and MS/MS collision induced dissociation experiments of selected peptides were performed. Proteins were identified by NCBInr database searching with peptide m/z values using MASCOT search engine (Matrix Science) (Perkins et al., 1999) with the following searching parameters: monoisotopic mass tolerance, 0.08 Da; fragment mass tolerance, 0.45 Da and one missing tryptic cleavage allowed. Significant scores ($p < 0.05$) were used as criteria for positive protein identification.

3. Results

3.1. *Mannheimia haemolytica* laboratory scale fermentation

During *M. haemolytica* laboratory scale fermentation, parameters such as glucose concentration, dissolved oxygen, optical density (OD_{600nm}) and pH were determined in samples taken every one hour of culture. This fermentation showed a kinetic profile compatible with typical bacterial growth in a batch system (Fig. 1B). Culture was harvested ten hours after fermentation start, at the beginning of the stationary growth phase.

Supernatant samples of a *M. haemolytica* laboratory scale culture were analyzed in order to evaluate the LKT accumulation in the supernatant. To identify LKT specific anti-LKT antibodies were produced. In preliminary evaluation experiments, we confirmed that these antibodies showed the expected reactivity against native and recombinant LKT and a good specific antibody titer. By means of these antibodies we identified LKT as a single defined band of approximately 110 kDa in culture supernatant samples of *M. haemolytica* (Fig. 1A). In parallel, the presence of LKT in this protein band was confirmed by MS analysis (MS/MS protein score 408, protein sequence coverage 36%).

The intensity of the specific LKT band detected by Western blot was determined by molecular imaging software. This analysis showed that LKT expression

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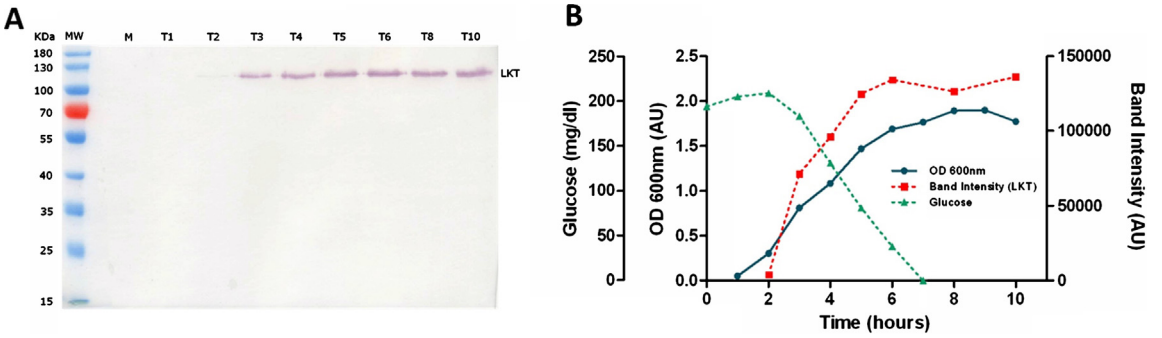

Fig. 1. *M. haemolytica* culture growth and LKT secretion profile. A. Anti-LKT Western blot of *M. haemolytica* culture supernatant samples from different fermentation time points. M: fermentation media, T1–T10: samples from culture kinetics (1–10 h). MW: Molecular weight marker. B. *M. haemolytica* culture growth curve and kinetics of Leukotoxin secretion. Optical density (OD) at 600 nm, glucose concentration (mg/dL) and band intensity of LKT expressed in arbitrary units (AU) are plotted versus culture growth time measured in hours.

correlates with growth kinetics, being associated with exponential growth phase of *M. haemolytica* (Fig. 1B), as previously reported (Chang et al., 1987).

Therefore, the presence of LKT was verified in the culture supernatant of the *M. haemolytica* production strain, during the exponential growth phase of the culture.

3.2. Proteomic characterization of *M. haemolytica* inactivated culture supernatant

For proteomic analysis, industrial antigen was prepared according to vaccine production protocol. Culture supernatant was obtained and concentrated by ultrafiltration. After SDS-PAGE electrophoresis and coomassie blue staining, selected bands were recovered for MS identification (Fig. 2A). This approach allowed the statistically significant identification of 11 proteins of *M. haemolytica* in the industrial antigen (Table 1). LKT was identified, in accordance with previous results of laboratory scale fermentation, among other relevant proteins. Some of these are outer membrane proteins (OMPs), which along with LKT are critical to achieve a protective immunity to the pathogen (Lo et al., 1991; Singh et al., 2011).

Subcellular *in silico* localization, predicted using free access PSORTb software (available at http://psort.hgc.jp/), indicated that most of the identified proteins are periplasmic (7/11), with others being extracellular (1/11) or located in the outer cell membrane (2/11).

Although most proteins coincided with their theoretical MW (Table 1), LKT was identified by MS in a higher than expected MW band (>230 kDa). This result was confirmed by Western blot of inactivated supernatant using anti-LKT antibodies

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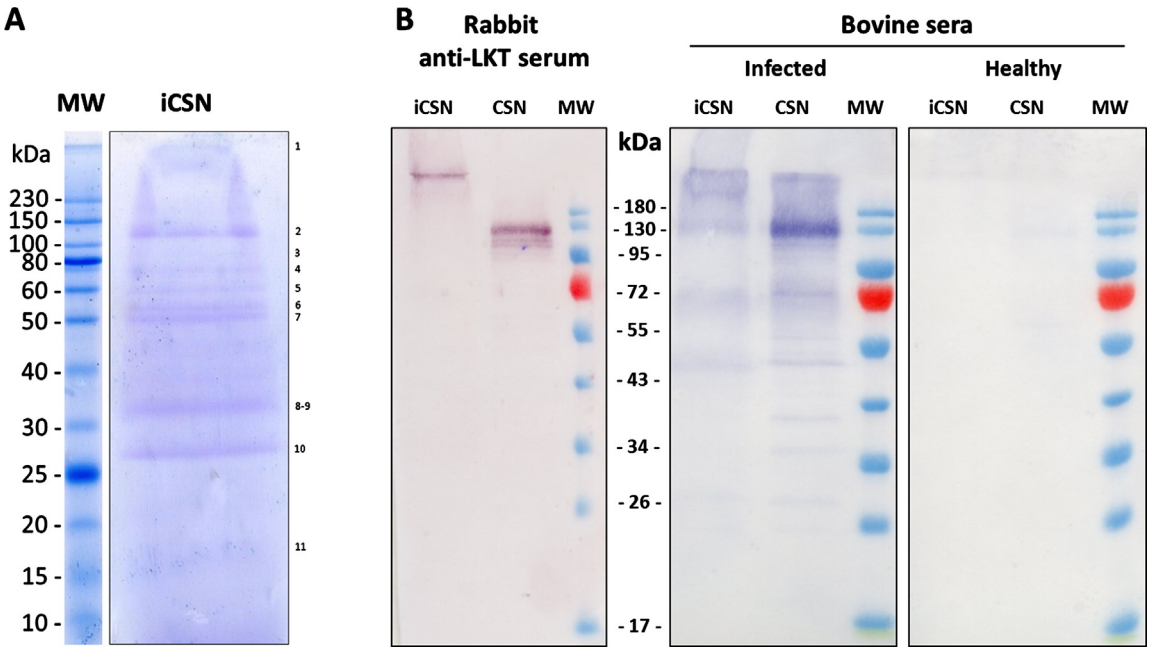

Fig. 2. Identification of *M. haemolytica* culture supernatant proteins. A. *M. haemolytica* proteins of inactivated culture supernatant (iCSN) resolved in SDS-PAGE and stained with coomassie blue. Bands corresponding to the proteins analyzed by MS (MALDI TOF/TOF) are numbered and referred to on Table 1. For complete image see Fig. S1 (Supplementary material). B. Analysis of culture supernatant proteins by Western blot. Left: *M. haemolytica* inactivated (iCSN) and non inactivated culture supernatant (CSN) proteins probed with anti-rLKT rabbit serum. Middle: Reactivity of bovine *M. haemolytica* infected serum against *M. haemolytica* iCSN and CSN. Right: Reactivity of bovine healthy serum against *M. haemolytica* iCSN and CSN. MW: Molecular weight marker.

(Fig. 2B). This behavior of LKT in electrophoresis could be explained by cross-linking caused by formaldehyde treatment leading to high molecular weight structures.

Immune reactivity of a serum obtained from a *M. haemolytica* infected calf against culture supernatant was addressed by Western blot. As shown in Fig. 2B several bands of both inactivated and non inactivated culture supernatant were specifically recognized by infected bovine serum and not by healthy bovine serum. Among them, LKT was the more reactive band.

4. Discussion

This work provides an exhaustive molecular characterization of an inactivated culture supernatant of *M. haemolytica* included in a line of polyvalent commercial bovine vaccines (Neumosan, Virbac Uruguay). Since *M. haemolytica* is the main etiological agent of BRD and is also responsible for a high proportion of the

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Table 1. Proteins identified in culture supernatant of *M. haemolytica*.

Gel reference (Fig. 2A)	Protein	Gene symbol	MS/MS scores*	ID (Uniprot)	M.W.	Subcellular Location	Additional Information
1	Leukotoxin A	lktA	121	I1W261	102.1 kDa	Extracellular	Involved in pathogenesis and calcium ion binding (Vougidou et al., 2013).
2	Serotype-specific antigen 1	ssa1	235	P31631	103.6 kDa	Outer Membrane	Associated to serotype change in the bovine upper respiratory tract after stress. May have protective vaccine potential (Lo et al., 1991; Ayalew et al., 2011).
3	Formate C-acetyltransferase	pflD	88	E2PBB6	86.7 kDa	Cytoplasmic	Piruvate metabolism, acetiltransferase activity (Lawrence et al., 2010).
4	2‘ 3'-cyclic-phosphodiesterase/ 3'-nucleotidase	cpdB	167	M9X363	72.8 kDa	Periplasmic	Nucleotide metabolism (Eidam et al., 2013).
5	Oligopeptide ABC superfamily transporter, binding protein	oppA	133	M9X4S4	61.0 kDa	Periplasmic	ABC transporter system component involved in molecule export through bacterial membrane (Eidam et al., 2013).
6	Extracellular solute-binding protein family 5	MHH_RS20960	211	A0A0B5BMJ5	57.9 kDa	Periplasmic	ABC transporter system component involved in peptide transmembrane transport (Harhay et al., 2013).
7	Heme-binding protein A	MHH_RS16715	168	A0A0B5BSE3	59.6 kDa	Periplasmic	Similar to antimicrobial peptide ABC transporter periplasmic binding protein SapA (Harhay et al., 2013).
8	Iron (Fe3+) binding protein	yfeA	212	S9Y6G0	33.0 kDa	Periplasmic	Cellular adhesion and iron transport (Hauglund et al., 2015).
9	TRAP transporter solute receptor TAXI family-periplasmic binding protein	MHH_RS16825	130	M9X2M0	34.5 kDa	Periplasmic	Extracytoplasmic solute-receptor-dependent secondary active transporter (Rabus et al., 1999; Eidam et al., 2013).
10	HisJ-like histidine ABC transport system-periplasmic binding protein	MHH_RS14805	328	M9WWE6	28.0 kDa	Periplasmic	ABC transporter system component involved in molecule export through bacterial membrane (Eidam et al., 2013).
11	Outer membrane protein P6	pal	121	M9X7K9	16.8 kDa	Outer Membrane	Belongs to ompA family (Eidam et al., 2013). Ortholog Omp P6 reported as immunogen in other species (Roier et al., 2013; Alvarez et al., 2015).

* For this search protein scores greater than 87 are significant ($p < 0.05$).

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disease associated morbid-mortality (Potter, 2015), this kind of information is of utmost importance.

Available data demonstrate that the protective immunity against *M. haemolytica* can be acquired by neutralizing antibodies against Leukotoxin A (Mosier et al., 1989), as well as antibodies against bacterial cell surface antigens (Shewen and Wilkie, 1988; Confer et al., 1995; Confer et al., 2009). In order to promote an effective immune response, the presence of these pivotal elements in a BRD vaccine formulation is crucial.

Based on previous reports that detected Leukotoxin A activity in bacterium-free culture supernatant from logarithmic-phase cultures (Chang et al., 1987), Neumosan line of vaccines includes inactivated culture supernatants of an industrial optimized *M. haemolytica* culture. In this work we confirmed that LKT secretion is linked to the exponential growth phase during the antigen production culture, representing one of the primary secreted components. In culture supernatant, LKT was detected as a single band with the expected MW (approx. 110 kDa) (Lo et al., 1985; Chang et al., 1987). Interestingly, after formaldehyde treatment, LKT was detected as a higher MW band (>230 kDa), suggesting that the toxin could be arranged as a high order structure, which could be fixed by this cross-linking agent (Klockenbusch and Kast, 2010). This finding is in agreement with previous reports that showed that the molecular weight of native LKT in culture supernatant was higher than 400 kDa (Chang et al., 1987).

Besides LKT identification, we delved further into the characterization of the culture supernatant protein profile. By MS we identified other 10 proteins of *M. haemolytica*. Most of them are periplasmic and outer membrane proteins, which due to their extracellular location and exposure to the host immune system could represent interesting candidates to play a key role in the protection against this pathogen. It is worth mentioning that we also identified a cytoplasmic protein involved in carbohydrate metabolism (Formate C-acetyltransferase). This finding could be explained by some cellular lysis that could occur during bacterial growth and inactivation (Derzelle et al., 2005), hence we cannot rule out that the culture supernatant may contain some cytoplasmic proteins.

Some of the outer membrane proteins identified in this work have been reported as important immunogens and have been evaluated as recombinant subunit antigens due to their protective vaccine potential (Ayalew et al., 2011; Guzman-Brambila et al., 2012). In particular, vaccination of mice and cattle with recombinant serotype specific antigen 1 (rSsa1) stimulated high antibody responses (Ayalew et al., 2011). Besides, a "Best-Reciprocal-Blast-Hit" approach was performed in order to determine that *M. haemolytica* outer membrane protein P6 identified in this work, is ortholog to Omp P6 of *H. somni* and *P. multocida*, both reported as

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immunogenic in recent reports (Roier et al., 2013; Alvarez et al., 2015). In that sense, the natural occurrence of these proteins in the inactivated vaccine antigen indicates that the vaccine contains the main components required for protection against *M. haemolytica*.

Different proteins belonging to the ATP-binding cassette (ABC) transporter superfamily were identified in the commercial antigen. ABC transport systems are essential for many processes of the cell, such as uptake of amino acids, peptides, carbohydrates and metal ions in an ATP coupled process. There is increasing evidence that these transport systems play either direct or indirect roles in the virulence and pathogenicity of bacteria (Garmory and Titball, 2004). In a recent work, other ABC transporters were identified as immunodominant proteins from *M. haemolytica* and *H. somni* in cattle immunized with a commercial cellular vaccine, confirming that the surface exposed transporters of these species are immunogenic (Alvarez et al., 2015). Interestingly, our results evidence that ABC transporters are relevant components in the vaccine antigen, and therefore could be potential targets for the development of antibacterial subunit vaccines (Garmory and Titball, 2004).

Finally, we also identified an iron-regulated periplasmic protein YfeA, previously described as immunodominant in animals immunized with a cellular *M. haemolytica* vaccine (Alvarez et al., 2015), as well as with an OMP-enriched fraction of *M. haemolytica* (Ayalew et al., 2010).

Taken together, these observations support the protective potential of several of the proteins indentified in the *M. haemolytica* antigen. As an additional outcome anti-LKT antibodies developed for LKT identification can be used in routine quantitative assays in order to establish acceptance criteria of antigen commercial batches.

Declarations

Author contribution statement

Paula Tucci: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Verónica Estevez, Lorena Becco: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Florencia Cabrera-Cabrera: Performed the experiments; Analyzed and interpreted the data.

Germán Grotiuz: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Eduardo Reolon: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Mónica Marín: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Competing interest statement

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Additional information

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