International Journal of Pharmaceutics 421 (2011) 94–98

International Journal of Pharmaceutics

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

Evaluation of the immunogenicity and *in vivo* toxicity of the antimicrobial peptide P34

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ARTICLE INFO

Article history:
Received 24 June 2011
Received in revised form 22 August 2011
Accepted 19 September 2011
Available online 21 September 2011

Keywords:
Antimicrobial peptide
In vivo toxicity
Bacillus
Bacteriocin

ABSTRACT

Immunogenicity and toxicity of antimicrobial peptide P34 were evaluated *in vivo*. BALB/c mice were inoculated intraperitoneally with peptide P34 alone and associated with Freund's adjuvant. For acute toxicity testing, different concentrations of the peptide P34 (82.5, 165.0, 247.5 and 330.0 mg/kg) were orally administered. To evaluate the sub-chronic toxicity the tested dose of 0.825 mg/kg/day of the peptide P34 or nisin were administered for 21 days. There were no hypersensitivity reactions or significant increase in antibody titer during the immunogenicity experiment or death of animals during the acute or sub-chronic toxicity tests. The LD_{50} was higher than 332.3 ± 0.76 mg/kg. No significant changes in serum biochemical parameters were observed in the animals treated with the peptide P34 unlike nisin-treated group showed a significant increase in alanine transaminase levels in comparison to controls. The group treated with 0.825 mg/kg/day of nisin showed histological changes in the spleen, skin and liver. In the group treated with peptide P34 histological changes in the spleen were observed, with the presence of megakaryocytes. Few studies report the use of animal models to evaluate the *in vivo* toxicity of antimicrobial peptides and such investigation is an essential step to ensure it safe use in foods.

1. Introduction

Species of *Bacillus* produce a large number of antimicrobial substances including several different peptides commonly referred as bacteriocins and bacteriocin-like substances (Riley and Wertz, 2002a,b). These include those produced by *Bacillus subtilis*, *Bacillus thuringiensis*, *Bacillus amyloliquefaciens*, *Bacillus stearothermophilus*, and other *Bacillus* species that have been recognized as industrially important for more than 50 years (Pedersen et al., 2002; Stein, 2005). These peptides have been often classified according their chemical and structural characteristics, based on their variable molecular mass and net charge (Von Döhren, 1995). Their proteinaceous nature implies a putative degradation in the gastro-intestinal tract of man and animals, suggesting that some bacteriocins could be used as food preservatives (Eckner, 1992; Cleveland et al., 2001; Deegan et al., 2006) and as therapeutic agents against pathogenic bacteria (Reddy et al., 2004). Although many strains of *Bacillus* have been safely used in food and pharmaceutical industry, there are relatively few specific studies on the toxicity of antimicrobial peptides from *Bacillus* (Mikkola et al., 2000; Vaucher et al., 2010a,b).

One of the requirements for the application of an antimicrobial peptide as food preservative would be the evaluation of its immunogenicity and also the *in vitro* and *in vivo* toxicity. The testing of a potential food antimicrobial would consider repeated and daily administration of the substance for a required time period to access a possible chronic toxicity (Pariza and Foster, 1983; Pariza and Cook, 2010). The route of administration should be the same proposed for use in humans (Food and Drug Administration, 1988; Post, 1996) and toxicological studies involving animals are a major component of safety assessment of bacteriocins (Moreno et al., 2000).

Bacillus sp. strain P34 produces the peptide P34, which has been previously characterized as a broad range antimicrobial substance that inhibits important food-borne pathogens like *Listeria monocytogenes* and *Bacillus cereus* (Motta et al., 2007a). Preliminary investigation on the *in vitro* toxicity of peptide P34 showed similar results to that obtained for nisin (Vaucher et al., 2010b). The objective of this study was to determine the acute toxicity of the antimicrobial peptide P34 and compare the effect of this peptide with nisin by the use of subchronic toxicity tests *in vivo*.

2. Materials and methods

2.1. Microorganism

The bacterium *Bacillus* sp. strain P34, previously isolated and characterized, was used for production of antimicrobial peptide

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0378-5173
doi:10.1016/j.ijpharm.2011.09.020

(Motta et al., 2007a). The organism was stored at −20 °C in Trypticase Soy Broth (TSB) medium containing 20% glycerol. The bacterium was propagated in fresh TSB medium before use.

2.2. Antimicrobial peptides

Bacillus sp. strain P34 was cultivated aerobically in 500 ml Erlenmeyer flasks containing 200 ml of TSB broth at 30 °C, 180 rpm for 24 h. The culture supernatant was obtained by centrifugation at 10,000 × *g* for 15 min at 4 °C. The supernatant was filtered through 0.22 μm filter membrane (Millipore, Billerica, MA, USA) and submitted to ammonium sulfate precipitation at 20% saturation. After centrifugation for 10,000 × *g* at 4 °C for 15 min, the pellet was resuspended in 10 mM sodium phosphate buffer pH 7.0 and loaded on a Sephadex G-100 column (GE Healthcare/Pharmacia Biotech, Uppasala, Sweden) of 0.8 cm of diameter and 23 cm of length. Fractions were eluted with the same buffer and collected in flow rate of 0.5 ml/min. Those fractions presenting antimicrobial activity were pooled and stored at 4 °C until used. The solution of peptide P34 has 12,800 AU/ml with a specific activity of 4920 AU/mg and purification factor of 80-fold. Six consecutive purifications were carried out to obtain about 180 mg of the peptide (Motta et al., 2007b). Subsequently, the peptide was lyophilized and the volume adjusted with PBS added 0.02 mol/l HCl to a concentration of 17.5 mg/ml. Nisaplin® was suspended in PBS buffer containing 0.02 mol/l HCl to a final nisin concentration of 12.5 mg/ml. The protein concentration was determined using the Folin–phenol reagent method (Lowry et al., 1951), using a calibration curve developed with bovine serum albumin as protein standard. The antimicrobial peptide P34 and nisin concentration was adjusted before the oral administration of mice with PBS with the addition of 0.02 mol/l HCl to a final concentration of 0.825 mg/kg per day. The PBS added of 0.02 mol/l HCl was used as control.

2.3. Experimental animals

Male BALB/c mice were used for the experiments. Animals were weighing between 20 and 35 g were provided by the animal house of the Federal University of Santa Maria (UFSM/RS), Brazil and kept in plastic boxes with food and water *ad libitum*. The animals were used in experiments after a period of 7 days of adaptation in captivity, with regular 12 h light–dark periods and ambient temperature of 20 °C (National Institutes of Health, 1996). The procedures used in the assays were approved by the Internal Committee of Animal Ethics-UFSM (Protocol number 68/2010), and conform to international standards of animal welfare, as specified by the CIOMS International Guiding Principles for Biomedical Research Involving Animals, Geneva, 1985.

2.4. Immunogenicity

Twenty male BALB/c mice, aged between 60 and 70 days were divided into two groups (peptide P34 and peptide P34 + Freund's complete adjuvant), each containing 10 animals. The mice were immunized intraperitoneally with 0.5 ml of the antimicrobial peptide P34 (50 μg/ml) and 0.25 ml peptide P34 (50 μg/ml) + 0.25 ml of Freund's complete adjuvant, respectively. After 14 and 28 days of the first inoculation, the mice received a booster with 0.5 ml of the preparations mentioned above. To verify the presence of antibodies against the peptide P34, the serum collected from mice tails was tested at 21 and 42 days after the first inoculation by indirect Enzyme-linked immunosorbent assay (ELISA) as described elsewhere (Del Pino et al., 1998). Titration of antigen, labeled anti-mouse antibody and mouse antisera were carried out and the assay was standardized with 12.5 μg/well of P34 antigen, a dilution of 1:2000 of anti-mouse antibody and 1:100 mouse antiserum.

2.5. Acute toxicity

Acute toxicity was assessed with six male BALB/c mice, by oral administration of increasing doses of the antimicrobial peptide P34 (82.5, 165.0, 247.5, 330.0 mg/kg). The route of administration was the same proposed for use in humans (Food and Drug Administration, 1988). After administration, the animals were kept under observation for a minimum of 48 h. The number of animals killed for each of the doses was noted and the LD_{50} calculated by the Up and Down method, which is one of the most used to reduce the number of animals used (Botham, 2004).

2.6. Subchronic toxicity

Eight BALB/c males were used for each of the treated groups (0.825 mg/kg/day of the antimicrobial peptide P34 or nisin) and control group. This concentration was chosen based on reported ADI (Acceptable Daily Intake) for nisin of 33,000 IU or 0.825 mg/kg of body weight (Hoover and Steenson, 1993). The animals were subjected to experimental protocol, lasting 21 days, the treatments daily orally using gavage. The scheme offered food and water *ad libitum*, and body weight was recorded at days 0, 7, 14 and 21.

2.7. Biochemical analysis

Laboratory analysis of biochemical parameters was performed on serum samples. Serum aspartate transaminase (AST), alanine transaminase (ALT), urea and creatinine were performed in automated biochemical analyzer (LabMax 240, Labtest, Brazil).

2.8. Histopathological analysis

After 21 days of oral administration of antimicrobial peptide P34 and nisin, the animals were sacrificed by cervical dislocation, and the organs examined macroscopically. Tissue samples of stomach, intestine, spleen, liver, kidney and skin of mice were collected and fixed in 10% buffered formalin and processed for paraffin embedding. The histological sections (3–5 μm) were stained with hematoxylin–eosin (HE) (Prophet et al., 1992). The slides were coded and analyzed at the Veterinary Pathology Laboratory (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil) by a veterinary pathologist who was unaware of the experimental conditions of each group.

2.9. Statistical analysis

The results were expressed as mean ± standard deviation of the groups and subjected to analysis of variance (ANOVA) and Tukey's test. The differences were considered statistically significant when $p < 0.05$.

3. Results and discussion

The immunogenicity of the antimicrobial peptide P34 was assessed by intraperitoneal administration into two groups of mice. No animal deaths or hypersensitivity reactions were observed during the experiment. A non-significant increase in antibody titer of group 1 mice that receive only the antimicrobial peptide P34 was observed at day 42 when compared with day zero (Fig. 1). Mice inoculated with peptide P34 + Freund's complete adjuvant (group 2) showed a significant increase in antibody titer in day 42 (Fig. 1).

Bhunia et al. (1990) evaluated the immunogenicity of pediocin PA-1 (AcH) to mice and found that it was not immunogenic for

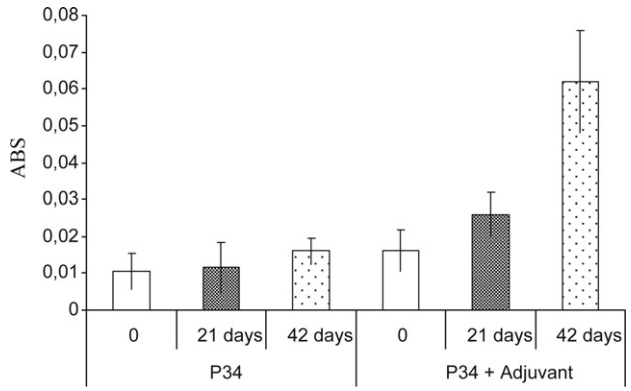

Fig. 1. Determination of antibody titer of mice administered intraperitoneally with the antimicrobial peptide P34 and its association with adjuvant after 42 days. The results represent the mean ± S.E.M. of absorbance of group 1 (Antimicrobial peptide P34 alone) and group 2 (Antimicrobial peptide P34 + Freund's adjuvant).

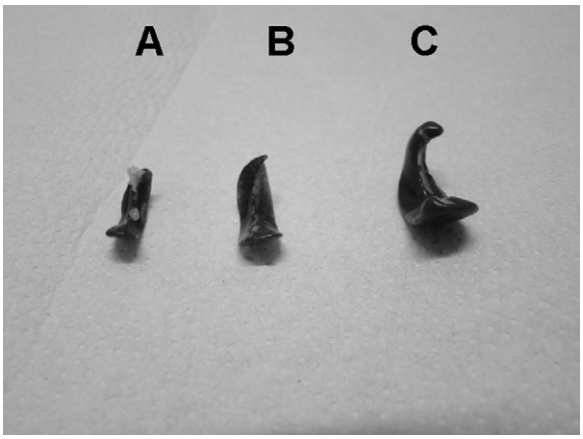

Fig. 2. Comparison of spleen size observed in mice control (A), administered with the antimicrobial peptide P34 (B) and nisin (C) after 21 days (sub-chronic toxicity).

animals. Short-term administration of diets containing nisin (Nisaplin) induced an increase of both CD4 and CD8 T-lymphocyte cell counts and also a decrease of B-lymphocyte counts. The macrophage/monocyte fraction isolated from peripheral blood became significantly increased after long-term administration (100 days) of Nisaplin-containing diets (De Pablo et al., 1999).

After the studies of immunogenicity of peptide P34, the investigation for oral acute toxicity in mice was performed with a single dose administration. In tests to determine the acute toxicity and LD_{50} of peptide P34, no deaths occurred following oral administration of any of the tested concentrations (82.5, 165.0, 247.5, 330.0 mg/kg). Thus, the LD_{50} was higher than 332.3 ± 0.76 mg/kg. These tests are crucial to evaluate antimicrobial peptides as potential food preservative and the concentration used should be equivalent to at least 100 times the estimated average human exposure or at least 2000 mg/kg body weight following guidelines established by the Organisation for Economic Cooperation and Development (1987). Although several antimicrobial peptides have been purified and characterized there are currently few studies on acute toxicity for comparison. Extensive toxicological studies showed that nisin intake does not cause toxic effects to the human body with a reported LD_{50} of 6950 mg/kg, which is similar to salt, when administered orally (Jozala et al., 2007). Pediocin PA-1 is another bacteriocin that has been used for the same purpose, and studies on its toxicity have been reported (Bhunia et al., 1990; Dabour et al., 2009), although its use was not yet recommended by WHO (Drider et al., 2006). Some authors have associated high LD_{50} of bacteriocins with digestive enzymes capable of rapidly inactivating these substances, being trypsin and chymotrypsin produced in the pancreas and released into the small intestine a prime example (Hara et al., 1962, Eckner, 1992; Cleveland et al., 2001, Deegan et al., 2006). In this regard, peptide P34 is sensitive to trypsin as well (Motta et al., 2007a). Claypool et al. (1966) evaluated the effect of nisin in milk chocolate consumed orally and noted that only ¼ of the original concentration was detected in saliva after a minute of use. Besides this, some bacteriocins can also be sometimes sensitive to ptyalin, being not detected in human saliva 10 min after the consumption of a liquid containing bacteriocin (Chandrapati and O'Sullivan, 1998).

In tests conducted to evaluate the sub-chronic toxicity no death of control or treated animals were observed. There were no significant differences in body weight gain among groups (data not shown). Blood samples were collected from control and treated mice and processed for examination of possible changes in the biochemical parameters one day before and one after the final administrations. The values for serum aminotransferases, creatinine and urea were similar among the treatments (Table 1). A significant increase ($p < 0.05$) in serum ALT levels was observed for the nisin-treated group after 21 days. These results suggest that the antimicrobial peptides may be continued ingested, although the increased level of ALT suggests a potential hepatotoxic effect of nisin.

Histopathological studies were performed with the stomach, intestines, liver, spleen, kidney and skin of control and treated animals. No histological changes were detected in the intestines and kidneys of any group. Signs of possible toxicity were observed in animals treated with 0.825 mg/kg/day of nisin, with histological changes in the spleen, skin and liver. An enlargement in gross organ size was observed in the spleen (Fig. 2), with a significant increase in weight (151.5 ± 2.81 mg versus 120 ± 1.71 mg of control).

Histological analysis of the spleen showed abundant presence of megakaryocytes in the groups treated with 0.825 mg/kg/day of both nisin and peptide P34 (Fig. 3). The histological change observed in the spleen with the presence of megakaryocytes indicates a possible inflammatory process. Puertollano et al. (2003) analyzed pro-inflammatory cytokines produced in spleen cells of mice in response to nisin, and reported increased levels of some cytokines involved in inflammation. In the liver of the nisin group, histological changes suggesting hepatic degeneration were observed (Fig. 4). The presence of neutrophils can be also related to an inflammatory response. The results observed in the nisin group could be associated to other components present in the commercial nisin preparation, which contains about 12% whey protein and 77% NaCl. Toxicity studies on basic milk protein fractions report the absence of adverse effects and conclude that it is safe for use as food ingredient (Kruger et al., 2007). Results from subchronic oral toxicity of a proprietary whey fraction indicate it is safe for consumption with a non-observed adverse effect level of 3000 mg/kg/day (Dyer et al., 2008). A recent study showed that nisin A administered to rats at 5% dietary level for 90 days cause no toxicological effects, although statistically significant increases of kidney weight, and incidences of minimal squamous cell hyperplasia of limiting ridge in the forestomach, were found in nisin A-treated group (Hagiwara et al., 2010). These changes were related to NaCl, since they were also noted in rats given diet containing similar amount of salt. Although similar alterations were not observed in this study, it seems feasible that inflammatory response could be associated to high salt intake. Post-mortem examination of salt-poisoned animals may include gastric irritation and histopathological lesions may be depending on species (Thompson, 2007).

Except for studies with nisin (Frazer et al., 1962; Hoover and Steenson, 1993), few reports on the use of animal models to

Table 1
Biochemical parameters obtained from the serum of BALB/c mice administered for 21 days with antimicrobial peptide P34 and nisin (subchronic toxicity).

Sample		AST (U/L)	ALT (U/L)	Creatinine (mg/dL)	Urea (mg/dL)
Day 0	Control	143.7 ± 20.6	69.2 ± 22.0	0.165 ± 0.06	62.6 ± 25.5
	Nisin	155.0 ± 24.7	75.4 ± 14.1	0.133 ± 0.03	51.3 ± 14.9
	P34	130.3 ± 23.4	60.7 ± 14.3	0.14 ± 0.03	47.0 ± 15.9
Day 22	Control	145.6 ± 18.6	79.5 ± 13.4	0.16 ± 0.03	60.0 ± 11.7
	Nisin	236.5 ± 27.7*	82.0 ± 10.6	0.26 ± 0.04	55.0 ± 12.4
	P34	156.6 ± 25.3	74.5 ± 8.2	0.24 ± 0.04	47.2 ± 7.6

AST, alanine aminotransferase; ALT, aspartate aminotransferase. The results are presented as mean ± standard deviation.
* $p < 0.05$ (ANOVA-Test of Tukey).

evaluate the *in vivo* toxicity and to assess the effects of bacteriocins in target organs are available (Mota-Meira et al., 2005). Nisin was tested in pregnant rats and the treated animals and their progeny did not show any clinical signs of toxicity when compared to the control animals (Gupta et al., 2008). Recently, Dabour et al. (2009) using *in vivo* experiments showed that repeated doses of pediocin PA-1 (250 mg/day for three consecutive days) resulted in a decrease of 2 log cycles of *L. monocytogenes* in artificially infected animals, and promotes the disappearance the pathogen in target organs of animals (spleen and liver) within 6 days. In the same study, they demonstrated that consumption of feed containing the purified bacteriocin did not affect the intestinal microflora, weight change or development of the animals.

The evaluation of *in vitro* and *in vivo* toxicity of an antimicrobial peptide is an essential step before it could be considered for use in food. The results presented here agree with previous investigation on the *in vitro* toxicity of peptide P34 on eukaryotic

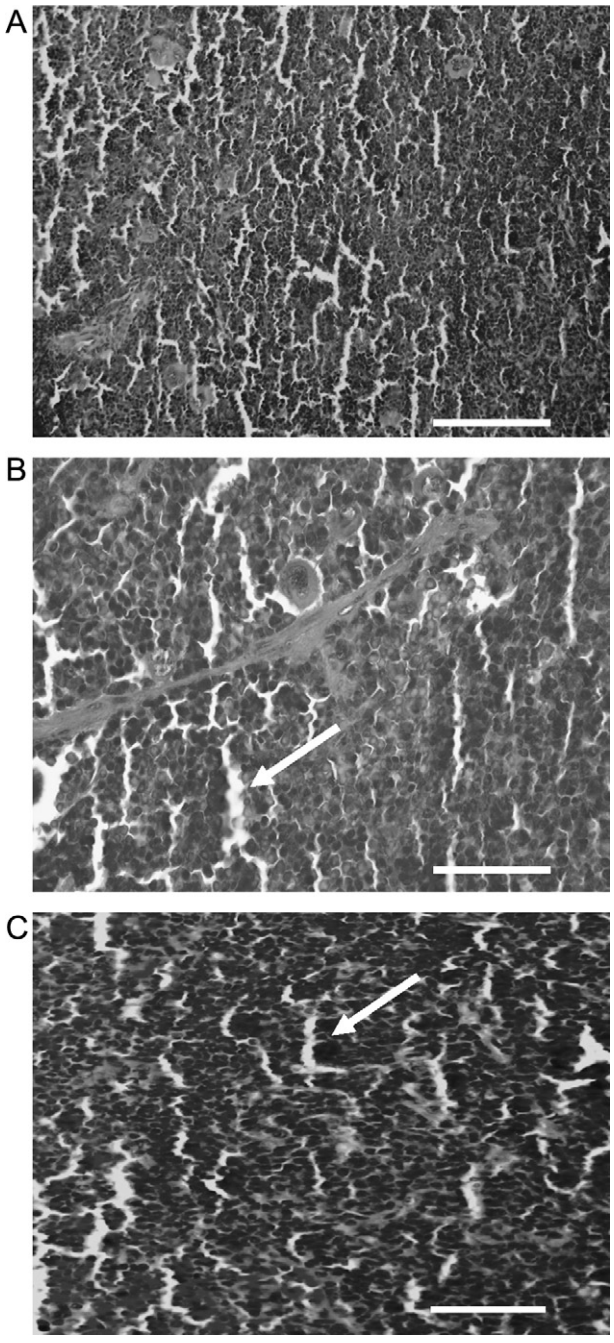

Fig. 3. Histological changes of mice spleen. Spleen sections of control animals (A) and mice receiving an oral dose of 0.825 mg/kg for 21 days of peptide P34 (B) or nisin (C) with the presence of megakaryocytes (arrow). Hematoxylin–eosin (HE). Bar = 100 μm.

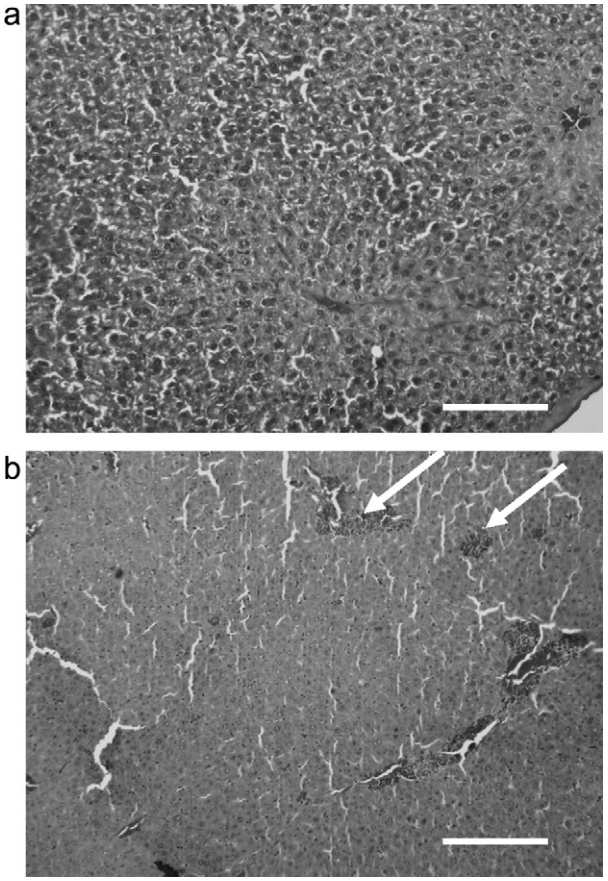

Fig. 4. The liver of nisin-treated mice can be observed hepatic degeneration and presence of neutrophils (arrow). Liver sections of control animals (A) and mice receiving an oral dose of 0.825 mg/kg for 21 days with nisin (B). Hematoxylin–eosin (HE). Bar = 100 μm.

cells, where similar results to that obtained for nisin were obtained (Vaucher et al., 2010b). VERO cells were treated with peptide P34 and nisin and the EC_{50} values in MTT and neutral red and lactate dehydrogenase assays were similar for both peptides. The peptide P34 revealed similar hemolytic activity on human erythrocytes (5.8%) when compared to nisin (4.9%). When the sperm parameters viability, motility and acrosomal exocytosis were evaluated, nisin and P34 also showed similar effects. The evaluation of the immunogenicity, acute and sub-chronic toxicity of the antimicrobial peptide P34 developed in this study corroborate the potential of this substance as an alternative food preservative.

Acknowledgements

Authors thank Dr. P.M. Roehe of Virology Laboratories (ICBS/UFRGS) for helpful support with ELISA assays and Dr. D. Driemeier of Department of Clinical Pathology and Veterinary (UFRGS) for performing the histological analysis. This work received financial support of CNPq, Brazil.

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