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Scientific Report

Adhesion profile of *Salmonella enterica* to the intestinal epithelium in the presence of probiotic microorganisms: an *in vitro* study

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1.0 Introduction

Animal feed is at the beginning of the food safety chain in the "farm-to-fork" model. The emergence of variant Creutzfeldt-Jakob disease has raised awareness of the importance of contaminated animal feed, but less attention has been paid to the role of bacterial contamination of animal feed in human foodborne illness. In the United States and also in South America, animal feed is frequently contaminated with non-Typhi serotypes of *Salmonella enterica* and may lead to infection or colonization of food animals. These bacteria can contaminate animal carcasses at slaughter or cross-contaminate other food items, leading to human illness. Although tracing contamination to its ultimate source is difficult, several large outbreaks have been traced back to contaminated animal feed. Improvements in the safety of animal feed should include strengthening the surveillance of animal feed for bacterial contamination and integration of such surveillance with human foodborne disease surveillance systems. A Hazard Analysis and Critical Control Point program should be instituted for the animal feed industry, and a Salmonella-negative policy for feed should be enforced. The reduction of the *Salmonella enterica* contamination could be achieved using probiotic microorganism included in feed that reduce the colonization of the intestinal epithelium for this pathogen, for a mechanisms of competition for the intestinal surface adsorption sites. The object of this study will be to test a probiotic ingredient on the reduction of the *Salmonella enterica* contamination of the intestinal epithelium, using an *in vitro* study based on the Caco-2 cells that is the validated international method for the simulation *in vitro* of the intestinal enterocytes using the Twell plate. The Caco-2 cells will be differentiated at 21 days and after that a culture of 5 different strains of *S. enterica* will be putted in contact with the simulated intestinal epithelium in presence or not of the probiotic ingredient. The percentage of adsorption of the *S. enterica* on the Caco-2 cells will be calculated with microbiological analysis, and the data obtained in the control experiments will be compared with the co-culture of *S. enterica*-probiotic microorganism to demonstrate if the ingredient has the property to reduce the risk associated to intake of the pathogenic bacteria by animals.

2. Materials and methods

2.1 Chemicals

The reagent grade chemicals and cell culture components used, mainly Dulbecco's

Modified Eagle's Medium (DMEM), penicillin, streptomycin, amphotericin B, HEPES, no essential amino acids (NEAA), Phosphate Buffer Saline (PBS), crude mucin (Type II), and trypsin were Sigma–Aldrich products (Sigma Co., St. Louis, Mo., USA). Fetal calf serum (FCS) was from Cambrex Company (Belgium). Peptone water (0.1%), De Man Rogosa Sharpe agar (MRS Agar), Tryptic Soy Agar (TSA Agar), Xylose lysine deoxycholate agar (XLD agar).

2.2. Cell culture of Caco-2 cells

The Caco-2 (ATCC HTB-37) cells were cultured in monolayer in 9 cm^2 polystyrene tissue culture dishes with DMEM supplemented with 25 mM HEPES, 1% NEAA, 100 U/ ml penicillin, 100 mg/ml streptomycin, 2.5 mg/ml amphotericin B, and 10% heat inactivated FCS. Incubation conditions were pH 7.4, 37ºC and 5% CO_2 in a 95% relative humidity atmosphere.

2.3 Bacterial strains and culture conditions

The probiotic product was prepared inoculating the suspension at $3.8 \cdot 10^7$ UFC/mL in 10 mL of PBS and incubated at 37ºC for 30´. For the *Salmonella* strains 5 serotypes of *Salmonella enterica* were used, and in particular *Salmonella enterica* CECT 4156, *Salmonella enterica* CECT 4371, *Salmonella enterica* CECT 7160, *Salmonella enterica* CECT 4396, and *Salmonella enterica* CECT 4300 that were obtained from the Spanish Type Culture Collection (CECT Valencia, Spain). These microorganisms were inoculated at 10^5 UFC/mL in 10 mL of TSB and incubated at 39ºC for 24 h. After that a co-culture of the five strains was prepared at 10^6 UFC/mL before the adhesion assay experiments.

2.4 In vitro bacterial adhesion assay

Before the adhesion assay, Caco-2 monolayers were treated with mucin to promote the adhesion of the microorganism to the intestinal epithelium cells (Perales et al., 2007). Crude mucin (Type II, Sigma–Aldrich) was diluted in PBS (pH 7.2). An aliquot (0.5 ml) of this solution was loaded into a polycarbonate six-well plates (Costar, Cambridge, MA, USA) and incubated at 37ºC during 1 h. To remove unbound mucin, each well was washed twice with PBS.

Adhesion assay was performed according to Laparra and Sanz (2009). For adhesion assays, the Caco-2 cells (previously treated with mucin) were seeded at $25x10^4$ cells/

cm^2 on six-well Transwell Permeable Supports, 12 mm diameter (Corning, NY, USA) and incubated at 37ºC in 5% CO_2 atmosphere in a humidified incubator until fully differentiated cells. Cell monolayers were carefully washed twice with PBS before probiotic bacteria and *Salmonella* were added.

Four different treatments were tested and in particular a) control of probiotic, b) control of *Salmonella*, c) co-culture of the probiotic-Salmonella and d) co-culture of the probiotic-Salmonella where the probiotic was pre-incubated alone with cells during 90 min., before *Salmonella* adding. The cells treated with the control experiments (the microorganisms were introduced in one mL of DMEM without antibiotics) were inoculated with $3.8 \cdot 10^7$ UFC/mL of probiotic and 10^6 UFC/mL of salmonella respectively to simulate the real suspension of probiotic microorganisms and pathogens presents in the poultry gastrointestinal tract. In the co-culture experiments the microorganisms were inoculated using the same suspensions applied for the controls trials.

The bacterial cell suspensions (10^6–10^8 CFU/ml) were incubated in culture grown medium during 90´ with a monolayer of fully differentiated Caco-2 cells, at 37ºC in an atmosphere of 95% air and 5% CO2, all monolayers were washed three times with PBS to release unbound bacteria.

After the incubation times used for each experiments, the cells were scratched by plate surface with PBS and with plastic handle, and the bacteria were counted by Standard Plate Count, in MRS agar for probiotic microorganisms and with TSB Agar with an overlay of XLD Agar for *Salmonella* counting (Meca et al., 2012; Beltran et al., 2016).

2.5 Statistical analysis

The data produced were analyzed with ANOVA, using a post-test of Tukey for the comparison of the mean with a significance level of $p<0.01$. Lowercase letters indicate significance differences between the probiotic groups, whereas the capital letter indicates significance differences between the Salmonella groups.

2.0 Results

The percentage of adhesion of the probiotic product to the intestinal epithelium in the control experiment was of 0.04%, whereas the percentage evidenced by the *Salmonella* was of 5.11%.

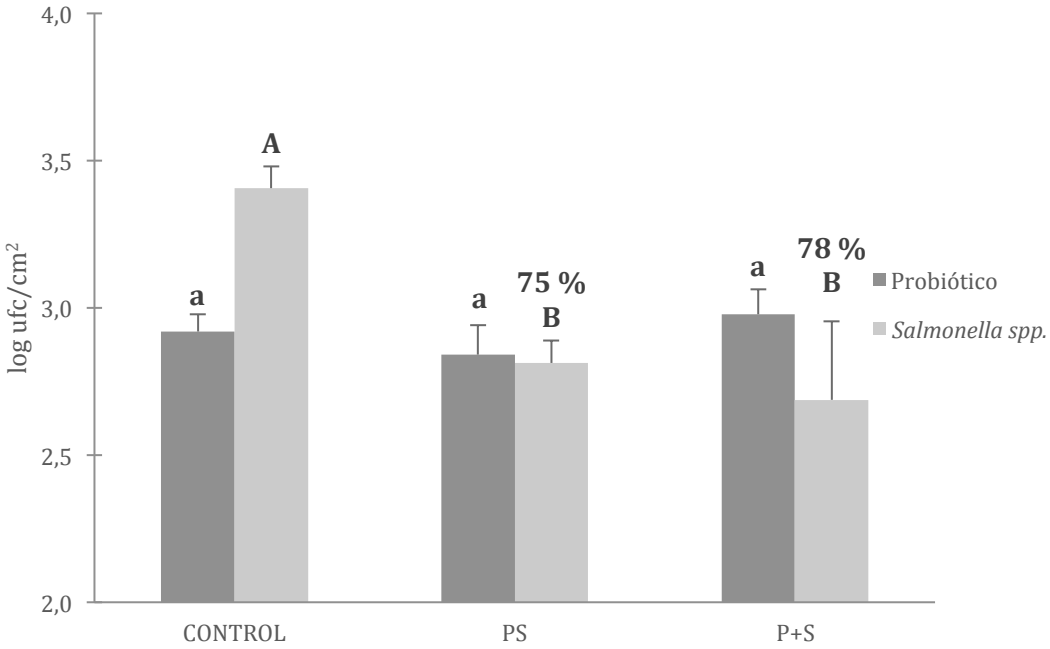

Figure 1. Adhesion results showed by the microorganisms tested. **Control** = Assay of the probiotic product and Salmonella without any competition **PS** = Assay developed using the probiotic and the Salmonella at the same time. **P+S** = Assay carried out pre-incubating the probiotic during 90 minutes with cells before *Salmonella* inoculation.

As evidenced in the figure 1, the probiotic product reduce (75%) with an important significance the adhesion of the pathogenic *Salmonella* strains in the cell system studied when the probiotic and the Salmonella were incubated at the same time (PS), and this reduction was higher (78%) than PS test, when the probiotic was pre-incubated during 90 min. with Caco-2 cells, before that the Salmonella strains were added to the cell system. Probably the pre-inoculum of the probiotic permits to the microorganisms to occupy more adhesion sites on the Caco-2 cells tissue and reducing in this way the quantity of the Salmonella strains that could be adhered.

4.0 References

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