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Original Article

Commercial bacterins did not induce detectable levels of antibodies in mice against *Mycoplasma hyopneumoniae* antigens strongly recognized by swine immune system

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ABSTRACT

Enzootic Pneumonia (EP) caused by *Mycoplasma hyopneumoniae* results in major economic losses to the swine industry. Hence, the identification of factors that provide protection against EP could help to develop effective vaccines. One such factor that provides partial protection are bacterins. Therefore, the aim of this study was to verify the induction of antibodies against fifteen *M. hyopneumoniae* antigens, strongly recognized by the swine immune system during natural infection, in mice vaccinated with six commercial bacterins. Each group of mice was inoculated with one bacterin, and seroconversion was assessed by indirect ELISA using recombinant antigens and *M. hyopneumoniae* 7448 whole cell extract. Sera from one inoculated group recognized antigen MHP_0067, and sera from four inoculated groups recognized antigens MHP_0513 and MHP_0580. None of the bacterins was able to induce seroconversion against the twelve remaining antigens. This absence of a serological response could be attributed to the lack of antigen expression in *M. hyopneumoniae* strains used in bacterin production. Additionally the partial protection provided by these vaccines could be due to low expression or misfolding of antigens during vaccine preparation. Therefore, the supplementation of bacterins with these recombinant antigens could be a potential alternative in the development of more effective vaccines.

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1. Introduction

Enzootic Pneumonia (EP) is a worldwide disease that results in major economic losses to the swine industry [1]. Commercial vaccines, which consists of inactivated whole-cell adjuvanted formulations (bacterins), induce partial protection against EP [2,3].

These vaccines provide protection by reducing lung damage, clinical signs and weight loss and improving production rates in vaccinated animals [4–7]. Although the partial protective mechanisms have not been fully elucidated, it has been described that these bacterins are able to induce both local and systemic immune responses [8–10]. However, they are not capable of avoiding transmission of the pathogen [11,12]. Therefore, identification of the factors that mediate the partial protection of bacterins can enable the development of highly effective vaccines against EP.

Comparative analysis of *Mycoplasma hyopneumoniae* strains allowed the identification of virulence factors possibly related to bacterial pathogenesis [13–16]. Previous work has shown that commercial bacterins did not induce antibodies against P97 adhesin and NrdF protein [17–20], virulence factors well characterized that were capable of promoting some level of protection in swine subjected to experimental challenge conditions [21–24].

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Furthermore, in corroboration with published literature, recent studies from our group also demonstrated that one commercial bacterin did not induce antibodies in mice against several *M. hyopneumoniae* surface antigens [19,20,25,26]. Hence in order to better understand the bacterins and their protective mechanisms, it is essential to investigate the profile of antibodies generated by these formulations. Therefore, the aim of this study was to verify the induction of antibodies against fifteen *M. hyopneumoniae* antigens in mice vaccinated with six commercial bacterins. These antigens were selected based on our previous studies that demonstrated their ability to be strongly recognized by swine immune system during *M. hyopneumoniae* natural infection [20,25–27].

2. Materials and methods

2.1. Commercial vaccines

Six commercial bacterins against EP were used in this study: Ingelvac Mycoflex® (Boehringer Ingelheim; 008/10), M+Pac® (Intervet; 00051097 023/11), Mypravac® Suis (Hypra, 15FH-1 002/11), Respisure® 1One (Pfizer, 002/2011), Resprotek™ One Shot (Bayer, 002/2012) and Serkel Pneumo (Vencofarma, 002/2010). These bacterins were randomly designed as A–F.

2.2. Selection and production of recombinant antigens

Fifteen proteins with probability of being virulence factors were selected from the *M. hyopneumoniae* 7448 database (GenBank, NC_007332.1) [14]. The following criteria were used for candidate protein selection: (i) likelihood of being a virulence factor based on genomic and proteomic comparative analysis between virulent and avirulent *M. hyopneumoniae* strains [14–16]; (ii) predicted cellular localization on the outer cell surface (transmembrane or secreted protein) [25,27]; (iii) high antigenicity [25,26]; (iv) high immunogenicity in mice [25,26]; (v) significant probability of having adhesin activity relative to the protein MHP_0198 (P97 adhesin) according to the analisis from Vaxign software [28]. The recombinant proteins corresponding to the fifteen selected antigens were produced according to Simionatto et al. [27].

2.3. Swine sera

Three pools of positive and negative swine sera (three sera/pool) were used to confirm antigenicity of recombinant antigens and as ELISA controls. Positive swine sera were obtained from animals aged approximately 3 months old, unvaccinated against EP, and positive diagnosis of EP. This diagnosis was based on clinical signs, positive serology assessed by indirect ELISA with crude extract of *M. hyopneumoniae* strain 7448 [27] and positive PCR from nasal swab [29]. Negative sera were obtained from a swine herd tested to be free from *M. hyopneumoniae* infection through PCR and serology.

2.4. Mice inoculation

Male BALB/c mice aged 6–8 weeks old (5 mouse/group) were immunized with commercial vaccines (one bacterin/group) or saline without adjuvant (100 µl) as negative control, via intramuscular injection. The mice received 5% of the dose recommended by the manufacturer for swine. When indicated, each animal was boosted with the same dose 21 days after the first innoculation. Blood samples were collected from the retro-orbital sinus at days 0 and 42 after first innoculation. At day 42, the animals were euthanized. All animal experiments were performed according to the guidelines of the Ethics Committee in Animal Experimentation

from the Federal University of Pelotas (project number 7722). The experiment was repeated twice.

2.5. Assessment of humoral immune response

Seroconversion of mice was evaluated through indirect ELISA using recombinant antigens or *M. hyopneumoniae* 7448 whole cell extract. A previous analysis was performed to determine the optimum amount of antigens (100 ng, 300 ng, 500 ng, 750 ng or 1 µg) to coat the plate and the mice sera diluition (1:25, 1:50, 1:100 or 1:200) to be used in the analysis. The antigen amount which resulted in highest reactivity before reaching the plateau, and the lowest sera diluition with no detection of background (detectable reactivity against negative sera) were selected to perform the ELISA. Microtiter plates were coated with recombinant antigens or with crude extract of *M. hyopneumoniae* strain 7448 (500 ng/well) diluted in 50 mM carbonate–bicarbonate (pH 9.6) and incubated overnight at 4 °C. Wells were washed three times with phosphate-buffered saline + 0.05% Tween 20 (PBS-T) and incubated for 2 h at 37 °C with 5% non-fat dry milk diluted in PBS (blocking solution). After three washes, wells were incubated for 2 h at 37 °C with mice or swine sera (1:50) diluted in blocking solution. After three washes, wells were incubated for 1 h and 30 min at 37 °C with goat IgG anti-mouse antibody (1:6000) or rabbit IgG anti-pig (1:4000) conjugated to horseradish peroxidase (Sigma Aldrich) diluted in PBS. Reaction was developed with o-phenylenediamine dihydrochloride (Sigma Aldrich) and hydrogen peroxide, after PBS-T washes. The color reaction was allowed to develop for 15 min and stopped with 50 µL of 2 M H_2SO_4. Absorbance was determined at 492 nm with plate reader ThermoPlate ELX800 (BioTek Instruments). The mean and standard deviation (S.D.) values for animal samples were calculated. Reactions were preformed in triplicate. Values of seroconversion were obtained by the quotient between the average absorbance of immune sera and non-immune sera (O.D. day 42/O.D. day 0 for mice and O.D. positive sera/O.D. negative sera for swine). Seroconversion was considered positive when increased four times.

2.6. Statistical analysis

GraphPad Prism 4 software systems (GraphPad Software) was used to perform the statistical analysis and build the graphics. Seroconversion data were submitted to ANOVA followed by Dunnett test to determine significant differences (C.I. 95%, $p < 0.05$) between sera of mice inoculated with the bacterins and the negative control. Unpaired Student t Test was used to determine significant difference between positive and negative swine sera.

3. Results

3.1. Recombinant antigen selection

According to the previously stipulated criteria, fifteen recombinant antigens were selected for this study (Table 1). The antigens MHP_0067, MHP_0223 and MHP_0596 do not have predicted adhesin activity. To confirm the antigenicity of these recombinant antigens, their reactivity with positive and negative swine sera was evaluated by ELISA. All recombinant antigens were strongly recognized by positive swine sera ($p < 0.05$) (Table 2), supporting our previous characterization demonstrating that recombinant antigens maintain reactive epitopes similar to those found in native proteins [25,26].

Table 1
Identification and characterization of recombinant antigens selected.

	Annotation	Identification	Selected fragment (aa)	Molecular mass (kDa)	Antigenicity with pig sera[1] ELISA[*]/western blot[**]	Immunogenicity in mice[1] ELISA[***]	Likely location in cell[2]	Likely adhesin activity (%)[2]
1	MHP_0067	DnaK chaperone	229–601	42	+/HC	+	Cytoplasm (99.7%)	18.7
2	MHP_0099	P95	564–911	43	+/C	+	Outer membrane (94.9%)	55.8
3	MHP_0107	P102[3]	648–948	38	+/C	+	Outer membrane (95.2%)	52.6
4	MHP_0223	NrdF beta chain	202–340	14	+/NR	+	Cytoplasmic (89.6%)	19.2
5	MHP_0234	Periplasmic sugar binding	12–340	39	+/HC	+	Unknown	52.8
6	MHP_0272	P97[3]	228–708	59	+/C	+	Outer membrane (94.9%)	43.4
7	MHP_0332	Subtilisin-like serine protease	58–264	25	+/C[†]	+	Outer membrane (94.9%)	43.6
8	MHP_0372	LppT protein	497–883	44	+/C	+	Outer membrane (94.9%)	54.5
9	MHP_0373	LppS protein	776–982	27	+/H	+	Unknown	42.6
10	MHP_0390	Hypothetical protein	4–214	26	+/HC	+	Cytoplasmic Membrane (100%)	54.2
11	MHP_0468	Hypothetical protein	118–467	42	+/NR	+	Cytoplasm (89.6%)	60.5
12	MHP_0513	P46	102–420	35	+/C	+	Periplasm (97.6%)	40.3
13	MHP_0580	Membrane nuclease lipoprotein	180–378	25	+/C	+	Unknown	57.7
14	MHP_0596	Hypothetical protein	110–379	31	+/C[†]	+	Outer membrane (94.9%)	21.3
15	MHP_0663	P146	754–1022	30	+/NR	+	Outer membrane (95.2%)	53.1

[1] Data obtained from previous work of our research group [25,26].

[2] Likely location in cell and significant probability of having adhesin activity, determined by Vaxign [28] online software; probability values for adhesin activity were considered positive when near or above the value predicted for the characterized adhesin MHP_0198 (P97), 49.1%.

[3] Paralog protein.

[*] ELISA of recombinant proteins against convalescent and SPF swine sera; + recombinant antigens that presented positive reaction with convalescent swine sera, with significant statistical differences compared to SPF sera ($p < 0.05$).

[**] Western blot of recombinant proteins against swine sera from SPF, convalescent and swine immunized with *M. hyopneumoniae* 7448 inactivated whole cell; H, recombinant proteins recognized by hyperimmune sera; C, recombinant proteins recognized by convalescent sera; HC, recombinant proteins recognized by both, hyper-immune and convalescent; C[†], recombinant proteins recognized by convalescent sera, but not tested against hyperimmune sera; NR, recombinant proteins with no reaction against convalescent or hyperimmune sera.

[***] ELISA of recombinant proteins against sera from immunized mice; + recombinant antigens that presented positive reaction with immunized sera, with significant statistical differences compared to non-immunized (saline) sera ($p < 0.05$).

Table 2
Mean and standard deviation of absorbance related to the reactivity of the negative control swine serum (CS−) and positive (CS+) with the selected recombinant antigens and the *M. hyopneumoniae* extract 7448.

Antigens	CS−	CS+	p
MHP_0067	0.336 ± 0.013	3.269 ± 0.112	<0.0001
MHP_0099	0.179 ± 0.019	1.366 ± 0.152	0.0003
MHP_0107	0.218 ± 0.021	1.477 ± 0.259	0.0011
MHP_0223	0.313 ± 0.014	1.269 ± 0.114	0.0006
MHP_0234	0.262 ± 0.029	1.161 ± 0.097	0.0001
MHP_0272	0.247 ± 0.015	1.024 ± 0.020	0.0005
MHP_0332	0.291 ± 0.015	1.193 ± 0.279	0.04
MHP_0372	0.415 ± 0.015	2.813 ± 0.148	<0.0001
MHP_0373	0.260 ± 0.039	1.395 ± 0.404	0.0085
MHP_0390	0.252 ± 0.020	1.586 ± 0.295	0.0015
MHP_0468	0.256 ± 0.026	1.763 ± 0.212	0.0003
MHP_0513	0.428 ± 0.017	2.596 ± 0.429	0.0009
MHP_0580	0.313 ± 0.008	1.291 ± 0.060	<0.0001
MHP_0596	0.360 ± 0.007	1.456 ± 0.342	0.0052
MHP_0663	0.210 ± 0.022	1.668 ± 0.208	0.0003
M. hyopneumoniae 7448	0.425 ± 0.011	3.232 ± 0.247	<0.0001

Fig. 1. Mean seroconversion of sera from mice inoculated with bacterins or saline, determined by indirect ELISA using *M. hyopneumoniae* 7448 whole cell extract as antigen. The vertical bars correspond to the standard deviations of the seroconversions. All reactions were performed in triplicate. Asterisks (*) indicate statistical difference ($p < 0.05$) between seroconversion of mice inoculated with the bacterins and the negative control group (saline), verified by ANOVA followed by Dunnett test.

3.2. Humoral immune response induced by bacterins in mice

To confirm the immunogenicity of the evaluated bacterins, the reactivity of sera from vaccinated mice against *M. hyopneumoniae* 7448 whole cell extract was assessed by ELISA. Except for bacterin F, all other bacterins showed significant reactivity in this analysis,
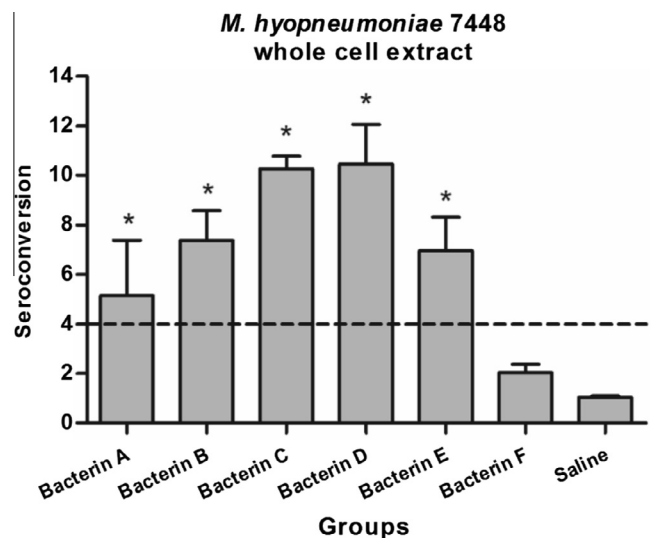
compared to the negative group (Fig. 1). Although this result suggests failure of the vaccination with the bacterin F, this vaccine induced significant seroconversion against one of the recombinant antigens. Hence bacterin F was selected for use in this study.

Furthermore, only one of the six bacterins was able to induce seroconversion against the antigen MHP_0067 (Fig. 2A), and four bacterins induced seroconversion against the antigens MHP_0513

(Fig. 2B) and MHP_0580 (Fig. 2C). Moreover, all the bacterins failed to induce seroconversion against the antigens MHP_0099, MHP_0107, MHP_0223, MHP_0234, MHP_0272, MHP_0332, MHP_0372, MHP_0373, MHP_0390, MHP_0468, MHP_0596 and MHP_0663 (data not shown). Bacterin E failed to induce seroconversion against all fifteen evaluated antigens.

4. Discussion

Based on the annotation of *M. hyopneumoniae* 7448 genome [30], proteins possibly associated with the pathogenesis of this bacteria and with high potential to be used as vaccines were identified. We cloned 71 of these candidate proteins, and 36 were successfully produced in *Escherichia coli*, purified and characterized for their antigenicity and immunogenicity [20,25,26]. Based on the initial characterization of these recombinant proteins, fifteen of these antigens which play a role in host–pathogen interactions, such as adhesion or tissue damage, and/or are strongly recognized by the swine immune system during natural infection were selected for further serological analysis. Since the commercially available bacterins result in partial protection against EP, we investigated their ability to induce humoral immune response in mice against the above mentioned 15 putative virulence factors of *M. hyopneumoniae*. Although the bacterins evaluated were able to induce antibodies against the crude bacteria extract, they did not induce seroconversion in mice against most of the selected antigens, suggesting that the bacterial strains used to produce these vaccines do not express them or that the vaccine preparation results in low expression or misfolding of these antigens. Furthermore, the adjuvants used in the formulation of commercial bacterins may not be suitable for the presentation of these antigens in particular. This result corroborates with findings of our previous studies, which demonstrated that the commercial bacterin used as control during experiments did not induce immune response against the antigens evaluated at that time [20,25,26]. The absence, low expression or misfolding of putative virulence factors in commercial bacterins, as well as the inappropriate processing of these antigens by presenting cell, could be related with the partial protection conferred to the vaccinated swines against EP.

Conditions of *in vitro* growth modulate *M. hyopneumoniae* expression profile. *Mycoplasma hyopneumoniae* 232 grown *in vitro* showed changes in transcriptome profile when compared with the same strain recovered after experimental *in vivo* infection in pigs [31]. Assunção et al. [32] identified antigenic variation of *M. hyopneumoniae* strains isolated from slaughterhouses according to the number of *in vitro* passages. Bacterins are produced with strains that suffer successive *in vitro* passages in optimal growing conditions, which may lead to a reduction or interruption of the gene expression required for natural infection, such as adhesins. *M. hyopneumoniae* require adhesins to attach to respiratory tract epithelial cilia, and antibodies capable of binding these adhesins may decrease the establishment of bacteria in the host tissues [33]. In a total of fifteen antigens evaluated in the present study, twelve (about 80%) were highly likely to have adhesin activity according to bioinformatic prediction. It is worth noting that none of the bacterins induced detectable levels of antibody against proteins P97 (MHP_0272) and P146 (MHP_0663), which are confirmed adhesin of *M. hyopneumoniae* [33–36]. Since most bacterins evaluated in this study are produced with avirulent J strain of *M. hyopneumoniae*, the results of this study also corroborate the findings of Li et al. [16], which showed through proteomic analysis that J strain has lower expression of genes associated with the synthesis of adhesins when compared to 232 strain. Absence or low expression of adhesins by vaccine strains may compromise neutralizing antibody induction and reduce vaccine efficacy, thereby allowing *M. hyopneumoniae* colonization and persistence in the host.
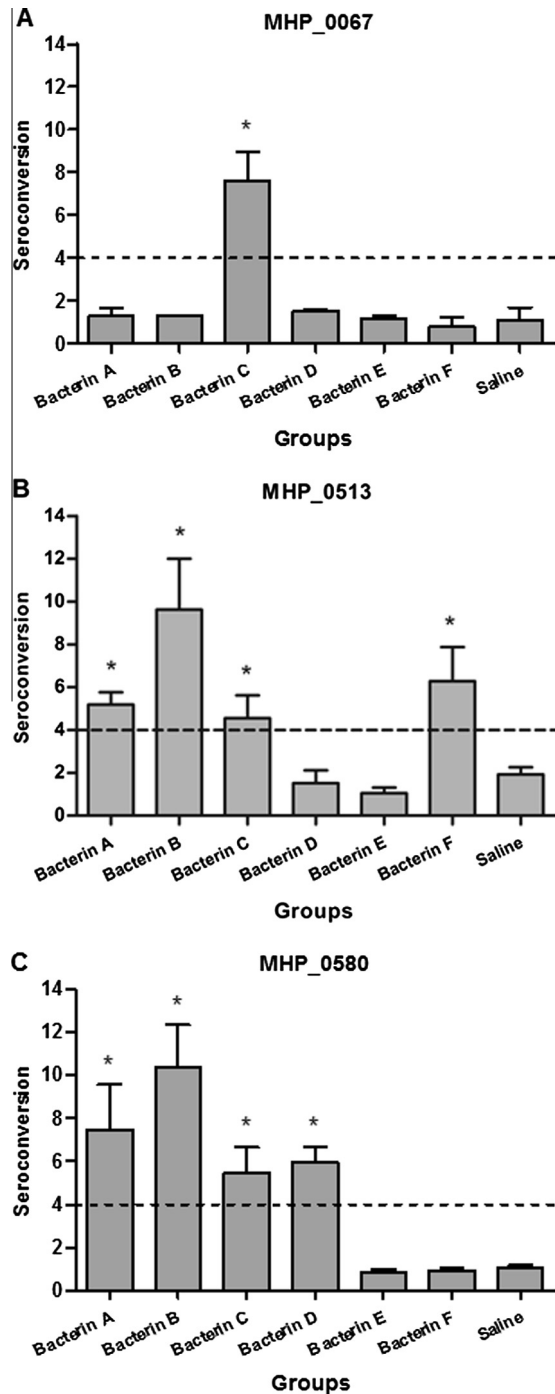

Fig. 2. Mean seroconversion of sera from mice inoculated with bacterins or saline, determined by indirect ELISA using MHP_0067 (A), MHP_0513 (B) or MHP_0580 (C) as antigen. The vertical bars correspond to the standard deviations of the seroconversions. All reactions were performed in triplicate. Asterisks (*) indicate statistical difference ($p < 0.05$) between seroconversion of mice inoculated with the bacterins and the negative control group (saline), verified by ANOVA followed by Dunnett test.

In an attempt to increase the level of protection against EP, research groups have investigated the use of attenuated strains of *M. hyopneumoniae*, but the results obtained so far are controversial. Villarreal et al. [37] showed that infection with an isolate of low virulence did not protect piglets challenged with a highly virulent isolate. On the other hand, vaccines based on the attenuated *M. hyopneumoniae* strain 168 administered by intrapulmonic and intramuscular routes conferred significant protection against experimental EP [38–40]. However, a comparison with an inactivated vaccine (bacterin) was not performed. Regardless of the effectiveness of this type of vaccine, it seems reasonable to hypothesize that the same limitations of bacterins, demonstrated in the present study, also occur in live attenuated vaccines, since they are produced from strains that have been cultivated *in vitro* through successive passages in culture medium. In fact, a comparative genomic analysis between the attenuated and the virulent variants of strain 168 showed the occurrence of mutations in CDS of diverse virulence factors, including adhesins after attenuation [41]. Many of the mutations identified generates truncated CDS, but the direct effects of the mutations on the protein expression was not evaluated. Therefore, it is necessary to determine the protein expression profile of virulence factors in attenuated strains and the antibody induction after immunization, in order to clarify whether the use of live attenuated vaccines could be a useful approach when the objective is to enhance the immunity against these antigens.

An alternative approach to improve vaccine efficacy is to increase virulence factor expression during *in vitro* growth by simulating the host lung enviroment during natural infection [42–44]. Supplementation of the medium with norepinephrine induced overexpression of some virulence genes of *M. hyopneumoniae* strain 232, including the adhesin P146 [43]; while increasing the incubation temperature of the culture promoted overexpression of DnaK chaperone by the same strain [44]. In this study, all bacterins failed to stimulate production of anti-P146 antibodies (MHP_0663) and only one of them stimulated the production of anti-DnaK (MHP_0067) antibodies. This strategy may be useful when the goal is to overexpress a few antigens; however, to induce the expression of several virulence factors it may be critical to provide different stimuli to bacteriale growth. In addition, the determination of optimal conditions for *in vitro* stimulation is dependent on further elucidation of the disease pathogenesis. Thus, supplementation of bacterins with recombinant antigens seems to be the best alternative to increase the level of protection against EP.

Adjuvants are essential compounds in vaccines, capable of enhance antigen processing and adaptive immune responses [45]. It has been shown that the use of different adjuvant formulations affects the immunogenicity and protective effect of attenuated and inactivated wholle-cell *M. hyopneumoniae* vaccines. [40,46]. Thus, evaluate the development of immune response against specific virulence factors and their correlation with protection against EP after immunization with *M. hyopneumoniae* associated to different adjuvants is necessary to verify if this could be a useful approach to enhance protection elicited by bacterins.

In this study we have demonstrated that commercial bacterins failed to stimulate seroconversion in mice against *M. hyopneumoniae* antigens that were strongly recognized by the immune system of pigs. This may be due to the low expression of these antigens during *in vitro* bacterial cultivation. Therefore, the addition of recombinant antigens to commercial bacterins could be a potential alternative for development of effective vaccines. However, future studies are necessary to evaluate the interactions between the recombinant antigens and commercial vaccines and the protective potential of these associations.

Conflict of interest statement

The authors state they have no conflict of interest.

Acknowledgments

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