Bacillus subtilis spores competitively exclude *Escherichia coli* O78:K80 in poultry

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Abstract

Newly hatched specific pathogen-free chicks were dosed with a suspension of *Bacillus subtilis* spores prior to challenge with *Escherichia coli* O78:K80, a known virulent strain associated with avian colibacillosis, 24 h later. A single oral inoculum of 2.5×10^8 spores was sufficient to suppress all aspects of *E. coli* O78:K80 infection. Colonisation of deep organs was reduced by a factor of over $2 \log_{10}$ whilst colonisation of the intestine, as measured by direct caecal count, was reduced over $3 \log_{10}$. Shedding of *E. coli* O78:K80 was measured by semi-quantitative cloacal swabbing and was reduced significantly for the duration of the experiment, 35 days. *B. subtilis* persisted in the intestine although with decreasing numbers over the same period. Challenge with the same dose 5 days after pre-dosing with spores overcame any suppressive effect of the spores. Crown Copyright © 2001 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Protection of human health is now a primary concern for food producers with the elimination of food borne pathogens from food animals and their products. In the poultry industry, interventions to reduce the burden of zoonotic pathogens include improved hygienic methods, vaccination and competitive exclusion. Each of these approaches is well established particularly in the developed world. The concept of competitive

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exclusion is well established with many commercially available products on the market (Collins and Gibson, 1999; Rowland, 1999). Many of these products are complex mixtures of bacteria derived from the gut of healthy birds and are given on hatching in order to establish a mature gut flora early in life. Chicks are immunologically naive (Jeurissen et al., 1989) and prone to rapid and persistent colonisation by both commensal and pathogenic bacteria in the first 3–4 weeks of life (Barrow et al., 1988). Recent studies by Holt et al. (1999) indicated that the avian immune system may be compromised by vaccination at day old and that an optimum time for delivery of vaccines was about 4 weeks of age. Up to that age, birds remain vulnerable to infections although maternally derived immunity may reduce colonisation by pathogens but this is unlikely to be fully protective and re-enforces the view that combined strategies to prevent infections are required.

In this laboratory, we are interested in the initial interaction between pathogenic bacteria and the intestinal epithelium of the host with the particular aim of devising strategies to prevent colonisation. Recent studies proved conclusively the essential role of flagella and type 1 and curli fimbriae for persistent gut colonisation by *E. coli* serotype O78:K80 (La Ragione et al., 1999, 2000a,b), one of the prevalent causes of avian colibacillosis. Interestingly, although *Salmonella enterica* serotype Enteritidis possesses orthologous structures and at least three other fimbriae as well, these surface structures played only minor roles in persistent gut colonisation (Allen-Vercoe and Woodward, 1999a,b; Allen-Vercoe et al., 1999; Dibb-Fuller et al., 1999). Other surface antigens such as LPS play a significant role in persistence (Turner et al., 1998). Vaccination of birds with either killed or rationally attenuated vaccines may reduce the systemic phase of infection and develop sIgA for mucosal protection. However, vaccines do not eliminate initial colonisation of the mucosal surfaces, particularly in the young bird (Dougan et al., 1988; Barrow et al., 1990; Barrow, 1996; Tims et al., 1990; Cooper et al., 1992, 1993, 1994a,b; Chatfield et al., 1993; Gast et al., 1992). The priority remains the development of strategies to eliminate this.

Milner and Schaffer (1952) observed that suppression of Salmonella infection in young birds corresponded to increasing age. Nurmi and Rantala (1973) formalised these observations by proposing the concept of "competitive exclusion" which has now been studied extensively for the past two and half decades. However, the only universally accepted fact concerning the mechanism of competitive exclusion is that protection depends upon oral administration of viable bacteria, especially anaerobes (Schneitz and Mead, 2000). With regard to the colonisation of the chick gut by Enterobacteriaceae, undefined complex cultures were more effective than defined probiotic treatments (Stavric, 1992) and the most effective exclusion agents were derived direct from chicken intestines (Cameron and Carter, 1992; Spencer et al., 1998). However, one of the current problems associated with complex competitive exclusion agents is the lack of definition both in terms of bacterial flora and of the effect mediated. Recent studies have demonstrated that *Bacillus* spp. (Kyriakis et al., 1999) and *B. subtilis* spores (Mazza, 1994) may be successful competitive exclusion agents. A spore monoculture has the advantage of being readily produced, having a long storage life and, in the case of *B. subtilis*, of being avirulent. Therefore, *B. subtilis* may be suitable for use in food producing animals if it is shown to be efficacious.

We wished to study the use of competitive exclusion to complement the use of vaccines. Also, we wished to place the emphasis upon the use of a defined monoculture that might permit the study of the underlying biological principles of exclusion. Here we report our findings on the effect of *B. subtilis* spores on the colonisation of the chick by the avian pathogen *E. coli* O78:K80, for which a well characterised model had been developed in this laboratory (La Ragione et al., 1999, 2000a,b).

2. Materials and methods

2.1. Bacteria and media

The nalidixic acid resistant derivative of an *E. coli* O78:K80 wild type (O78:K80 *nal*[r]) used in these studies was described previously (La Ragione et al., 1999). The *B. subtilis* strain used in this work was an isogenic derivative of strain PY79 (Youngman et al., 1984) containing a chromosomally inserted *cat* gene, encoding resistance to chloramphenicol (5 µg ml^{-1}). A derivative resistant to 12.5 µg ml^{-1} chloramphenicol (*B. subtilis* PY79[hr]) was prepared by streaking and restreaking on Nutrient agar (NA) plates supplemented with increasing concentrations of chloramphenicol. There was no discernible difference in growth rate in Nutrient broth (NB) between either PY79 or PY79[hr]. *E. coli* O78:K80 *nal*[r] was stored frozen at $-80°$C in heart infusion broth supplemented with glycerol (30%, w/v) and working cultures were grown on Luria Bertani agar and stored at 4°C. *B. subtilis* PY79[hr] was stored as spore suspensions (Youngman et al., 1984) and spore suspensions were used directly as inocula for chick experiments. *E. coli* inocula for chick experiments were grown overnight in static NB culture at 37°C. For selection of bacteria from chick experiments, samples were plated on NA supplemented with nalidixic acid (15 µg ml^{-1}) or chloramphenicol (10 µg ml^{-1}) as required.

2.2. E. coli O78:K80 chick model

Chickens. Newly hatched chickens were obtained from a specific pathogen-free white leghorn flock. Chicks were housed on wood shavings and fed standard rations and tap water ad libitum. Chicks were observed regularly and weighed. All animal experiments followed Home Office approved procedures.

Dosage regimes: *Experiment 1*. One group of 30 birds was dosed by intra-gastric intubation as described previously (La Ragione et al., 2000a) within 18 h of hatching with 2.5×10^8 *B. subtilis* PY79[hr] spore units suspended in 0.1 ml sterile water. This and a control group of 30 birds that had not received spores were dosed by intra-gastric intubation at 36 h of age with 10^5 cfu *E. coli* O78:K80 *nal*[r] suspended in 0.1 ml PBS. At 24 and 48 h post-inoculation with *E. coli* O78:K80, five birds from both groups selected at random were killed and bacteriological analysis of tissue samples done. This experiment was performed three times and the results combined for statistical analysis.

Dosage regimes: *Experiment 2*. One group of 30 birds was dosed orally within 18 h of hatching with 2.5×10^8 *B. subtilis* PY79[hr] spore units as above. This and a control group

of 30 birds that had not received spores were dosed orally at 144 h of age with 10^5 cfu *E. coli* O78:K80 *nal*[r] suspended in 0.1 ml PBS. At 24 and 48 h post-inoculation with *E. coli* O78:K80, five birds from both groups selected at random were killed and bacteriological analysis of tissue samples done. This experiment was performed once.

2.3. Enumeration of E. coli in organ homogenates

Animals were killed by cervical dislocation and liver, spleen and caeca were removed surgically to sterile 1 oz MacCartney glass bottles. Livers and spleens were weighed. Each organ was homogenised in PBS (10 ml) and the viable count in homogenates was determined by plating dilutions made in PBS (pH 7.2) on NA supplemented with nalidixic acid. The limit of detection was 200 cfu. Residual homogenate was enriched by addition to 200 ml NB, incubated for 24 h at 37°C and subcultured on NA supplemented with nalidixic acid.

2.4. Enumeration of E. coli and B. subtilis by cloacal swabbing

The semi-quantitative methods of Smith and Tucker (1975, 1980) were used. Duplicate cloacal swabs were taken at regular intervals from 24 h after challenge with *E. coli* O78:K80. One swab was spread directly on NA plates supplemented with antibiotics as required which were incubated at 37°C overnight for *E. coli* and for 3 days for *B. subtilis*. The other swab was immersed in NB for enrichment by growth at 37°C for 24 h prior to plating as before. Results were expressed as heavy (confluent growth), medium (>200 cfu direct plating), or light (<200 cfu direct plating).

2.5. Statistical analysis

For statistical analysis of invasion, colonisation and persistence of *E. coli* O78:K80 *nal*[r], Experiment 1 was repeated twice and total counts for specific organs calculated per tissue per time and data combined. The number of chicks colonised was assumed to follow binomial distribution and differences between mutants and wild type was compared over time for liver and spleen using a generalised linear model. For statistical analysis of faecal shedding, levels of shedding were given numerical scores (heavy: 3, medium: 2, light: 1) to allow use of analyses of variance techniques. Comparisons were made between levels of shedding separately at each time point and for each bacterium. The probabilities were calculated using the StatXact software program (CYTEL Software, MA, USA).

3. Results

The aim of the first experiment was to test whether pre-dosing newly hatched chicks with *B. subtilis* PY79[hr] spores suppressed colonisation and invasion by *E. coli* O78:K80 upon subsequent challenge. To do this, hatchlings up to 18 h of age received 2.5×10^8 spores by intra-gastric intubation prior to oral dosing with 10^5 cfu of *E. coli* O70:K80 *nal*[r]

24 h later, at 36 h of age. A control group of hatchlings was dosed with the *E. coli* alone at 36 h of age. This experiment was performed on three occasions, each with very similar findings, and the data were summated for statistical analysis.

The numbers of *E. coli* O78:K80 *nal*[r] recovered from the liver, spleen and caeca of chicks 24 and 48 h after challenge are shown in Fig. 1. Chicks that had received a pre-dose of *B. subtilis* PY79[hr] spores showed significantly lower recovery of *E. coli* ($p < 0.01$) for all organs at each time point.

Shedding of *E. coli* O78:K80 *nal*[r] was monitored by cloacal swabbing at weekly intervals for 7 weeks and the data are shown graphically in Fig. 2. Of the birds pre-dosed with *B. subtilis* PY79[hr], 85% were colonised of which only 12.5% showed heavy shedding 1 day after challenge (Fig. 2a). Interestingly, for the duration of the experiment, about 15–20% of birds failed to shed detectable levels *E. coli* O78:K80 *nal*[r] whilst the remainder shed at medium to low levels. By the end of the first week, 100% of birds that did not receive a *B. subtilis* PY79[hr] pre-dose were colonised with 65% showing heavy shedding 1 day after challenge (Fig. 2b). All birds in this group continued to shed *E. coli* O78:K80 *nal*[r] for the duration of the experiment but the number of birds shedding heavily declined steadily over 7 weeks. For the pre-dosed birds, swabs were also plated onto media selecting for the *B. subtilis* PY79[hr] in order to test whether this obligate aerobe persisted in the chicken. Surprisingly, *B. subtilis* PY79[hr] was recovered from the chickens for the duration of the experiment, albeit in declining numbers over the 7-week period (Fig. 2c). The numbers of *E. coli* O78:K80 *nal*[r] recovered from the caeca of chickens from both *B. subtilis* PY79[hr] pre-dosed and control groups were determined at the end of the experiment. The mean number of *E. coli* O78:K80 *nal*[r] recovered from birds pre-dosed with *B. subtilis* PY79[hr] was 3.1×10^4 cfu/g (S.D. 3.4×10^3) caecal material and 3.2×10^7 cfu/g (S.D. 4.46×10^6) caecal material from control birds ($p = 0.001$).

To test whether the effect of spores was lasting, a new experiment was done but the *E. coli* O78:K80 *nal*[r] challenge of 10^5 cfu was given 6 days after the pre-dose with *B. subtilis* PY79[hr] spores. No statistically significant differences between numbers of bacteria in liver, spleen and caeca at 24 h or in caeca at 35 days were observed (data not shown). The shedding of *E. coli* O78:K80 from these birds was monitored also. All birds from both control and pre-dosed groups were colonised. However, suppression of shedding by pre-dosing was noted for up to 7 days ($p < 0.05$) only with no statistically significant differences thereafter.

4. Discussion

B. subtilis PY79[hr] spores given to day old chicks 24 h prior to challenge resulted in a significant reduction of colonisation by *E. coli* O78:K80 *nal*[r] of the caecum and the deep tissues. The extent of colonisation by *E. coli* in the controls was similar to those described previously (La Ragione et al., 2000a,b) which suggested that this model was appropriate for these studies. In the experiments described here, 2.5×10^8 spores given to hatchlings as a single oral inoculum was sufficient to suppress all aspects of *E. coli* colonisation. Initial invasion and colonisation of deep organs was reduced by a factor of over $2 \log_{10}$ whilst colonisation of the intestine, as measured by semi-quantitative cloacal swabbing
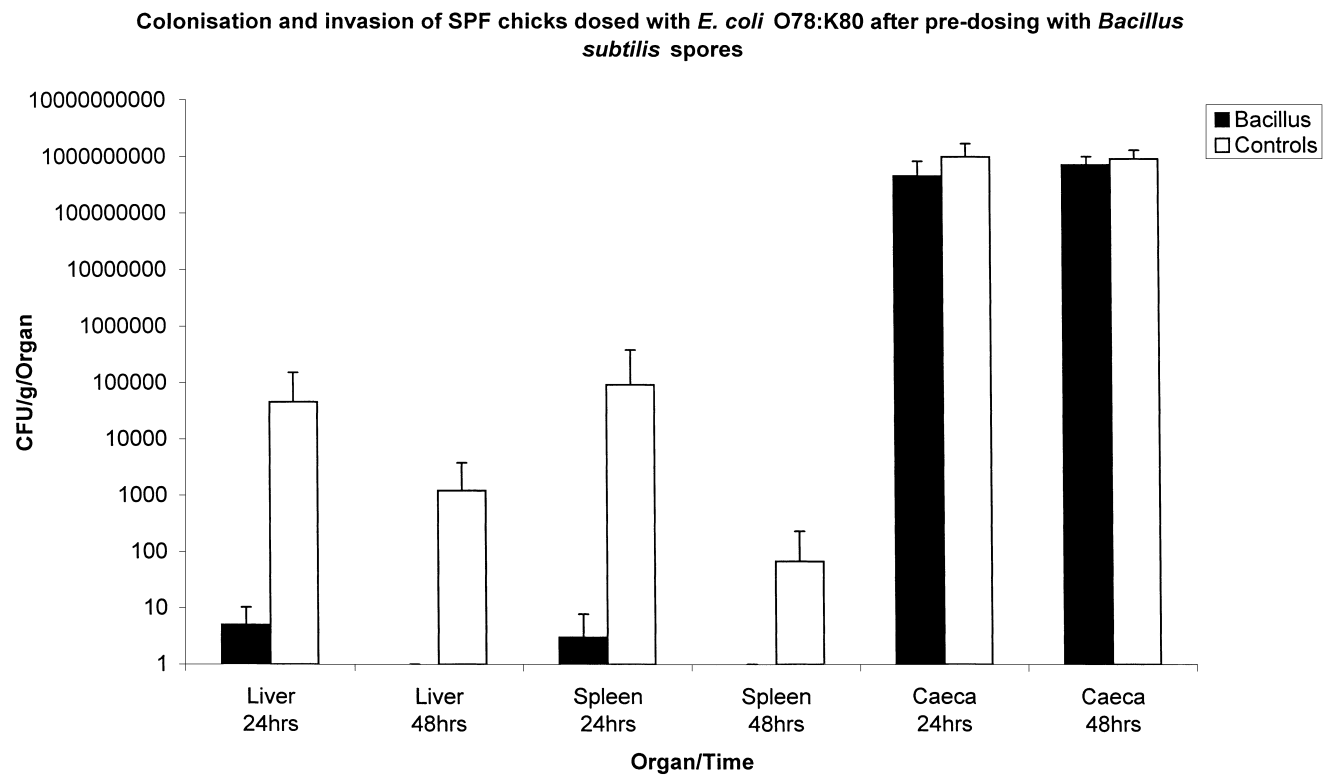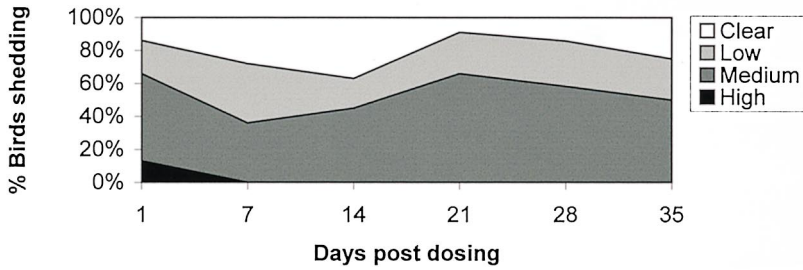
Colonisation and invasion of SPF chicks dosed with *E. coli* O78:K80 after pre-dosing with *Bacillus subtilis* spores

Fig. 1. Numbers of *E. coli* O78:K80 recovered from liver, spleen and caeca of SPF chicks with and without pre-dosing with *B. subtilis* spores.

**Persistence of *E. coli* O78:K80 in SPF chicks after dosing at
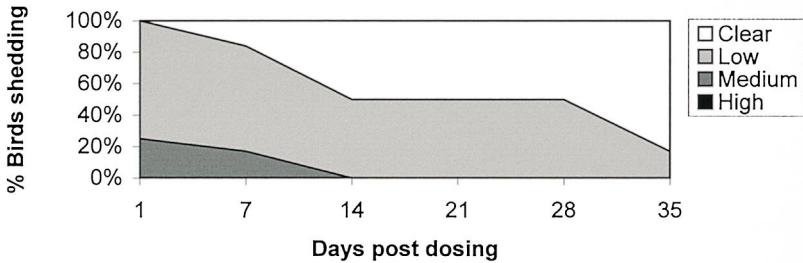day old with *Bacillus subtilis***

(a)

Persistence of *E. coli* O78:K80 in SPF chicks (control)
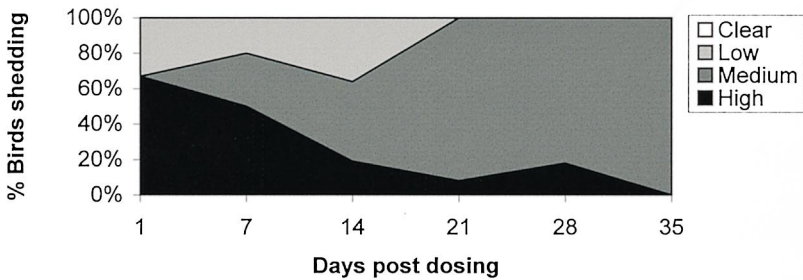
(b)

**Persistence of *Bacillus Subtilis* in SPF chicks challenged with
E. coli O78:K80 (1 day model)**

(c)

Fig. 2. Graphs to show shedding of *E. coli* O78:K80 *nal*[r] and *B. subtilis* from chicks dosed with *E. coli* alone (b) and both *E. coli* and *B. subtilis* (a) and (c).

and caecal counts at the end of the experiment, was reduced over $3 \log_{10}$. These changes are dramatic given that recent studies have shown that some intestinal population changes induced by probiotics may only be detected by sophisticated molecular methods (Netherwood et al., 1999).

A number of key issues arise from these observations. Firstly, the effect was immediate as evidenced by reduction of invasion that is known to occur within 24 h in the model used (La Ragione et al., 2000a,b). *E. coli* associated with colibacillosis are serum resistant and, therefore, the low numbers of *E. coli* O78:K80 *nal*[r] recovered from the internal organs suggested inhibition of invasion. Secondly, the initial suppressive effect was lasting with shedding levels significantly lower for the duration of the experiment, 35 days. Indeed some birds did not shed at all, implying freedom from *E. coli* O78:K80 *nal*[r]. However, shedding may be intermittent and swabbing is only an approximate method for detection. Importantly, up to 15% birds appeared to be resistant to re-infection from the environment into which other colonised birds were shedding. Thirdly, spores remained resident in the chick for the duration of the experiment although in decreasing numbers over time. It would be of interest to determine accurate numerical values of *B. subtilis* as this may give clues as to their status either remaining as spores or germinating and replicating in an anaerobic environment. The decline in *B. subtilis* may be due to mechanical removal by peristalsis or turnover of receptor sites. Fourthly, although the initial protective effect did persist, challenge 6 days after pre-dose overcame any suppressive effect. It was noteworthy that a dose of 10^5 cfu given at day 6 to chicks resulted in colonisation of the gut with O78:K80 *nal*[r] even in the presence of a developing flora. This indicated that the O78:K80 strain used in these studies was an effective coloniser. Taken collectively, the data are sufficiently promising to investigate giving spores in drinking water, e.g., over an extended period, possibly until immediately prior to vaccination at 4 weeks of age as suggested by Holt et al. (1999). Whether it is feasible to deliver sufficient numbers spores to chicks in the commercial poultry industrial environment requires examination. However, future experiments with Salmonella serotypes and other intestine associated pathogens of avian species are worthy of consideration.

Competitive exclusion agents are considered to exert their effect by one or more of four general principle actions namely the creation of a restrictive physiological environment, competition for bacterial receptor sites, elaboration of antibiotic like substances and/or depletion of essential substrates (Schneitz and Mead, 2000). Further investigation is required to establish the mechanism of the protective effect of spores, especially as spores are considered metabolically inert. It is possible that spores blocked adherence sites whereas suppression by physiological factors may only be invoked if germination occurred. Given an appropriate nutrient source and terminal electron acceptor such as nitrate, *B. subtilis* is capable of germination and growth in an anaerobic environment (Priest, 1994). Thus, *B. subtilis* may also exert metabolic effects upon *E. coli* in the chick gut but, as an obligate aerobe, germination may lead to death or at best limited survival of the bacterium. The decline in *B. subtilis* in the gut over time was consistent with this.

An attractive hypothesis is that spores may stimulate macrophage infiltration and thereby modulate the immune responses in the naive bird. It is known that naive 1-day old chicks dosed orally with as few as 10^2 cfu of *E. coli* O78:K80 are readily colonised within 24 h with numbers reaching 10^8 cfu/g of whole caecum and 10^3 cfu in the liver and spleen (La Ragione et al., 2000b). That spores caused a highly significant reduction in invasion may indicate that uptake sites were blocked and/or phagocytic cells were primed to engulf and kill invading *E. coli* O78:K80. Additionally, some birds dosed with spores

remained free from *E. coli* even when maintained in an environment likely to enhance natural environmental challenge. This may indicate that an immune response may be an important lasting component of the protective effects observed.

Acknowledgements

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