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Colonisation of poultry by *Salmonella* Enteritidis S1400 is reduced by combined administration of *Lactobacillus salivarius* 59 and *Enterococcus faecium* PXN-33

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ABSTRACT

Salmonella Enteritidis remains a significant issue within the poultry industry and one potential solution is to use probiotic bacteria to prevent *Salmonella* colonisation through competitive exclusion (CE). We demonstrate that combined administration of *Lactobacillus salivarius* 59 and *Enterococcus faecium* PXN33 were effective competitive excluders of *Salmonella* Enteritidis S1400 in poultry. Two models were developed to evaluate the efficacy of probiotic where birds received *Salmonella* Enteritidis S1400 by a) oral gavage and b) sentinel bird to bird transmission. A statistically significant ($p < 0.001$) 2 log reduction of *Salmonella* Enteritidis S1400 colonisation was observed in the ileum, caecum and colon at day 43 using combined administration of the two probiotic bacteria. However, no *Salmonella* Enteritidis S1400 colonisation reduction was observed when either probiotic was administered individually. In the sentinel bird model the combined probiotic administered at days 12 and 20 was more effective than one-off or double administrations at age 1 and 12 days. *In vitro* cell free culture supernatant studies suggest the mechanism of *Salmonella* Enteritidis S1400 inhibition was due to a reduction in pH by the probiotic bacteria. Our current study provides further evidence that probiotics can significantly reduce pathogenic bacterial colonisation in poultry and that mixed preparation of probiotics provide superior performance when compared to individual bacterial preparations.

1. Introduction

Salmonella is a major cause of food poisoning that accounted for an estimated 88,715 confirmed cases in the EU in 2014 (Osimani et al., 2016). Public health concerns over Salmonellosis remain due to several prominent out-breaks, including a reported 250,000 cases in Minnesota in 1994 and a recent hospital outbreak of 287 cases in the UK (Hennessy et al., 1996; Inns et al., 2015). Contaminated eggs and poultry meat are a major source of food poisoning with 46.1% and 6.4% of Salmonellosis being attributed to eggs and broiler meat respectively (Osimani et al., 2016). Since the introduction of EU legislation, member states have targeted the reduction of *Salmonella* in poultry. However, there was a considerable increase (15.3%) in reported *Salmonella* cases in the EU between 2013 and 2014 (EFSA and ECDC, 2015) despite regular use of vaccines in the layer sector and improved barrier security in the broiler meat sector.

Growth promoting antibiotics have been used previously to increase bird weight gain and led to a passive control strategy for *Salmonella* species (de Oliveira et al., 2004). However, with the increasing emergence of antimicrobial resistance, withdrawal of antibiotics in animal feed came into force in 2006 (European-Commission, 1998). Probiotics and prebiotics remain an appealing alternative control measure due to the potential competitive exclusion (CE) of pathogens, improved feed conversion rates and relatively low additional cost to production (Carter et al., 2009). Performance of probiotic preparations varies and there is a continuing need for product development and safety evaluation. Since 1972 several successful undefined *Salmonella* Entertitdis CE products have been developed including Aviguard and BROILACT (Nurmi and Rantala, 1973; Nuotio et al., 1992; Nakamura et al.,

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2002). Concerns with safety and the spread of antibiotic resistance has led to the development of defined preparations such as the FM-B11 (Higgins et al., 2008; Vicente et al., 2008; Prado-Rebolledo et al., 2016). Multi-species and single strain probiotic cultures have been shown to reduce *Salmonella* in poultry although complex cultures are significantly more effective (La Ragione and Woodward, 2003; Timmerman et al., 2004; Chapman et al., 2011). Our work aimed to evaluate the efficacy of novel probiotic preparations to reduce *Salmonella* Enteritidis S1400 colonisation in chickens.

2. Materials and methods

2.1. Bacterial strains, culture and enumeration

Enterococcus faecium PXN-33 and *Lactobacillus salivarius* 59 were kindly supplied by Probiotics International Ltd. *Salmonella* Enteritidis (S1400 Nal^r) was used for *in vivo* model challenges and has been described previously (Clifton-Hadley et al., 2002; La Ragione and Woodward, 2003). *Salmonella* Braenderup H9812 was used as the PFGE standard and was obtained from the Animal and Plant Health Agency (APHA) culture collection. *Escherichia coli* O111 and NM B171, *E. coli* O127:H6 EC2348/69 from the APHA culture collection was used as the controls for adhesion assays. Lactobacilli were grown for 48 h micro-aerophilically using BBL® GasPaks® (Becton and Dickinson™ Oxford, U.K.) on de Man, Rogosa, Sharpe agar (MRS). Enterococci were grown micro-aerophilically on Slanetz and Bartley (SB) agar at 37 °C for 16 h. *S.* Enteritidis was grown for 16 h aerobically on brilliant green agar (BGA). Broth cultures for enterococci, lactobacilli and *Salmonella* were cultured in Heart Infusion Broth (HIB), MRS and Luria-Bertani without glucose (LB-G), respectively with agitation for 16 h at 37 °C, unless stated otherwise in the methods. Prior to experimental dosing of birds, broth cultures were centrifuged at 1700*g* for 10mins at room temperature and adjusted to the appropriate bacterial counts in 0.1 M phosphate-buffered saline (PBS) (pH 7.2).

For culture of probiotic and *S.* Enteritidis S1400 isolates from *in vivo* studies circa 1 g of tissue was added to 9mls 0.1 M PBS (pH 7.2), homogenized using a CAT S620® (SLS) tissue macerator, serially diluted, plated and enumerated after incubation in a 5% CO_2 atmosphere at 37 °C for 24 h: MRS, Slanetz and Bartley (SB) agar and BGA plates (supplemented with 15 μg of nalidixic acid for selection of S1400) were used to culture lactobacilli, Enterococci and *S.* Enteritidis S1400, respectively.

2.2. Probiotic adherence to avian in vitro organ culture (IVOC) gut tissue

IVOC assays were performed as previously described with some modifications (Allen-Vercoe and Woodward, 1999; La Ragione et al., 2000). Day old SPF White Leghorn chicks (SPAFAS®) were killed by cervical dislocation. Approximately 2 cm sections of tissue from the crop, duodenum, jejunum, ileum, caeca and colon, were removed aseptically and placed in pre-warmed (42 °C) sterile Ringer's solution for immediate use. The tissue loops were sliced down the longitudinal axis to expose the epithelial surface. Tissue loops were washed in sterile Ringer's solution twice, placed in new 10 ml of sterile pre-warmed Ringer's solution, inoculated with 100 μl of 5×10^8 cfu/ml of *L. salivarius* 59 or *E. faecium* PXN33 bacteria and incubated aerobically at 42 °C with shaking for 2 h. The tissues were subsequently rinsed in Ringer's solution three times and homogenized and bacterial counts were determined. Assays were performed using three chicks from which two duplicate intestinal sections were aseptically removed and used in the association assays (adhesion and invasion). Experiments were repeated on two separate occasions. The bacteriological procedures were as described above.

2.3. Adherence of probiotic to human cell monolayers

Tissue culture assays were performed essentially as described previously with minor modifications (Dibb-Fuller et al., 1999). Briefly, HEp-2 and CaCo-2 cells were reconstituted in Dulbecco's Modified Eagles Medium D5671 (DMEM) (Sigma) supplemented with foetal calf serum (10% v/v, Autogenbioclear), non-essential amino-acids (1% v/v, Sigma) and gentamicin (50 μg/ml, Sigma) and grown to confluency in 24 well micro-titre plates. HEp-2 and CaCo-2 mono-layers were washed twice in HBSS and inoculated with 5×10^7 CFU/ml *L. salivarius* 59 and *E. faecium* PXN-33 Mono-layers were then incubated at 37 °C supplemented with 5% CO_2 in air for 3 h. The supernatant was removed and the mono-layers were washed (×3) to remove non-adherent bacteria. Mono-layers were disrupted with 1% Triton X-100 (Sigma) and adherent bacteria numbers were determined by plating serial dilutions.

2.4. Scanning electron microscopy of HEp-2 cells

Mono-layers were grown on in 24 well plates and prepared for bacterial adherence as described above. Supernatant was removed from the mono-layers and fixed for 16 h in 3% (v/v) glutaraldehyde in 0.1 M PBS (pH 7.2). Samples were washed in 0.1 M PBS (pH 7.2) and post fixed in 1% (w/v) osmium tetroxide, washed in PBS, dehydrated in ethanol and placed in hexamethyldisizane. Samples were subjected to critical point drying with liquid carbon dioxide. Air dried specimens were fixed to aluminium stubs with silver conductive paint, sputter coated with gold and examined using a Stereo-scan S250 MarkIII SEM at 10–20 KV.

2.5. General in vivo poultry methods

Mixed sex SPF white leghorn chicks were used in all *in vivo* studies. All chicks were hatched and transferred to sterile Wey-isolators maintained under negative pressure (source; APHA, Weybridge). A commercial antibiotic free feed (complete mash diet-chick crumbs; Zootechnical Products) and water were sterilized and made available to the chicks *ad libitum*. All licensed procedures were approved by the local ethics committee and performed under the jurisdiction of project licenses 70/6435 and 70/5282 at the APHA.

2.6. Probiotic feeding trial after S. Enteritidis S1400 oral challenge

The probiotic feeding trial used in this study was performed as described previously with minor modifications (Pascual et al., 1999). One hundred day old chicks were divided randomly into 4 groups of 25 birds housed in Wey-isolators. Probiotic and *S.* Enteritidis S1400 administration was performed by oral gavage in a final volume of 0.1 ml PBS. Four randomized groups of birds were dosed by oral gavage with 1×10^9 cfu of probiotic in 100 μl 0.1 M PBS (pH 7.2) or 100 μl of PBS only for control birds; **Group 1**) PBS by oral gavage, **Group 2**) *E. faecium* PXN-33, **Group 3**) *L. salivarius* 59 or **Group 4**) a 50:50 preparation of both probiotic strains (combined group). At 2 days old all the birds in the 4 groups were dosed with 5×10^4 cfu of *S.* Enteritidis (S1400 Nal^r). To confirm colonisation of the chicks by *S.* Enteritidis S1400 10 birds from each group selected at random were cloacally swabbed at 3, 6, and 8 days of age and plated on BGA (supplemented with nalidixic acid). At 2 days of age and 3 days of age 3 birds were sacrificed to determine probiotic and *S.* Enteritidis S1400 colonisation, respectively. At 3, 6, 24 and 43 days of age 3 birds were killed by cervical dislocation and subjected to *post-mortem* examination. *Circa* one gram of the ileum, caecum and colon were aseptically sampled for bacteriology. Probiotic colonisation was determined at days 24 and 43 days of age by growth on MRS agar or SB agar for lactobacilli and