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Short communication

Evaluation in broilers of the probiotic properties of *Pichia pastoris* and a recombinant *P. pastoris* containing the *Clostridium perfringens* alpha toxin gene

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ABSTRACT

The probiotic properties of *Pichia pastoris* and of a recombinant *P. pastoris* containing the *Clostridium perfringens* alpha toxin gene were evaluated in broilers. One-day-old chicks randomly divided in four groups were fed with commercial feed devoid of antibacterials. The control group (1) received plain food, while the other groups were supplemented with either *P. pastoris* (2), the recombinant *P. pastoris* (3) or *Bacillus cereus* var. Toyoi (4). At day 49, live weights, feed efficiency and seroconversions were higher ($P < 0.05$) in the supplemented groups than in the control groups. Group 3 showed the best results, while group 2 had lower weight gain than groups 3 and 4 although food conversion was better than in group 4. Seroconversions were not different ($P > 0.05$) among the supplemented groups. Adverse reactions were not observed in histopathologic evaluation. We concluded that *P. pastoris* and the recombinant *P. pastoris* could be used as probiotics in broilers.

1. Introduction

The high frequency of potentially pathogenic bacteria for animals and humans present in products of animal origin and the increase of their antibiotic resistance, questioned the use of antibiotics as growth promoters (AGP) in animals feed and supported the recommendations of the Swann Committee (1969) and the ban of AGP in animal production by the European Community (Council of the European Union, 2003).

The suppression of AGP from feedstuffs favoured the emergence of diseases that were efficiently controlled by them. One of the most important is Avian Necrotic Enteritis (ANE) caused by *Clostridium perfringens*, whose clinical form produces high mortality in chickens 2–5 weeks old (Engström et al., 2003) and its subclinical form may reduce productivity in 33% (Lovland and Kaldhusdal, 2001; Van der Sluis, 2000). The control of ANE was considered the greatest challenge to the poultry industry after the antibiotic ban (Phillips, 2002; Kaldhusdal, 2003).

Probiotics appear as the most promising among the several alternatives to control the disease (Patterson and Burkholder, 2003). Several microorganisms studied during the last decades showed probiotic properties (Gil de los Santos and Gil-Turnes, 2005; Gil-Turnes et al., 2007). Among those studied by our group, *Bacillus cereus* var. Toyoi and *Saccharomyces boulardii* increased productivity and controlled infectious diseases in swine (Zani et al., 1998) and broilers (Gil de los Santos et al., 2005), and modulated immune responses in mice (Conceição et al., 2000; Coppola et al., 2005).

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Seeking for new probiotics, our group studied the properties of *Pichia pastoris* yeast, extensively used to express heterologous proteins (Macauley-Patrick et al., 2005; Li et al., 2007; Bollok et al., 2009; Ramón and Marín, 2011). The validation in broilers of *P. pastoris* and a recombinant *P.* pastoris containing the gene of *C. perfringens* type A alpha toxin as probiotics, and in the induction of immunity against the toxin are reported in this paper.

2. Material and methods

2.1. Animals and treatments

Ross^{TM} P8 one-day-old female broilers were randomly allocated in four groups of ten animals each. All the animals received a commercial feed devoid of antibacterials. Group 1 (control) received non supplemented feed, group 2 feed supplemented with $1 \times 10^6\,g^{-1}$ viable cells of *P. pastoris* strain KM71H, group 3 feed containing $1 \times 10^6\,g^{-1}$ viable cells of recombinant *P. pastoris* KM71H containing the *C. perfringens* type A alpha toxin gene (Gil de los Santos, 2007), and group 4 feed containing $1 \times 10^6\,g^{-1}$ viable spores of *B. cereus* var. Toyoi. Feed and water were offered *ad libitum*. The animals were handled in accordance with the Brazilian Animal Experimentation National Protection Law. The experiments were done in triplicate.

2.2. Feed efficiency

Individual weight gains were estimated subtracting from the live weights at 49 days old the weight of the same animal one-day old. Feed conversions were estimated dividing the weight gain of the group by the quantity of feed consumed by the group during the experiment.

2.3. Antibody response

Individual blood samples were collected on days 1, 10, 20, 30 and 49 of the experiment. Antibody titres were assessed by ELISA following accepted protocols (Gil de los Santos, 2007), using *C. perfringens* alpha toxin (*C. perfringens* phospholipase C type I, Product No. P7633, Sigma–Aldrich Inc., St. Louis, USA) as antigen. Briefly, Polystyrene microtiter plates (Greiner Labortechnik, Germany) were coated with 0.5 μg of the antigen suspended in carbonate–bicarbonate buffer pH 9.6 and incubated overnight at 4 °C. Sera diluted 1:50 were added in duplicate to the wells and incubated for 1.5 h at 37 °C. After washing with PBS-T, rabbit anti-chicken (IgY) peroxidase conjugated antibodies (Sigma–Aldrich Inc., St. Louis, USA) were added and incubated for 1.5 h at 37 °C. After washing with PBS-T, OPD-chromogen substrate (1 mL Phosphate-citrate buffer pH 4.0, 1 μL 30% hydrogen peroxide, 0.4 mg ortophenylene diamine) was added. Absorbancies were measured in an ELISA spectrophotometer (Dynatech MR 700) at 492 nm. Seroconversions were estimated dividing the absorbancies at 10, 20, 30 and 49 days by those of day 1 (Gil-Turnes et al., 1999).

Dot-ELISAs were performed using 5 μg of either the commercial antigen mentioned above or a recombinant *C. perfringens* alpha toxin produced at our laboratory (Gil de los Santos, 2007) placed on a 0.45 μm nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences UK Ltd., Buckinghamshire, UK) and dried at room temperature. The membranes were blocked for 1 h with 5% non fat dry milk under agitation, washed with PBS-T and 3 μL of a pool of sera of each group of the 49 days sample were added and incubated for 30 min at 37 °C. After another washing with PBS-T, rabbit anti-chicken (IgY) peroxidase conjugated antibodies (Sigma–Aldrich Inc., St. Louis, USA) were added and incubated for 1.5 h at 37 °C. The reactions were visualized with DAB-chromogen substrate (9 mL Tris–HCl 50 mM, 1 mL nickel sulphate 0.3%, 10 μL of 30% hydrogen peroxide and 6 mg 3.3-diaminobenzidine tetrahydrochloride).

2.4. Pathology

At the end of the experiment all the animals (120) were euthanized and necropsied. After the macroscopic evaluation, samples from liver, lungs, kidneys, spleen, heart, Bursa of Fabricius, brain, pancreas, proventriculus, crop and small and large intestines were collected and submitted for histopathological analysis. The samples were processed to paraffin embedding, stained by the haematoxylin–eosin method and evaluated by light microscopy.

2.5. Statistical analysis

The statistical analysis of data was performed by ANOVA using the Statistix software version 8 (Analytical Software, Tallahassee, FL, USA). Data reported are the means of the three replicates.

3. Results

The higher weight gain was obtained by the animals fed with recombinant *P. pastoris* (2.36 kg), followed by those who received *B. cereus* var. Toyoi (2.28 kg), *P. pastoris* (2.17 kg) and non-supplemented feed (2.12 kg) (Table 1). The best feed conversion was obtained by the group that received recombinant *P. pastoris* (2.35), followed by the *P. pastoris*, *B. cereus* Toyoi and control groups (2.41, 2.5 and 2.58, respectively).

Seroconversions estimated with the commercial *C. perfringens* alpha toxin were significantly higher ($P < 0.05$) in the recombinant *P. pastoris*, *P. pastoris* and *B.* Toyoi groups (1.49, 1.44 and 1.34, respectively) than in the control group (1.12) (Table 1). Sera of the animals of all the groups reacted

Table 1
Weight gain, feed conversion and seroconversions of each group.

Group	Weight gain (kg)	Feed conversion (kg)	Seroconversions
1. Control	2.12^{c}	2.58	1.12^{c}
2. *Pichia pastoris*	2.17^{c}	2.41	1.44^{a}
3. Recombinant *P. pastoris*	2.36^{a}	2.35	1.49^{a}
4. *Bacillus cereus* var. Toyoi	2.28^{b}	2.50	1.34^{ab}

Different letters in the same column indicate significant differences ($\alpha = 0.05$).