A crude capsular polysaccharide extract as a potential novel subunit vaccine with cross-protection against the most prevalent serovars of *Glaesserella (Haemophilus) parasuis* in the Czech Republic

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Abstract: *Glaesserella (Haemophilus) parasuis* is a part of the normal flora of the respiratory tract of pigs. However, under certain conditions it can also induce severe systemic disease with high morbidity and mortality leading to gross economic losses in the pig industry. The most prevalent serovars in pig herds in the Czech Republic are the virulent serovars 1, 4, 5 and 13. The currently available commercial vaccines are inactivated vaccines with certain limitations, such as no or poor cross-serovar protection. Therefore, the aim of the present study was to construct a subunit vaccine with a crude capsular polysaccharide extract (cCPS) isolated from *G. parasuis* CAPM 6475 (serovar 5) and evaluate its immunogenicity in a mouse model. Mice were immunised subcutaneously with two doses of the constructed vaccine in a 14-day interval and challenged intraperitoneally with various *G. parasuis* strains (serovars 1, 4, 5, 13) at 21 days after the second immunisation. The results of the ELISA test showed that the boost dose of the vaccine induced the production of IgG antibodies in high levels. On the basis of the death cases, the pathological findings and the bacterial isolation, the mice immunised with the cCPS were partially protected against the challenge with the homologous serovar 5 as well as with heterologous serovars 1, 4 and 13 of G. *parasuis.* The cross-reaction of the mixed serum from the immunised mice with the tested serovars was seen in the western-blotting also. Moreover, the most abundant protein found in the cCPS by mass spectrometry was catalase, a protein of molecular weight 55 kDa that may correspond to the strongest reaction seen in the western-blotting. Our findings indicated that the crude capsular polysaccharide extract may provide an effective immunogenicity in preventing a *G. parasuis* infection caused by the most prevalent serovars in the Czech Republic. However, the evaluation of the efficacy needs to be performed in pigs before any conclusions can be drawn.

Keywords: catalase; cross-serotype protection; mouse model; respiratory pathogen

Glaesserella parasuis, formerly known as *Haemophilus parasuis,* is a small Gram-negative bacterium from the family *Pasteurallaceae* (Biberstein and White 1969; Inzana et al. 2016). Based on the immunodiffusion test of the *G. parasuis* strains with specific antisera, Kielstein and Rapp-Gabrielson (1992) proposed a uniform scheme for the designation of 15 serovars and reference strains associated with different likely serovar-related virulence. Avirulent serovars are commensal organisms of the upper respiratory tract (Moller and Killian 1990), while virulent serovars may cause pneumonia (Little 1970), acute septicaemia (Peet et al. 1983) or Glässer's disease characterised by fibrinous polyserositis, polyarthritis and meningitis (Amano et al. 1994). Howell et al. (2013) proposed that the capsular polysaccharides are the main determinant of the serovar in *G. parasuis.* However, the virulence differs, not only among the different serovars, but also within each serovar, which has been confirmed by genotyping methods and, thus, assumes further factors of virulence in this bacterium (Costa-Hurtado and Aragon 2013; Howell et al. 2013; Howell et al. 2014; Zhang et al. 2014; Turni et al. 2018). The most prevalent serovars among the *G. parasuis* strains isolated from sick pigs in the Czech Republic were serovar 4, 5, 13, 1 and the non-typeable isolates (Nedbalcova et al. 2005).

Vaccination is one of the most effective ways how to control and eradicate infectious diseases. The currently available commercial vaccines against *G. parasuis* are inactivated vaccines with certain limitations, such as no or poor cross-serovar protection (Liu et al. 2016). In addition, some components of the inactivated vaccines may generate side effects (Liu et al. 2009). Therefore, a subunit vaccine containing antigens that induce the expression of major histocompatibility complex or co-stimulatory molecules on the antigen-presenting cells to improve the antigen uptake, processing or presentation, may be promising in inducing an immune response protective against more *G. parasuis* serovars (Liu et al. 2016). *G. parasuis* may avoid the host immune system mechanisms thanks to a capsular polysaccharide presented on its surface, especially by incorporating a glycolyl-neuraminic acid into the bacterial carbohydrates (Perry et al. 2013). Therefore, the antibodies against the capsular polysaccharide of *G. parasuis* may help the host to recognise and phagocyte this bacterium. On the other hand, the capsular polysaccharides are

T-lymphocyte independent antigens and the structural similarities of the bacterial polysaccharides with glycolipids and glycoproteins (e.g., glycolylneuraminic acid) of the host may be a reason for their poor immunogenicity (Weintraub 2003). Moreover, the structure of the capsular polysaccharide in the *G. parasuis* serovar 5 and 15 has the same main chain with various side chains (Perry et al. 2013), but the gene content of the loci encoding biosynthesis of the capsular polysaccharide in these two serovars differs significantly as was also observed in another 13 serovars of *G. parasuis*, except serovar 5 and 12 that were identical (Howell et al. 2013). Based on these facts, we hypothesised that a crude capsular polysaccharide extract (cCPS) containing proteins from a virulent *G. parasuis* strain (serovar 5) could be used as a vaccine with good immunogenic properties leading to cross-serotype protection. Therefore, we prepared a vaccine with the cCPS and tested its safety and efficacy in a mouse model after the experimental challenge with the most prevalent *G. parasuis* serovars in the Czech Republic.

MATERIAL AND METHODS

Bacterial strains. The reference strains of *G. parasuis* kindly provided by the Université de Montréal (Canada) used in this study were the following: strain No. 4 of serovar (s.) 1, strain SW 124 of s. 4, strain Nagasaki of s. 5 and strain 84-17975 of s. 13. The field strain CAPM 6475 of serovar 5 originated from the brain of a pig with meningitis (Nedbalcova et al. 2011).

The strains were grown on chocolate agar plates (LabmediaServis, Czech Republic) at 37 °C for 18 hours. After incubation, the bacteria were harvested by resuspending in a calcium-magnesium free Dulbecco's phosphate-buffered saline (D-PBS; Lonza, Switzerland) and the density of the final inoculum was adjusted to 3×10^9 CFU/ml. This concentration was confirmed by the ten-fold dilutions plated on the chocolate agar plates.

Preparation of a crude capsular polysaccharide extract. A crude capsular polysaccharide extract (cCPS) was obtained from the *G. parasuis* strain CAPM 6475 by methanol extraction (Adlam et al. 1984). An overnight culture of *G. parasuis* grown on a chocolate agar plate was resuspended in D-PBS and incubated in a water bath at 60 °C for 1 hour. Thereafter, the bacterial suspension

was centrifuged at 9500 × *g* at 4 °C for 1 h to remove the cells following the addition of formaldehyde to a final concentration of 0.2% (v/v). Subsequently, the supernatant was concentrated to 1/50 of the volume using a VIVAFLOW 200 filter 5000 MWCO PES (Sartorius Stedim Biotech, Germany) and three concentrate volumes of methanol (PENTA, Czech Republic) and sodium acetate (PENTA,Czech Republic) to 1 % (w/v) were added. After 24 hours of the precipitate gravity settling, the supernatant was filtered with a VIVAFLOW 200 filter 0.2 µm PES (Sartorius Stedim Biotech, Germany) and three concentrate volumes of acetone (PENTA, Czech Republic) were added. After 36 hours, the supernatant was discarded and the precipitate was resuspended in an aqua pro injection and stored at −20 °C. The concentration of the proteins in our cCPS was determined with a Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, USA) according to the manufacturer's instructions.

LC-MS/MS analysis of the cCPS. Fifty micrograms of the proteins (measured with the Pierce Pierce™ BCA Protein Assay Kit, Thermo Scientific, USA) were used for each of the four replicates (four cCPS preparations) with the FASP (filter-aided sample preparation) method (Wisniewski et al. 2009). Each sample was washed five times with 8 M urea (Serva, Germany) in Vivacon 500 centrifugal tubes (Sartorius Stedim, Germany) with a 10 000 MWCO membrane filter. Dithiothreitol (10 mM, Sigma-Aldrich, USA) and iodoacetamide (50 mM, Serva, Germany) in a triethylammonium bicarbonate buffer (25 mM, Sigma-Aldrich, USA) were used for the reduction and alkylation, respectively. The proteins were then digested with trypsin (Promega, USA) in a 1 : 50 ratio, for one hour, at 37 °C and then overnight at 25 °C. After centrifugation, the eluate with the digested peptides was evaporated (DNA120 SpeedVac, Thermo Savant, USA), the peptide pellet was resuspended in a 0.1% aqueous formic acid (Sigma-Aldrich, USA) which serves as the mobile phase for the liquid chromatography (UltiMate 3000 RSLCnano, Dionex -Thermo Scientific, USA). For the separation and elution of the peptides, 2-hours gradient with increasing (0 min − 4%, 4 min − 4%, 98 min − 45%, 98.5 min − 90%, 112 min − 90%, 112.5 min − 4%, 120 min − 4%) concentrations of acetonitrile (0.1% formic acid in acetonitrile, Sigma-Aldrich, USA) at a flow rate of 300 nl/min was used. The peptides were separated on a 25 cm column (Acclaim PepMap RSLC C18, 2 µm, 100 Å, 75 µm I.D., Thermo Scientific, USA). The uHPLC was connected to an EASY-Spray ion source and an OrbitrapVelos Pro mass spectrometer (Thermo Scientific, USA). A survey scan over the m/z range 390–1700 was used to identify the protonated peptides with charge states of at least 2, which were automatically selected for the data-dependent MS/MS analysis and fragmented by collision with helium gas. Ten fragment mass spectra after each full scan were recorded. The measured spectra were then searched using the Proteome Discoverer (version 1.4, Thermo Scientific, USA) software with Sequest HT (Thermo Scientific, USA) as a searching algorithm. Oxidation of the methionine and deamidation of the asparagine and glutamine as a dynamic, and carbamidomethylation of the cysteine as a static modification was used. The precursor and fragment mass tolerances were set up as 10 ppm and 0.5 Da, respectively. The Uniprot database for the *Glaesserella parasuis* strain (from February, 2016) was used in the Sequest algorithm. Only peptides with a false discovery rate of less than 0.01 were considered as well identified.

Immunisation and experimental challenge. All the animal experiments were carried out in strict accordance with the recommendations of the Czech guidelines for animal experimentation and were approved by the Branch Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic (MZe 1539).

Seventy-two female BALB/c mice at the age of six weeks were randomly divided into 12 groups (six mice per group). Six groups were immunised intramuscularly with 0.2 ml of the cCPS (50 µg of proteins) emulsified in the complete Freund's adjuvant (Sigma Aldrich, USA). After 14 days, the immunised mice were re-vaccinated with the same concentration and volume of the cCPS emulsified in the incomplete Freund's adjuvant (Sigma Aldrich, USA). At 21 days, following the boost dose of the cCPS, the mice were challenged intraperitoneally with 1.5×10^9 CFU of various *G. parasuis* strains (Table 1) resuspended in 0.5 ml of D-PBS. Two groups, one immunised with the cCPS and one non-immunised, were injected intraperitoneally with 0.5 ml of D-PBS (Lonza, USA). The mice were monitored daily for morbidity and death until the end of experiment. After seven days, the surviving mice were necropsied to observe the gross lesions. Samples from lungs, liver and spleen were

collected aseptically for the bacterial recovery performed on a blood agar with a nurse strain of *Staphylococcus aureus* by incubation for 18 hours at 37 °C. The total score of the strain pathogenicity was evaluated by summation of the score for the death cases (a point per death), the pathological lesions represented as splenomegaly (a point per splenomegaly) and the bacterial recovery from three organs (a point for recovery per organ).

Antibody detection by Enzyme-linked immunosorbent assay. The level of the specific antibodies against the cCPS in the sera of the mice before the immunisation and at 21 days after the second immunisation was determined by a homemade enzyme-linked immunosorbent assay (ELISA). The cCPS antigen was dissolved in a carbonate-bicarbonate buffer (pH 9.6) in a concentration of 1 μg/ml that was subsequently dispensed (100 μl/well) into the wells of a 96-well polystyrene microtitre plate (Gama Group, Czech Republic). After the overnight incubation at 4 °C, the wells were rinsed four times with 300 μl of diluting solution containing PBS with 0.05% Tween. The non-specific binding sites of the antigen were blocked with 0.5% casein and 10% sucrose (in 250 μl of the PBS per well) for 30 min at room temperature (RT). The tested sera were prediluted 300 × in a solution of PBS with 0.5% Tween and 0.5% casein hydrolysate and dispensed to the wells (100 μl per well). After incubation of the plate for one hour at RT, the wells were rinsed four times (PBS with 0.05% Tween). 100 μl of Donkey anti-mouse IgG labelled with horseradish peroxidase (Jackson Immunoresearch Europe Ltd, UK) prediluted to 1 : 30 000 in the PBS with 0.05% Tween and 0.5% casein hydrolysate was added to the wells and incubated for another 1 h at RT. Afterwards, the plate was rinsed four times (PBS and 0.05% Tween) and 100 μl of the TMBComplete 2 solution (TestLine, Czech Republic) was added into each well. The reaction was stopped after 15 min of incubation at RT by adding 50 μl of 1 M sulfuric acid into each well. The absorbance was read at 450 nm using a multi-detection microplate reader Synergy H1 (BioTek, USA).

Western-blotting. To prove the cross-reactivity of the antibodies with all the tested *G. parasuis* strains, a western-blotting was performed. The bacteria were harvested after the overnight cultivation on the chocolate agars at 37 °C and resuspended in the D-PBS to the optical density of 2.5 McFarland. Afterwards, 10 μl of each bacterial strain as well as the cCPS antigen were resolved on 12.5% SDS-PAGE (Laemmli 1970) and blotted on a PVDF membrane (Amersham, Sigma-Aldrich, USA). The membrane was then incubated overnight at 4 °C in a blocking solution (1% casein in PBS-T). After blocking, the membrane was incubated with 85× diluted sera of the mice before or after the second immunisation with the cCPS (1% casein in PBS-T) for one hour at RT, washed in the

Table 1. The evaluation of the pathological lesions, bacterial recovery and death cases in the groups of mice immunised or non-immunised with the cCPS and challenged with the *G. parasuis* strains

Immunisation	Infection (*G. parasuis* strain)	Pathological lesions	Bacterial recovery	Death cases	Total score
Yes	No.4 (s. 1)	1	0	1	2
No	**No.4 (s.1)**	**6**	**2**	**1**	**9**
Yes	SW 124 (s. 4)	4	0	0	4
No	**SW 124 (s. 4)**	**5**	**3**	**0**	**8**
Yes	Nagasaki (s. 5)	0	0	0	0
No	**Nagasaki (s. 5)**	**6**	**15**	**0**	**21**
Yes	84-17975 (s. 13)	4	3	1	8
No	**84-17975 (s. 13)**	**4**	**18**	**0**	**22**
Yes	CAPM 6475 (s.5)	4	2	0	6
No	**CAPM 6475 (s.5)**	**6**	**12**	**1**	**19**
Yes	D-PBS	0	0	0	0
No	**D-PBS**	**0**	**0**	**0**	**0**

PBS with 0.05% Tween-20 (PBS/T, Serva) and incubated with the Donkey anti-mouse HRP-conjugated IgG (Jackson Immunoresearch Europe Ltd, UK) for another hour at RT. The secondary antibodies were diluted to 1 : 1000 in PBS/T. The specific protein bands were visualised with 3,3'-diaminobenzidine (Sigma-Aldrich).

Statistical analysis. Data were analysed using the Wilcoxon matched-pair singed rank test in GraphPad Prism (GraphPad Software, Inc.). The data are expressed as the means ± SEM. A *P*-value less than 0.05 was considered significant.

RESULTS

Evaluation of the immunisation efficacy after the experimental challenge

Two days after the challenge, one mouse died from the immunised groups challenged with serovar 1 and 13 and one mouse died from the non-immunised group challenged with serovar 1. The rest of the mice survived until the end of experiment. The total score for pathological lesions and bacterial recovery in each group is shown in Table 1. There were differences between the immunised and the non-immunised groups infected with the same *G. parasuis* strain. The cCPS immunisation led to the significant decreasing of the *G. parasuis* loading in the target tissues, especially in the groups challenged with the strains of serovar 5 and 13. Based on the results, the groups immunised with the cCPS were partially protected against the most prevalent serovars in the Czech Republic, unlike the non-immunised mice.
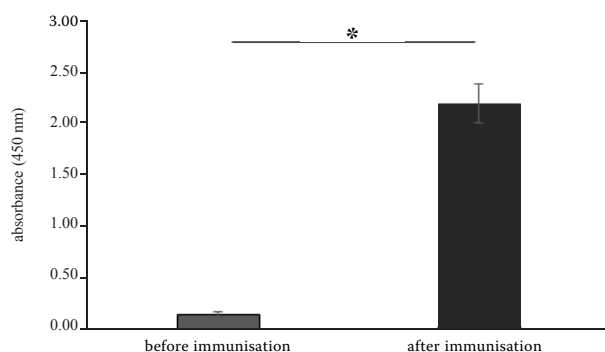

Figure 1. The ELISA test for the detection of the antibodies against the cCPS in the mixed mouse sera before and after the immunisation. The significant difference between the groups is denoted by an asterisk (*P* < 0.05)

Immunogenicity of the cCPS and its cross-reactivity

Based on the results of the ELISA test (Figure 1), the immunisation of the mice with the cCPS induced the production of specific IgG antibodies at very high levels. The absorbance of the mixed sera from the mice after the second immunisation with the cCPS was significantly higher than the absorbance of the mixed sera from mice before the immunisation.

Moreover, the antibodies reacted with all the tested *G. parasuis* strains in the western-blotting with the strongest reaction with the protein of a molecular weight of about 50 kDa (Figure 2).

Because the method of the cCPS preparation was not very specific, we, thus, expected a protein contamination in the final product. The crude capsular polysaccharide extract contained more than 100 bacterial proteins according to the mass spectrometry analysis (Table 2). The most abundant protein found in the cCPS was Catalase (Uniprot accession number U4RLR7) with a molecular weight of 55 kDa. From the western-blotting results (Figure 2), in the area about 50 kDa, we saw
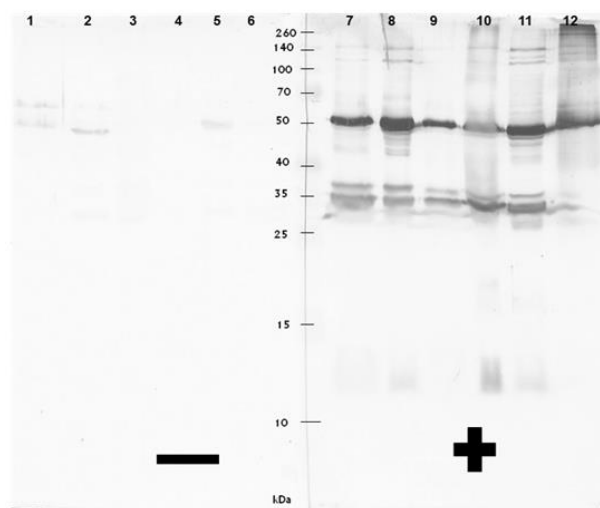

Figure 2. The western-blotting of the cCPS and lysates of the selected serovars of *G. parasuis* with pooled sera from the immunised or non-immunised mice. The antigens are as follows: lanes 1 and 12 = the cCPS; lanes 2 and 11 = strain CAPM 6475 (s. 5); lanes 3 and 10 = strain No. 4 (s. 1); lanes 4 and 9 = strain SW 124 (s. 4); lanes 5 and 8 = strain Nagasaki (s. 5); lanes 6 and 7 = strain 84-17975 (s. 13). The primary antibodies: (–) the pooled serum from the non-immunised mice, (+) the pooled serum from the immunised mice

Table 2. The *G. parasuis* proteins identified by the mass spectrometry analysis of the crude capsular polysaccharide extract. Only the ten proteins with the highest abundance are shown

Accession number	Protein name	ΣCoverage	Σ# Unique Peptides	Σ# Peptides	Σ# PSMs	# AAs	MW [kDa]	calc. pI
U4RLR7	Catalase	81.54	34	34	1557	482	54.9	7.01
B8F7C3	Acyl carrier protein	40.79	4	4	1153	76	8.5	4.03
U4SVA8	Peptidase M16 inactive domain protein	47.76	12	45	722	980	109.9	6.30
U4RN70	Thioredoxin	97.12	8	8	641	104	11.5	5.08
A0A0E1RNV3	Soluble cytochrome b562	70.16	17	17	614	124	13.8	9.20
U4RW17	Peptidase M16	33.27	1	34	569	980	109.9	6.24
U4S275	Maltose ABC transporter periplasmic protein	70.81	23	23	489	394	42.4	5.82
U4S0K9	Superoxide dismutase [Cu-Zn]	62.78	5	8	483	180	19.0	7.36
A0A0E1RPP7	Amino acid ABC transporter substrate-binding protein	77.31	24	24	450	260	28.1	8.59
U4S3M6	Cell division protein ZapB	100.00	13	13	419	72	8.4	4.84

AA = amino acid; calc pI = calculated isoelectric point; MW = molecular weight; PSM = peptide spectrum match

a strong band on the membrane with the sera from the cCPS-immunised mice compared to the non-immunised animals. Moreover, the antibodies from the immunised animals reacted with the proteins of different molecular weights that were not seen with sera from the non-immunised mice.

DISCUSSION

Capsular polysaccharides are the virulence factors in many bacteria, including *G. parasuis.* They are very heterogenic in their structure and the gene content of the biosynthesis loci as was documented for various strains of *G. parasuis* (Weintraub 2003; Howell et al. 2013; Perry et al. 2013; Howell et al. 2014). Capsular polysaccharide has been used in vaccines against encapsulated bacteria, but it often needs another immune stimulant to enhance the protection of a host against the infection (Weintraub 2003). Therefore, we prepared a crude capsular polysaccharide extract from the *G. parasuis* CAPM 6475 strain that also contained proteins from this bacterium.

The cCPS was able to induce a strong humoral response in the mice as seen from a high level of IgG in the serum after the second immunisation. In the western-blotting, these antibodies were able to react with all the tested *G. parasuis* strains. Moreover, the most abundant protein in the cCPS was a catalase with the molecular weight of 55 kDa, which may correspond to the strongest reaction of the immunised mice serum with the band at a molecular weight of approximately 50 kDa seen in the western-blotting. Li et al. (2017) detected a catalase among the secreted proteins from the *G. parasuis* Nagasaki strain that reacted with the anti-Nagasaki pig convalescent serum. The cross-reaction of the sera from the immunised mice seen in the western-blotting was proven also *in vivo* when the mice were intraperitoneally infected with various *G. parasuis* strains. The total score for pathological lesions, bacterial recovery and death cases in the immunised groups of mice were lower than in the non-immunised groups.

By mass spectrometry, we detected more than one hundred proteins in our cCPS. Some of these proteins have been previously determined as viru-

lence-related factors of *G. parasuis*, like the outer membrane proteins (ompp5, omp p2), lipoproteins, virulence-associated autotransporters (VtaA) or cytolethal distending toxins (CDT) (Costa-Hurtado and Aragon 2013; Zhang et al. 2014).

The immunogenic properties of VtaA were used in the experimental challenge where pigs immunised with recombinant VtaA from *G. parasuis* were partially protected against the lethal dose of the *G. parasuis* Nagasaki strain (Olvera et al. 2011). Antibodies against the lipoprotein plp4 and cytholethal distending toxins partially protected the animals against the *G. parasuis* challenge (Liu et al. 2016). The haeme-binding protein A, the Oligopeptide permease ABC transporter membrane protein, the ABC transporter periplasmic protein (likely the Maltose ABC transporter periplasmic protein in our cCPS), the Iron (Fe^{3+}) ABC superfamily ATP binding cassette transporter binding protein and the Outer membrane protein (omp) p2, also detected in our cCPS, induced a humoral as well as a cellular immune response in the mice, protected them partially against the challenge with the *H. parasuis* Nagasaki strain and effectively reduced growth of *G. parasuis* in the mice's organs (Li et al. 2017). Furthermore, we identified the Peptidoglycan-associated outer membrane lipoprotein PalA, omp p2 and omp p5 that were proven as immunogenic in mice, but only PalA and omp p2 protected them against the challenge with the *G. parasuis* SH0165 (serovar 5) (Zhou et al. 2009). On the other hand, PalA had negative effects on the protection when piglets were immunised against *Actinobacillus pleuropneunomiae* (van den Bosch and Frey 2003). We also detected the outer membrane protein 26 that was identified as immunogenic with a protective efficacy against a lethal dose of the *G. parasuis* serovar 5 in a guinea pig model (Li et al. 2016). Furthermore, we also identified the superoxide dismutase (SOD), thioredoxin and glutaredoxin that are other virulence-associated proteins (Guo et al. 2016; Li et al. 2017; Wen et al. 2018). The immunisation of mice with SOD elicited a strong humoral immune response and even provided significant protection against a lethal dose of the *G. parasuis* serovar 4 or 5 (Guo et al. 2016).

Taken together, the crude capsular polysaccharide extract from the *G. parasuis* CAPM 6475 strain was able to elicit a strong humoral response in the mice. Moreover, as a decrease in the bacterial loading may be related to the vaccine efficiency (Li et al.

2015; Zheng et al. 2017), we conclude that such an immunisation of mice was associated with their partial protection. However, the evaluation of the efficacy needs to be performed in pigs before any conclusions can be drawn.

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