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Quantification of FITC-labelled probiotic *Lactobacillus salivarius* DSPV 001P during gastrointestinal transit in broilers

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RESEARCH ARTICLE

Abstract

The knowledge related to the fate of probiotics in the complex environment of the intestinal microbiota in broilers is just beginning to be elucidated; however, it is not yet well understood. A good method to investigate the mechanisms by which probiotics mediate their effects is to mark probiotic bacteria and trace them. The aim of this research was to develop a new method to estimate *in vivo* fluorescein isothiocyanate (FITC)-labelled *Lactobacillus salivarius* DSPV 001P counts during passage through the gastrointestinal tract (GIT) of broilers. Forty-five, 1 d old Cobb broilers were used in this trial. Programmed necropsies were performed 30 min, 6 h, and 12 h after the administration of the probiotic bacterium, and samples of liver, crop, duodenum, caecum, and bursa of fabricius were collected. To determine the spatial and temporal transit of *L. salivarius* DSPV 001P in broilers, the number of bacteria as well as its respective fluorescent signal produced by FITC were measured. In order to observe the relationship between the variables, a logistic regression analysis was applied. The amount of fluorescence could be used as an indicator of fluorescent probiotic bacteria in the crop and duodenum 30 min after probiotic bacterium supplementation. In addition, the fluorescent signal could be used to estimate bacterial counts in caecum 6 and 12 h after *L. salivarius* DSPV 001P administration. To the best of our knowledge, this research is the first *in vivo* trial to employ the bacterial FITC-labelling technique in order to enumerate probiotic bacteria during gastrointestinal transit in broilers.

Keywords: FITC-labelling technique, lactic acid bacteria, gastrointestinal tract, monitoring

1. Introduction

The utilisation of native microorganisms with probiotic capacity could provide an efficient alternative for the prevention of some animal illnesses (Rosmini *et al.*, 2004b). Probiotics could improve sanitary conditions and enhance performance in broilers, being a reliable option to replace antimicrobial growth promoters (Asghar *et al.*, 2016; Blajman *et al.*, 2015b). It is imperative to understand the interactions between the administered bacteria and the host intestinal system. However, the knowledge related to the fate of probiotics in the complex environment of the intestinal microbiota is just beginning to be elucidated, though it is not yet well understood (Yu *et al.*, 2007). A good method to investigate the mechanisms by which probiotics mediate their effects is to mark probiotic bacteria and trace them (Maldonado Galdeano and Perdigón, 2004). Different types of substances have been applied to label bacteria (Bloemberg *et al.*, 2000; Edelman *et al.*, 2012; Zhang *et al.*, 2010). Among them, fluorochromes as FITC, rhodamine, and phycoerythrin (Bachner and Bruner, 2002) are commonly used. FITC is a highly useful fluorescent tag for studying the localisation of living cells (Vinderola *et al.*, 2004). So far, FITC was qualitatively used to study the distribution of bacteria (Schmuch *et al.*, 2015). Nevertheless, the development of a new method that enumerates administered bacteria would represent an alternative to the reference technique (standard plate

count), and facilitate the *in vivo* monitoring in terms of both spatial and temporal distribution. The aim of this research was to develop a new method to estimate fluorescein isothiocyanate (FITC)-labelled *in vivo L. salivarius* DSPV 001P counts in different sections of the GIT of broilers at different time points to gastrointestinal content and tissue fluorescent signal.

2. Materials and methods

Fluorescent labelling of bacteria

The indigenous bacterium *L. salivarius* DSPV 001P with *in vitro* probiotic properties (Blajman *et al.*, 2015a), had been previously isolated from the gut of a healthy broiler and identified and conserved at the Laboratory of Food Analysis, Institute of Veterinary Science of the Litoral (ICIVET, Santa Fe, Argentina). *L. salivarius* DSPV 001P was made resistant to rifampicin in order to be able to trace down the bacteria during the *in vivo* study (Blajman *et al.*, 2015a).

Fresh cultures of the strain were obtained. Bacteria were harvested by centrifugation at 5,000×*g* for 10 min and washed twice with phosphate-buffered saline (PBS) solution (pH 7.2). The pellet was resuspended in PBS solution with FITC (Sigma, St. Louis, MO, USA) (1 mg/ml) and incubated for 2 h at 37 °C in the dark. Labelled bacteria were washed six times with PBS solution to remove unincorporated FITC. The final pellet was resuspended in PBS to a concentration of $10^{9.5}$ cfu/ml (Burns, 2012; Medici *et al.*, 2004).

Experimental design

Forty-five, 1 d old Cobb broilers were randomly assigned to five experimental groups of nine broilers: the control group (C-G) and four inoculated probiotic groups (P-G). Each P-G received the labelled probiotic strain through gavage in different concentration: 10 log cfu, 9.5 log cfu, 8.5 log cfu, and 7.5 log cfu. The C-G fed 1 ml of PBS as placebo. Broilers received a volume of 3.16 ml to achieve a concentration of 10 log, and 1 ml for the remaining concentrations. All procedures used in this study were approved by the Ethics and Security Committee of the Faculty of Veterinary Science, Universidad Nacional del Litoral and consistent with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, Federation of Animal Sciences Societies (FASS, 1999).

Necropsies

Programmed necropsies were performed in three broilers from each experimental group (15 total broilers) 30 min, 6 h, and 12 h after the administration of the probiotic bacterium. Broilers were euthanised by cervical dislocation. The liver, crop, duodenum, caecum, and bursa of fabricius were collected using sterile instruments.

Quantification of probiotic bacteria in gastrointestinal content and tissue with standard plate count technique

Samples of 0.1 g (wet weight) from distal crop wall, crop content, distal duodenum, distal caecum, and bursa of fabricius were homogenised in 1/4 Ringer solution (Biokar, Allonne, France), and De Man, Rogosa, Sharpe Broth (De Man *et al.*, 1960) with rifampicin (MRS_{rif}) agar plates were spread to recover only the administered probiotic strain. Petri dishes were incubated at 37 °C for 72 h in anaerobic conditions (10% CO_2 and 90% H_2; Indura, Buenos Aires, Argentina), and the characteristic colonies were counted. To measure translocation in the internal medium, samples of 0.1 g (wet weight) from the left lobe of the liver was homogenised in 1/4 Ringer solution and spread in MRS_{rif}. Cfu counts among the liver and GIT of broilers were expressed as the arithmetic mean ± standard deviation (SD). Differences between treatments were assessed using a generalised linear model with doses (10, 9.5, 8.5, 7.5 log cfu, and control group), time (30 min, 6 h, and 12 h), and GIT section (crop, duodenum, caecum, and bursa of fabricius) as independent variables. Analysis was performed using INFOSTAT version 2011 (InfoStat Group, FCA, Universidad Nacional de Córdoba, Argentina).

Quantification of probiotic bacteria in gastrointestinal content and tissue with fluorescence technique

To determine the spatial and temporal transit of *L. salivarius* DSPV 001P in broilers after a single oral administration, the fluorescent signal produced by FITC was measured. Samples of 0.1 g (wet weight) from the left lobe of the liver, distal crop wall, crop content, distal duodenum, distal caecum, and bursa of fabricius from each experimental group were first added to the appropriate wells of a black 96 well microtiter microplate (Greiner Bio-One 655 076) and read in a multimode microplate reader (Synergy HT; BioTek Instruments, Inc., Winooski, VT, USA). A piece of each organ was collected using sterile instruments, minimising the possible bacterial contamination between samples, and serous membrane was placed in contact with the microplate bottom. The mucosa of hollow organs was exposed in the microplate and reading was performed directly on it. The relative fluorescence units (RFU) were quantified using Gen5 software (BioTek) for Windows version 2.01. FITC levels were measured at excitation wavelength of 485 nm and emission wavelength of 528 nm (Tellez *et al.*, 2015). The fluorescence was determined by reading the top of the well with a gain set of 35 and a 'moderate shake' for 3 s. Statistical analysis was carried out applying logistic regression analysis, in order to observe the relationship between the variables (RFU vs microbiological counts). The evaluation of logistic parameters was performed and the

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coefficients of determination (R^2) were estimated. When results for RFU or microbiological counts were less than 1 log order cfu/g, data were excluded to relate the variables. Analysis was performed using INFOSTAT version 2011.

Fluorescent detection of probiotic bacterium in histological slices

Samples from the left lobe of the liver, distal crop, distal duodenum, distal caecum, and bursa of fabricius were removed for histological preparation following Sainte-Marie technique (Sainte-Marie, 1962) with some modifications for paraffin inclusion. Initially, samples of 1×1 cm in hollow organs and 1×1×0.3 cm in liver were removed with scissors and dissecting forceps. Then, the samples were immersed in 70% alcohol, stored overnight under refrigeration, and reduced to 0.5×0.5 cm. Subsequently, tissues were dehydrated by immersing through a series of alcohol with increasing concentrations (from 70% to absolute ethanol), infiltrated with xylene, and embedded in paraffin. The blocks were properly trimmed and sections of 5 μm thickness were sliced with microtome (Leica RM2245, Nussloch, Germany) and examined for fluorescence microscopy (CI Eclipse; Nikon, Tokyo, Japan). Fluorescence-stained sections were examined at 400× magnification using the following excitation/emission filter combinations (filter set for FITC): 450~480 nm exciter filter and 515 nm barrier filter and 505 nm dichroic mirror (Heo and Song, 2011). The images were taken with a digital camera (DCM900, ScopeTek Opto-Electric Co., Ltd., Hangzhou, China PR) using Minisee capture software for Windows version 1.1.3.0. Interactions between observed 'clusters', aggregates or isolated fluorescent bacteria and the different tissue layers from the GIT of broilers were described.

3. Results

Quantification of probiotic bacteria with standard plate count technique

Once the bacteria were administered, *L. salivarius* DSPV 001P values in crop, duodenum, caecum, and bursa of fabricius of broilers from the P-G were monitored. The administered bacterium was not present in the liver; neither bacterial translocation to the internal medium was found. After just 30 min of supplementation, strain was recovered from broilers' samples. In the crop, colonisation was 6.82 log cfu/0.1 g (SD 1.173 log cfu/0.1 g) for the wall and 7.35 log cfu/0.1 g (SD 1.07 log cfu/0.1 g) for the content. Total probiotic counts in crop were higher 6 and 12 h after bacterium administration. Throughout the studied period the level of *L. salivarius* DSPV 001P in duodenum decreased from 6.70 log cfu/0.1 g (SD 1.61 log cfu/0.1 g) to 4.75 log cfu/0.1 g (SD 1.09 log cfu/0.1 g). No probiotic bacterium was detected in the caecum of broilers who had received 9.5 log cfu, 8.5 log cfu, and 7.5 log cfu. However, in broilers treated with 10 log cfu *L. salivarius* DSPV 001P, the strain was found at levels of 4.52 log cfu/0.1 g caecum (SD 0.94 log cfu/0.1 g) 30 min after supplementation. Strain intake resulted in an increase in the level of *L. salivarius* DSPV 001P to 6.57 log cfu/0.1 g (SD 1.14 log cfu/0.1 g) and 6.86 log cfu/0.1 g (SD 0.88 log cfu/0.1 g) 6 and 12 h after probiotic supplementation, respectively. *L. salivarius* DSPV 001P could be recovered from bursa of fabricius 6 h following probiotic administration with a maximum value of 4.10 log cfu/0.1g. Also, the strain reached a maximum value of 5.39 log cfu/0.1 g 12 h after bacterium supply. There were significant differences between counts in terms of GIT section, doses and time. Total cell counts of *L. salivarius* DSPV 001P were significantly higher (P<0.05) in crop wall and crop content in comparison with duodenum and caecum. Regarding the administered dose, the highest cell count was observed in broilers receiving 10 log/cfu (P<0.05). Although the strain behaved differently in each assessed organ, in general, counts were higher 12 h after bacterium administration (P<0.05).

Quantification of probiotic bacteria with fluorescence technique

In crop and duodenum, the fluorescent signal reached a peak 30 min after administration, whereas it declined after 6 and 12 h. It took more time for the strain to reach the caecum and bursa of fabricius: 6 and 12 h after oral administration fluorescent signal became intense throughout these organs. The regression models and the R^2 obtained when comparing the standard plate count technique and the fluorescence technique are presented in Figure 1. The R^2 was 0.75 and 0.73 30 min after FITC labelled *L. salivarius* DSPV 001P administration in crop wall and crop content, respectively. The curve correctly describes the relationship between fluorescence and the reference technique (Figure 1A, 1B). In duodenum, results show an important correlation (R^2=0.88) between RFU and microbiological counts 30 min after probiotic supplementation (Figure 1C). This meant that 88% of the variability in the response could be explained by the model. In caecum, we obtained a value of R^2=0.80 and R^2=0.72 6 h and 12 h after bacteria supply, indicative of a strong relationship between the model and the response variable (Figure 1D, 2E). In all cases mentioned the R^2 was significant (P<0.05), which demonstrates the existence of a good data concentration around the regression curve. Thus, the calculated data is very close to the observed data (Figure 2). However, no correlation between the standard plate count technique and the fluorescence technique were found in caecum 30 min after probiotic bacteria supply and in crop and duodenum 6 h and 12 h after *L. salivarius* DSPV 001P administration. No fluorescence was detected in the caecum of broilers who had received 9.5 log cfu, 8.5 log cfu, and 7.5 log cfu. On average, the fluorescence in broilers treated with 10 log cfu *L. salivarius* DSPV 001P was 25 RFU 30 min after supplementation. The fluorescence

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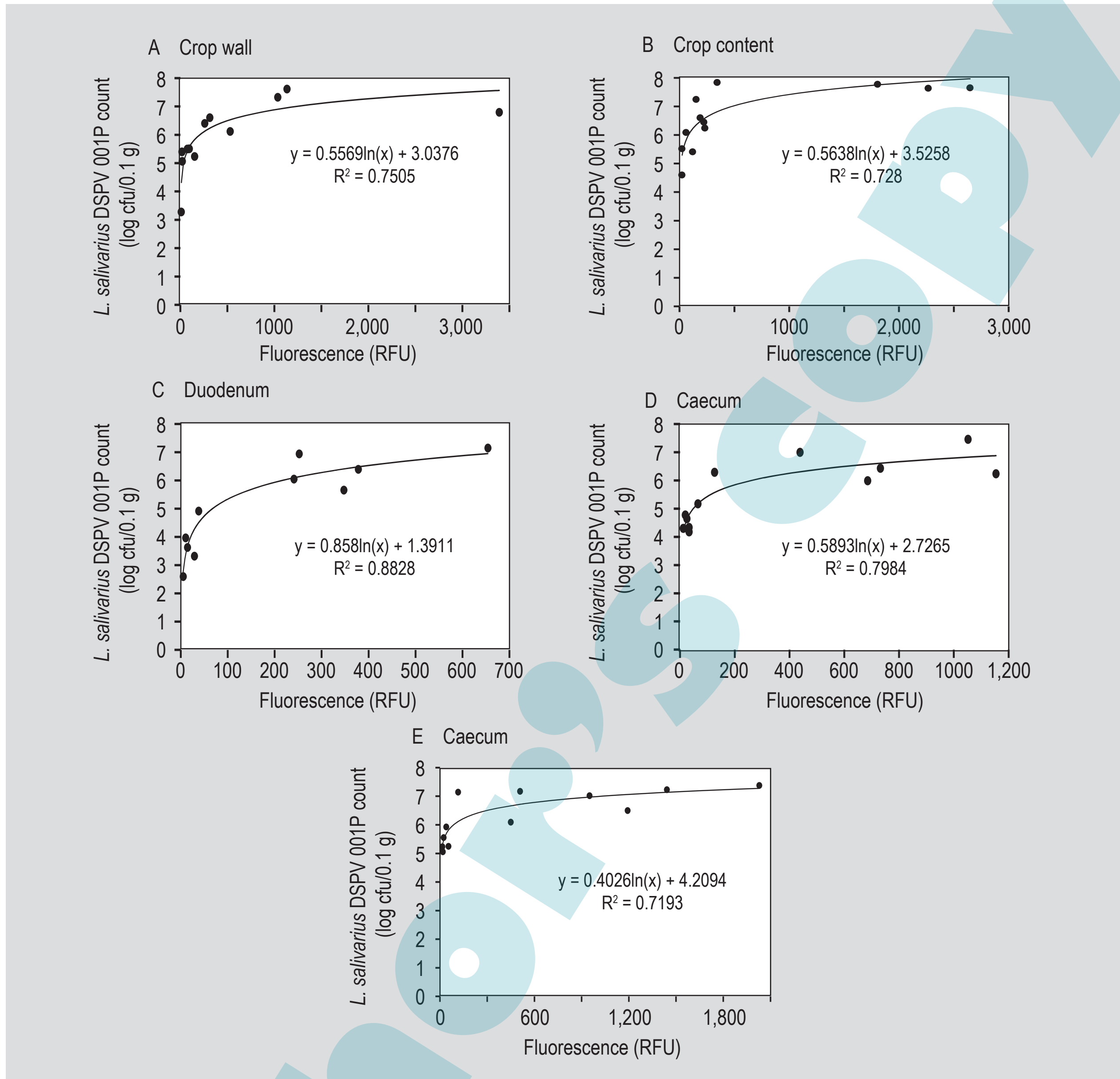

Figure 1. Correlation between *Lactobacillus salivarius* DSPV 001P (log cfu/0.1 g) counts vs fluorescence (RFU) at different time points and measured in different organs. Four dose trials (10 log cfu, 9.5 log cfu, 8.5 log cfu, and 7.5 log cfu) with 3 replicates each one were performed. (A) Crop wall after 30 min FITC labelled *L. salivarius* DSPV 001P administration; (B) Crop content after 30 min FITC labelled *L. salivarius* DSPV 001P administration; (C) Duodenum after 30 min FITC labelled *L. salivarius* DSPV 001P administration; (D) Caecum after 6 h FITC labelled *L. salivarius* DSPV 001P administration; (E) Caecum after 12 h FITC labelled *L. salivarius* DSPV 001P administration.

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obtained in bursa of fabricius was 54 RFU (6 h) and 60 RFU (12 h) in broilers treated with 10 log cfu. There was no fluorescence at 30 min regardless of the dose used and at 6 h and 12 h in broilers that received less than 10 log cfu. In any case the fluorescent signal was found below 2.5 log cfu/0.1 g, this value being the lower quantification limit of the technique.

Fluorescent detection of *Lactobacillus salivarius* DSPV 001P in histological samples

After 30 min of labelled probiotic bacterium administration, 'clusters' or bacterial aggregates were observed in the lumen of crop, duodenum, and caecum. Fluorescent bacteria were distinguished in the mucosa and submucosa of crop (Figure 3). In duodenum, fluorescent lactobacilli were embedded in the intestinal mucus and in the lumen, even though some

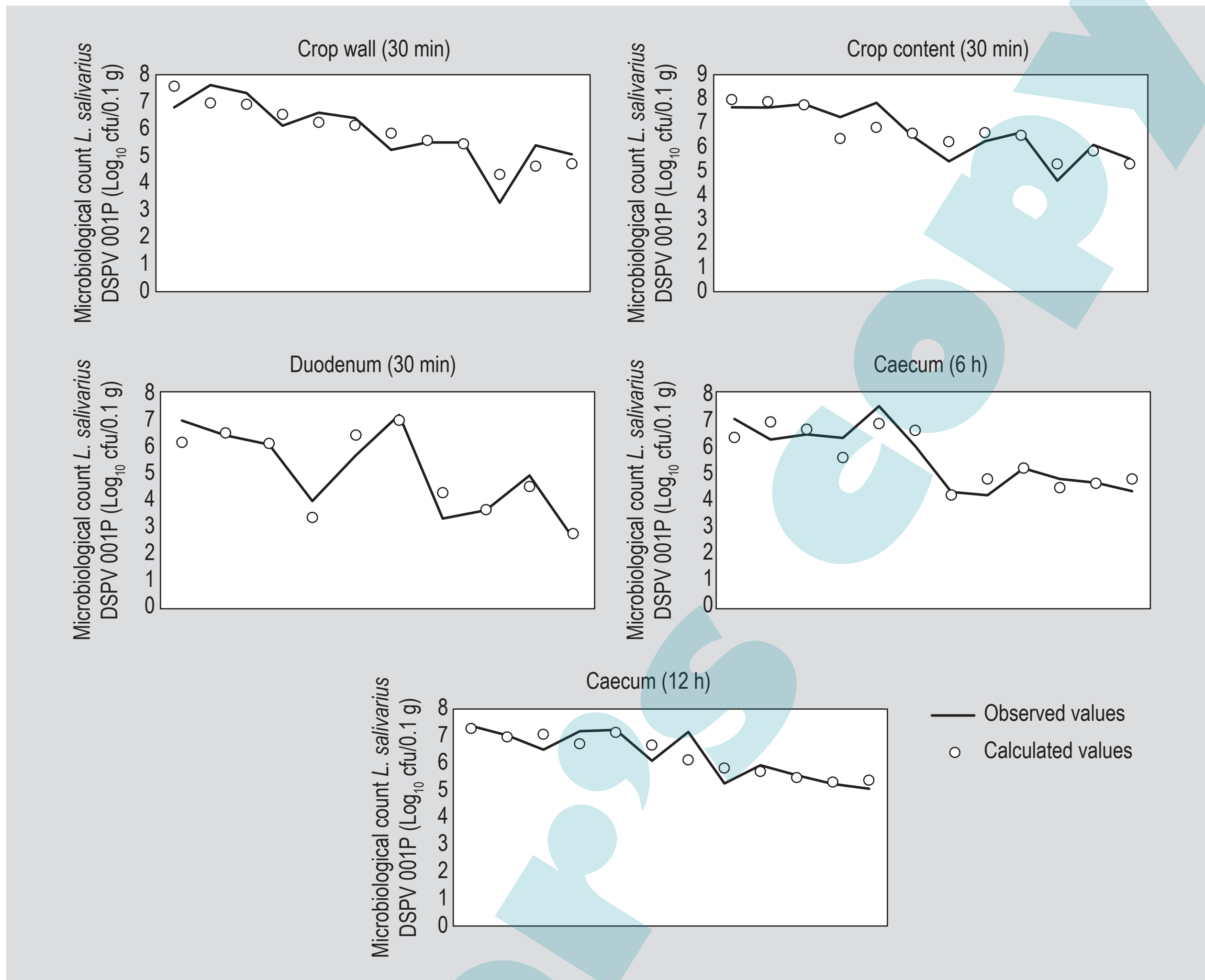

Figure 2. *Lactobacillus salivarius* DSPV 001P (log cfu/0.1 g) counts at different time points and measured in different organs. Observed values from the plate count technique vs calculated values from the equation obtained by the logarithmic model. Observed values are indicated by solid lines, while calculated values are indicated by circles.

bacteria seemed to be closely associated with epithelial cells. Labelled bacterium internalised in lamina propria and muscularis mucosae were also observed (Figure 4). A greater amount of bacterial aggregates was observed in broilers receiving 10 and 9.5 log cfu. Smaller aggregates and isolated bacteria were visualised in those broilers receiving 8.5 and 7.5 log cfu. Fluorescent bacteria were detected in the caecum of broilers that received 10 log cfu *L. salivarius* DSPV 001P. Meanwhile, only a few bacteria were found in the caecum of those that received 9.5 log cfu and no bacteria were observed in the caecum of broilers that received a lower bacterial probiotic concentration (Figure 5). No bacteria were found in the bursa of fabricius 30 min after probiotic supplementation (Figure 6). Bacterial translocation to the liver was not found in either group (Figure 6).

There was a noticeable increase in the number of fluorescent bacteria observed in mucosa, submucosa, and muscular layer of P-G caecum 6 h and 12 h post-administration (Figure 5). In addition, bacteria were observed in the bursa of fabricius of broilers receiving 10 log cfu (Figure 6). It should be noted that lower amounts of fluorescent bacteria were seen in the lumen of the crop. However, a higher degree of labelled bacteria was visualised in mucosa and submucosa of the crop 6 h after probiotic supplementation (Figure 3). Moreover, only a few bacteria were noticed in duodenum (Figure 4). There was no bacterial translocation to the liver in either group (Figure 6). No labelled probiotic bacterium was found in the C-G.

4. Discussion

In this study, a new method was used to estimate microbiological counts *in vivo* in different sections of the GIT of broilers. We monitored the number of *L. salivarius* DSPV 001P as well as its respective fluorescent signal in broilers' GIT at different time points after the oral

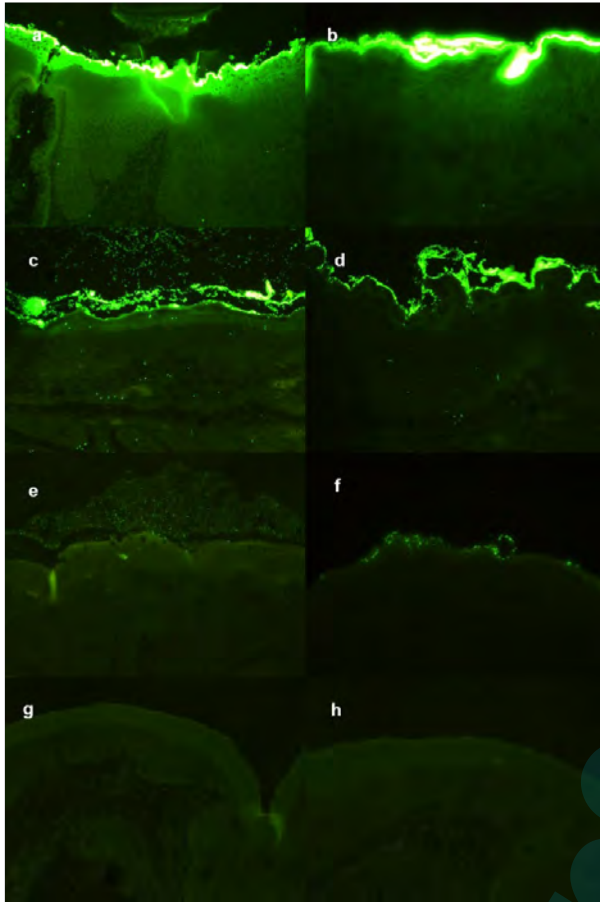

Figure 3. Histological slices of crop of broilers (magnification 400×) that received 10 log cfu FITC labelled bacteria. Fluorescence in the crop 30 min (a, b); 6 h (c, d); and 12 h (e, f) after *Lactobacillus salivarius* DSPV 001P administration. Crop from control broiler (g, h (absence of labelled probiotic bacterium)).

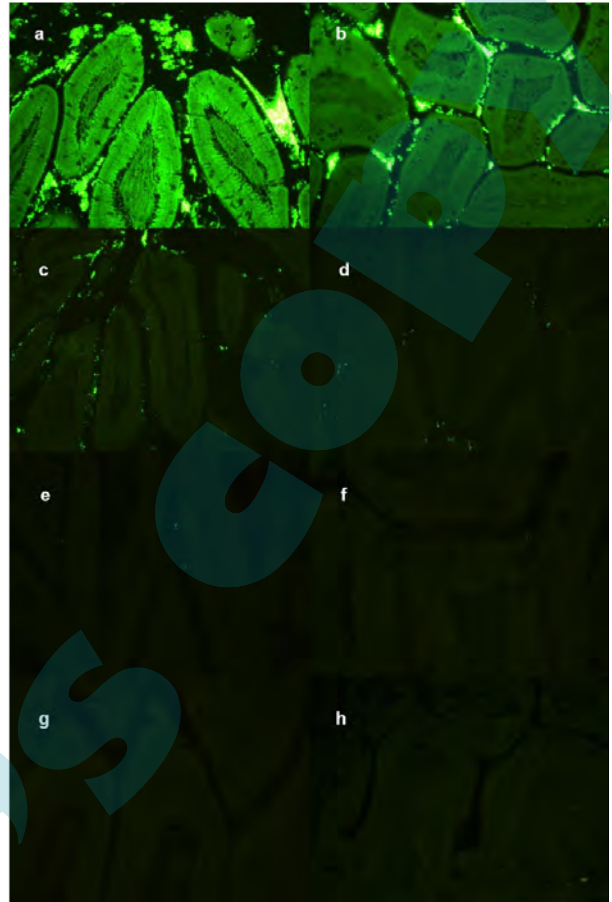

Figure 4. Histological slices of duodenum of broilers (magnification 400×) that received 10 log cfu FITC labelled bacteria. Fluorescence in the duodenum 30 min (a, b); 6 h (c, d); and 12 h (e, f) after *L. salivarius* DSPV 001P administration. Duodenum from control broiler (g, h) (absence of labelled probiotic bacterium).

administration of probiotic bacterium. It was revealed that the standard plate count technique can be simplified on the microplate fluorescent technique. We have also shown that the data obtained using an automated method to estimate bacterial counts significantly correlated with the reference technique. Importantly, LAB fluorescence could be detected in broilers after a single oral administration of labelled probiotic bacterium. The obtained curve described appropriately the relation between the two variables. However, the estimation curve adjustment depends on the GIT section used, the doses and the time point. The amount of fluorescence could be used as an indicator of fluorescent probiotic bacteria in the crop and duodenum 30 min after probiotic bacterium supplementation. In addition, the fluorescent signal could be used to estimate bacterial counts in caecum 6 and 12 h after *L. salivarius* DSPV 001P administration. At 30 min, the fluorescent signal achieved a maximum in crop and then started to decline. However, the number of viable bacteria increased with time. In fact, the number of *L. salivarius* DSPV 001P reached a peak in crop 12 h post administration, although the fluorescent signal corresponded to a low level. This means that externally administered LAB could not only persist but also replicate actively or colonise permanently the crop of broilers (Blajman *et al.*, 2015a). Thirty min post probiotic administration, a high fluorescent signal associated with a significantly high number of bacteria was observed in duodenum. Nevertheless, the signal weakened and bacterial counts declined after 6 and 12 h. Due to the low pH in stomach and rapid passage of intestinal contents, duodenal bacterial counts are low (Rinttilä and Apajalahti, 2013). These results may therefore be attributed, at least in part, to the adverse conditions to which ingested probiotic microorganisms are exposed in that compartment of the GIT (Silva Rocha *et al.*, 2014). At 30 min, fluorescence was found in broilers' caecum that received 10 log cfu. The number of viable bacteria increased in caecum with time, reaching its maximum level after 12 h. This peak correlated perfectly with the maximum level of fluorescent signal. Due to the infrequent emptying, retention time in the caecum

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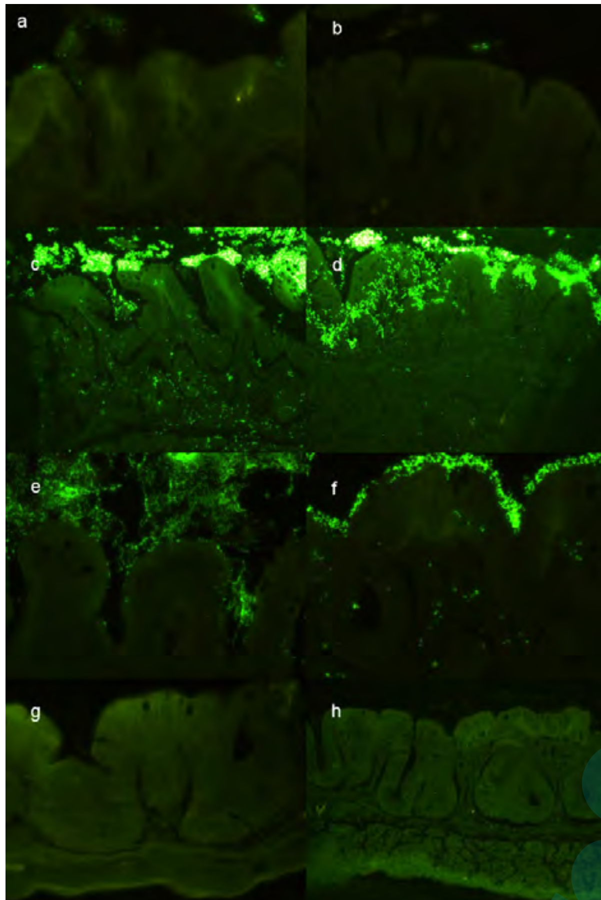

Figure 5. Histological slices of caecum of broilers (magnification 400×) that received 10 log cfu FITC labelled bacteria. Fluorescence in the caecum 30 min (a, b); 6 h (c, d); and 12 h (e, f) after *Lactobacillus salivarius* DSPV 001P administration. Caecum from control broiler (g, h) (absence of labelled probiotic bacterium).

is usually long, as indicated by the fact that caecal content was not significantly reduced after 24 h of feed deprivation (Warris *et al.*, 2004; Hinton *et al.*, 2000). Another point to be mentioned is that continuous antiperistaltic movements of the colon have been observed, and those aforementioned antiperistaltic movements will transport material into the caecum in a very short time (Svihus *et al.*, 2014).

Histological samples led us to check that probiotic bacterium used in this work were able to interact in different degrees with the gastrointestinal mucosal cells and in some cases to make contact with submucosa and muscular layer cells, on the basis of a visual evaluation of the slices (Vinderola *et al.*, 2004). It could be useful to understand the behaviour of microorganisms and their influence on host, considering that little is known about the fate of lactobacilli when administered in a complex microbial environment *in vivo* (Hashemzadeh *et al.*, 2015).

Colony counts indicated that this strain was not able to translocate to liver or, in case it did, the host immune system may eliminate bacteria before they could be detected (Frizzo *et al.*, 2010). Also, the translocation phenomenon to liver was not observed in the histological slices after the probiotic administration during the different periods of time assayed, thus strengthening the hypothesis that the strain is probably safe to be added as feed additive in the broilers' diet (Blajman *et al.*, 2015a). However, considering that the limit of quantification of the fluorescence technique is not so low (2.5 log cfu/0.1 g), absence of translocation could not be confirmed by this method.

On the one hand, the fate of the labelled probiotic strain could be monitored through GIT of broilers, whereas on the other hand, the short time during which the new method of

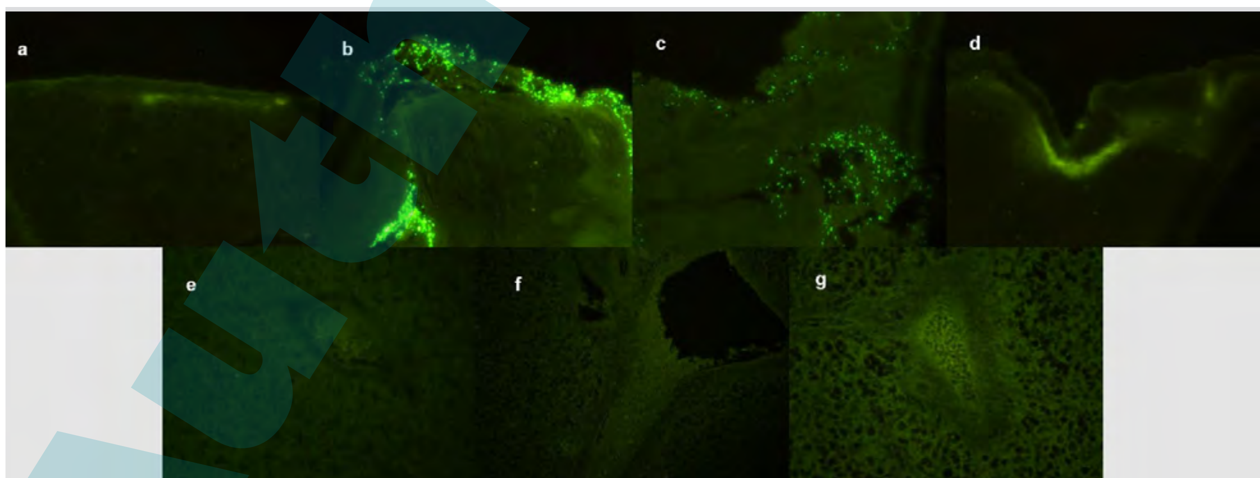

Figure 6. Histological slices of bursa of fabricius of broilers (magnification 400×) that received 10 log cfu FITC labelled bacteria. Fluorescence in the bursa of fabricius 30 min (a); 6 h (b); and 12 h (c) after *Lactobacillus salivarius* DSPV 001P administration. Bursa of fabricius from control broiler (d) (absence of labelled probiotic bacterium). Histological slices of liver of broilers that received 10 log cfu FITC labelled bacteria (e, f, g).

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quantification is effective represents a limitation that can be improved. FITC has a short half-life in living systems (Kasugai *et al.*, 2000) and it gradually loses its signal as bacteria growth. This explains what happened in crop and duodenum 12 h post probiotic administration. Tracing labelled *L. salivarius* DSPV 001P for an extended period could be interesting to complement the data generated. Considering our information, a possible support of more stable fluorophores that can be transmitted to replicate probiotic bacteria in the GIT should be evaluated. In this regard, it would be very useful to test this quantification method for a longer time using probiotic strains expressing green fluorescent protein (GFP) (Hashemzadeh *et al.*, 2015; Schultz *et al.*, 2005). Furthermore, the use of different fluorophores at each probiotic strain could enable an *in vivo* monitoring of a multi-strain inoculum.

Based upon the speediness, results in the microplate fluorescent technique are available within only 30 min from the moment of sampling, in contrast to the tedious preparation media and the 72 h of incubation required for the standard plate count method (Davis, 2014). Furthermore, the fluorescence technique allows the analysis of a high number of samples within a short period of time. This is especially useful when the number of samples to be analysed is large and laboratory human resources are limited (Rosmini *et al.*, 2004a). The method could be preferred when availability of results is critical, as only 30 min are required for the analysis but a multimode microplate reader is necessary.

5. Conclusion

To the best of our knowledge, this research is the first *in vivo* trial to employ the bacterial FITC-labelling technique in order to enumerate probiotic bacteria during gastrointestinal transit in broilers. This model may be useful to monitor the fate of a strain when administered to broilers, simplifying and automating the analysis of bacterial probiotic counts through the GIT. More studies should be conducted in order to test the effectiveness of the method on the study of different microorganism-host interactions.

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