In vivo broiler experiments to assess anti-Campylobacter jejuni activity of a live Enterococcus faecalis strain

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ABSTRACT Bacterial gastroenteritis caused by thermotolerant Campylobacter species, mainly Campylobacter jejuni, has been the most reported zoonotic disease in many developed countries in recent years. Reducing Campylobacter shedding on the farm could result in a reduction of the number of campylobacteriosis cases. In 2 independent broiler seeder experiments, in which broiler chickens were orally inoculated with 2 amounts of Enterococcus faecalis MB 5259, we established whether a live E. faecalis strain was capable of reducing cecal Campylobacter colonization in broiler chickens. In previous in vitro experiments it has been demonstrated that this E. faecalis MB 5259 displays anti-Campylobacter activity. The effect of pH and bile salts on E. faecalis MB 5259 showed that growth and survival of E. faecalis MB 5259 can be impaired during passage through the gastrointestinal tract of broiler chickens. Despite these results E. faecalis MB 5259 was capable of colonizing the broiler ceca. Contrary to the in vitro experiments, in which E. faecalis MB 5259 inhibited C. jejuni MB 4185 growth, no inhibition was observed in the in vivo experiments independent of the inoculum size.

Key words: live probiotic, Campylobacter jejuni, colonization, Enterococcus faecalis, broiler chicken

INTRODUCTION

From 2005 onward, the most reported zoonotic disease in many developed countries has been bacterial gastroenteritis. A total of 198,252 confirmed human cases in the European Union (EU) in 2009 have been caused by various thermotolerant Campylobacter species, mainly C. jejuni (EFSA, 2010b, 2011). Campylobacter jejuni is widespread in the environment with the alimentary tracts of birds and mammals as their principal reservoirs. Because broiler ceca can be colonized to a high degree and can carry a high C. jejuni load up until slaughter, broiler carcasses and derived food products are an important source of human Campylobacter infection. This high C. jejuni load can increase the chance of contamination of meat products (Rasschaert et al., 2006; Rosenquist et al., 2006). Consumption of improperly prepared contaminated poultry products is one of the major causes of campylobacteriosis in humans (Friedman et al., 2004). The European Food Safety Authority (EFSA) reported that, in 2008, an average of 76% of broiler carcasses in EU member states were contaminated with Campylobacter species (EFSA, 2010c). The proportion of Campylobacter positive fresh broiler meat samples (at slaughter, processing, or retail) amounted to approximately 31% in the EU for 2008 and 2009 (EFSA, 2011). One quantitative risk assessment model indicated that reducing Campylobacter shedding at farm level by 1, 2, or 3 log units could result in a 55, 84, or 94% reduction in the number of campylobacteriosis cases, respectively (Messens et al., 2007). Another risk assessment (Rosenquist et al., 2003) stated that a 100-fold reduction of the Campylobacter numbers on broiler chicken carcasses could lower the number of human campylobacteriosis cases 30-fold.

Even though most Campylobacter infections are mild, self-limiting, and usually resolve within a few days without antibiotic treatment, severe or prolonged infections such as Guillain–Barré syndrome, reactive arthritis, irritable bowel disease, and inflammatory bowel disease can occur (Havelaar et al., 2000; Gradel et al., 2009). These sequelae develop particularly in the young, elderly, pregnant, and immune-compromised individuals, in which case therapeutic intervention is usually needed (Allos, 2001; Blaser and Engberg, 2008; EFSA, 2010a).

Except for adequate biosecurity measures (Lin, 2009; Hermans et al., 2011b), which are important to reduce environmental exposure, no other measures for on-farm control of Campylobacter are commercially available.
Measures under study usually strive to increase poultry’s host resistance to *Campylobacter* and consequently reduce carriage in the gut. These measures include the use of competitive exclusion (Zhang et al., 2007), vaccination (de Zoete et al., 2007), medium-chain fatty acids (Hermans et al., 2012), or antimicrobial compounds such as bacteriocins (Line et al., 2008). In the literature, at least 2 *Enterococcus* species, *E. faecalis* (Nazef et al., 2008) and *E. faecium* (Svetoch et al., 2008), have been shown to inhibit *C. jejuni* in vitro by bacteriocin production, although only Svetoch et al. (2008) reported an in vivo influence on *C. jejuni* in therapeutic broiler trials when applying purified bacteriocin.

The aim of this study was to establish whether a live *E. faecalis* strain with demonstrated in vitro anti-*Campylobacter* activity (Robyn et al., 2012) is capable of reducing cecal *Campylobacter* colonization in broiler chickens. This was examined in 2 independent broiler seeder experiments, in which broiler chickens were orally inoculated with 2 amounts of *E. faecalis* MB 5259.

**MATERIALS AND METHODS**

*Effect of pH and Bile Salts on Survival/Growth of E. faecalis MB 5259*

The *E. faecalis* MB 5259 strain is of dairy origin; thus, we tested the probability of survival as well as growth of *E. faecalis* MB 5259 during passage through the broiler gastrointestinal tract using an in vitro experiment. Conditions of the general animal gastrointestinal tract were simulated (Van Coillie et al., 2007) for this experiment. The simulation consisted of inoculating $5 \times 10^6$ colony-forming units (cfu)/mL of the *E. faecalis* MB 5259 strain, anaerobically grown overnight at 37°C in de Man, Rogosa, and Sharpe (MRS) broth (CM0359, Oxoid, Basingstoke, UK), into MRS broth containing 0.5% bile salts extracted from purified fresh bile (BS: B3301, Sigma Aldrich, St. Louis, MO), or in MRS broth containing 0.5% bile salts from bovine and ovine origin (BO: B-8381, Sigma Aldrich) or in MRS broth at pH 3. These were subsequently incubated anaerobically at 37°C for 24 to 48 h.

To test for survival of *E. faecalis* MB 5259, samples were taken from all 3 volumes after 4 h of incubation. Ten-fold dilutions of these samples in Ringer’s solution were plated on MRS agar and anaerobically incubated at 37°C for 24 h. To test for growth of *E. faecalis* MB 5259, the OD$_{610nm}$ was measured after 0 and 24 h of incubation using a Genesys 10 Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

**Experimental Birds**

Day-of-hatch Ross broiler chickens of both sexes purchased at a local farm were raised in 6 groups in the experimental facility of the Faculty of Veterinary Medicine of Ghent University until the age of 21 d. Chickens were provided with feed and water ad libitum. Hus-
**Oral Inoculation of Chicks with E. faecalis MB 5259**

Day-of-hatch broiler chicks ($n_1 \sim 70$, $n_2 \sim 66$) were randomly divided into 6 separately housed groups. In both experiments, birds of 3 groups were inoculated orally on a daily basis with 0.5 mL of Ringer’s solution starting from d 1. These birds served as the control. Chicks of the other 3 groups were inoculated orally on a daily basis, at the same time and starting at the same age, with 0.5 mL of Ringer’s solution containing approximately $1 \times 10^4$ (first experiment) or $1 \times 10^8$ (second experiment) cfu of the *E. faecalis* MB 5259 strain until the end of the experiment. At the age of 15 d, 2 chicks of each group were orally inoculated with 0.5 mL of approximately $2.0 \times 10^4$ cfu of *C. jejuni* strain MB 4185. After inoculation, these birds were immediately placed back into their respective groups to spread *C. jejuni* to the other animals of the same group (seeder model; Van Deun et al., 2008a; Hermans et al., 2011a). At 21 d of age, all chicks were euthanized by injection of T61 (Intervet, Brussels, Belgium) in the vein wing (500 μL per chick), and the ceca were aseptically collected for *C. jejuni* enumeration (see below).

**Cecal C. jejuni MB 4185 and E. faecalis MB 5259 Enumeration**

Ceca were weighed and diluted 1:9 wt/vol in NB2 with modified Preston *Campylobacter* selective supplement and *Campylobacter*-specific growth supplement for *C. jejuni* MB 4185 enumeration and MRS broth containing 20 μg/mL of rifampicin for *E. faecalis* MB 5259 enumeration. After homogenization, a 10-fold dilution series ($10^{-1}$ to $10^{-6}$) was made in Ringer’s solution. Of each dilution, 100 μL was spread on mCCDA plates for enumeration of *C. jejuni* and on MRS agar containing 20 μg/mL of rifampicin for enumeration of *E. faecalis*. After 24 to 48 h of incubation at 41.5°C under microaerobic conditions for *C. jejuni* and 24 h at 37°C under anaerobic conditions for *E. faecalis*, colonies showing the morphology of *E. faecalis* MB 5259 or *C. jejuni* were counted. For each group of chickens, DNA of colonies selected from these selective MRS plates was isolated (Flamm et al., 1984). This DNA was subsequently used in a (GTG)$_5$ PCR reaction (Svec et al., 2005) to confirm these colonies as *E. faecalis* MB5259.

**Statistical Analyses**

Data were analyzed using Statistica (Statsoft, Tulsa, OK) software. The significance level was set at 0.05. *Campylobacter* counts were log-transformed before statistical analysis. A 1-way ANOVA was carried out to compare the means of the log-transformed numbers in chicken cecal contents of all groups (treated and control groups) of the seeder model and to compare the means of the log-transformed numbers found after exposure to acidic environment and to the different types of bile salts. Significant differences were assessed by Fisher post hoc tests.

**RESULTS**

**Effect of pH and Bile Salts on Survival/Growth of E. faecalis MB 5259**

Survival of *E. faecalis* MB 5259 is somewhat impaired under acidic circumstances and in the presence of bile salts from a nonovine and nonbovine source as shown in Figure 1A. Compared with the initial inoculum of *E. faecalis* MB 5259, numbers were reduced 1.3 and 0.9 log$_{10}$ after 4 h of anaerobic incubation in MRS broth containing BS or in MRS broth at pH = 3, respectively. Reduction in MRS broth at pH = 3 was statistically significant. In contrast, numbers of *E. faecalis* MB 5259 in standard MRS broth, acting as control, or in MRS broth containing BO were respectively 2.7 and 2.1 log$_{10}$ greater than the initial inoculum after 4 h of anaerobic incubation, which was a statistically significant difference. Additionally, after 4 h of anaerobic incubation, differences in *E. faecalis* MB 5259 numbers in standard MRS broth and MRS broth containing BO were significantly greater than *E. faecalis* MB 5259 numbers in MRS broth containing BS and in MRS broth at pH = 3. These results indicated there was a net loss of 3 log in *E. faecalis* numbers as *E. faecalis* grew 2 log under noninhibitory circumstances and lost 1 log under inhibitory circumstances.

*Enterococcus faecalis* MB 5259 did not grow at pH = 3 or in the presence of BS (Figure 1B), as the optical density (OD$_{610 \, \text{nm}}$) value measured after 24 h of anaerobic incubation at both circumstances was the same as the initial OD$_{610 \, \text{nm}}$ value. The OD$_{610 \, \text{nm}}$ value measured in standard MRS broth or in MRS broth containing BO, on the other hand, was 20 times greater than the initial OD$_{610 \, \text{nm}}$ value, which was significantly different. Additionally, after 24 h of anaerobic incubation, OD$_{610 \, \text{nm}}$ values in standard MRS broth or in MRS broth containing BO were significantly greater than OD$_{610 \, \text{nm}}$ values in MRS broth at pH = 3 or in MRS broth containing BS.

**In Vivo Trials with E. faecalis MB 5259**

The 3 groups of 21-d-old broilers in the seeder model that had been orally inoculated on a daily basis with either $1 \times 10^4$ or $1 \times 10^8$ cfu of the *E. faecalis* MB 5259 strain did not have significantly lower ($P > 0.05$) cecal *Campylobacter* MB 4185 counts compared with the control groups of both experiments. In the first in vivo experiment, in which broiler chickens were orally inoculated with $1 \times 10^4$ cfu of the *E. faecalis* MB 5259 strain, the log$_{10}$ value of the number of *C. jejuni* colonies found in the ceca amounted to 8.03, 7.81, and 8.01 in the control groups and 8.23, 7.41, and 8.23 in the experimental groups (Figure 2A). In the second in vivo experiment, in which broiler chickens were orally inoculated with $1 \times 10^8$ cfu of the *E. faecalis* MB 5259 strain, the log$_{10}$ value of the number of *C. jejuni* colonies found in the ceca amounted to 7.69, 7.46, and 7.81 in the control
groups and 7.70, 7.59, and 7.90 in the experimental groups (Figure 2B). For both experiments, there was no statistically significant difference in the number of \textit{C. jejuni} MB 4185 cfu/g of cecal contents found in seeder animals compared with nonseeder animals.

For chickens inoculated with $1 \times 10^4$ cfu of the \textit{E. faecalis} MB 5259 strain, feed uptake and average weight per group was not significantly different between the groups (data not shown). In the second experiment, one group of chickens inoculated with $1 \times 10^8$ cfu of the \textit{E. faecalis} MB 5259 strain had an average weight significantly lower than the average weight of one control group and one other group inoculated with strain MB 5259 (Figure 3).

The mean cecal \textit{E. faecalis} MB 5259 numbers (in log$_{10}$ cfu/g of cecal content) detected after the second experiment (inoculation with $1 \times 10^8$ cfu of the \textit{E. faecalis} MB 5259) on MRS agar plates containing 20 μg/mL of rifampicin amounted to 5.37, 5.52, and 5.05 for the 3 groups of broilers, respectively. They were not significantly different ($P > 0.05$). All mean cecal \textit{E. faecalis} MB 5259 numbers from the first in vivo experiment were significantly different from all mean cecal \textit{E. faecalis} MB 5259 numbers from the second in vivo experiment.

**DISCUSSION**

Different studies revealed that using competitive exclusion organisms could reduce the \textit{Campylobacter} load in chickens (Soerjadi et al., 1982; Soerjadi-Liem et al., 1984; Aho et al., 1992; Schoeni and Doyle, 1992). The problem with the use of undefined bacterial mixtures is that the antagonistic activities of the supplied bacteria were not well understood and presented a potential risk of introducing avian or human pathogens into the food chain (Stavric, 1992). In our study, a defined
E. faecalis MB 5259 strain with proven in vitro anti-Campylobacter activity (Robyn et al., 2012) was tested in 2 in vivo experiments to specify results in live broilers. To our knowledge, no in vivo experiments using live E. faecalis strains to inhibit cecal Campylobacter colonization have been done before. Administration of probiotic bacteria (Willis and Reid, 2008; Vandeplas et al., 2009; Higgins et al., 2010) or purified antimicrobial components (Line et al., 2008) to broilers by feed or drinking water could prevent colonization of chickens by human pathogenic bacteria such as Campylobacter.

To effectively influence Campylobacter, these probiotics or antimicrobial components must reach the broiler intestinal site colonized by the targeted human pathogenic bacteria (i.e., the cecal environment for C. jejuni). This implies that they have to survive passage through the digestive tract. When these antimicrobial products are used to prevent colonization, they might also work proximal to the intestinal site normally colonized by the targeted human pathogenic bacteria.

As evidenced by the effect of pH and bile salts on survival/growth of the E. faecalis MB 5259 strain in an in vitro experiment, growth and survival of E. faecalis MB 5259 during passage through the gastrointestinal tract of broiler chickens will be somewhat impaired. Nonetheless, the in vitro experiment also illustrates the acidic pH and bile salts will probably not kill all E. faecalis MB 5259 cells, even after 24 h of incubation at pH 3 or in the presence of bile salts. Because a total transit through the broiler gastrointestinal tract takes around 4 to 9 h, depending on the feed and age of the broilers (Sundu, 2009; Sedghi et al., 2010), the results mentioned above indicate that E. faecalis MB 5259 might be capable of surviving the transit and may reach the broiler cecal environment alive. Theoretically, this allows the E. faecalis MB 5259 strain to colonize the ceca, given it is capable of adapting itself to survival in that specific environment.

In the in vivo experiments cecal colonization levels were lower than or equal to the initial E. faecalis MB 5259 inoculum. When inoculation was high (1 × 10^8 cfu), cecal inoculation levels were 3 to 2.5 log_{10} lower than the initial inoculum. But when the initial inoculation level was low (1 × 10^4 cfu), the number of E. faecalis MB 5259 found to colonize the cecal environment were only 0.31 to 0.01 log_{10} lower. Because this cannot solely be explained based on growth and survival impairment by bile salts and acidic pH, supplementary influences may be acting on the cecal colonization of...
E. faecalis MB 5259. Low colonization levels can be a result of the dairy origin of the E. faecalis MB 5259 strain, which could prevent it from fully colonizing the cecal environment, or competitive exclusion by other cecal microorganisms may be responsible. Strain fitness might also be impaired due to rifampicin resistance mutations in the E. faecalis MB 5259 strain, which would lead to lower cecal colonization, although no growth impairment in in vitro experiments was identified.

A different reason for the low number of E. faecalis MB 5259 identified in the broiler ceca might be underreporting of the respective strain due to loss of rifampicin resistance of some isolates during the in vivo experiment (Pascual et al., 1999).

Contrary to the previous in vitro experiments, in which E. faecalis MB 5259 inhibited C. jejuni MB 4185 growth by 1 to 2 log, no evidence for similar inhibition was identified in the in vivo experiments. Independent of the inoculum size, there was no statistically significant difference in cecal numbers of C. jejuni in control broilers or experimental animals. A statistically significant difference in weight between one group of broilers receiving a daily inoculum of 1 × 10^8 cfu E. faecalis MB 5259 and all other groups was seen, but no reason for this effect could be identified.

Some reports indicate that a mixture of different strains, rather than an individual strain, would be more effective for use as a probiotic (Stavric, 1992; Timmerman et al., 2004). If the E. faecalis MB 5259 strain would be combined with either other strains showing anti-C. jejuni activity or with prebiotics selective for E. faecalis, evidence for in vivo inhibition might be found. In literature, authors agree that a probiotic displays a greater effect if it is part of a preparation including prebiotics than when it is used alone (Jung et al., 2008; Awad et al., 2009; Vandeplas et al., 2009; Baffoni et al., 2012). Coating or microencapsulating the probiotic strain might also help the controlled release of the strain in the gut after passage through the gastric barrier (Kailasapathy, 2002; Del Piano et al., 2010). It might also help survival of the strain in feed, when storage as part of feed is needed (Baffoni et al., 2012).

As with a described Lactobacillus (P93) strain (Chaveerach et al., 2004), which also exhibits in vitro probiotic properties against C. jejuni and C. coli strains, the mode of inhibition by the E. faecalis MB 5259 strain is not exactly known, because excluding the effect of organic acids and hydrogen peroxide and adding proteases to block bacteriocin substances did not influence anti-Campylobacter activity in vitro. Other recent in vitro studies have clearly shown that non-bacteriocin producing strains can exhibit anti-Campylobacter or anti-Helicobacter activity, although it is not always clear by which mode of inhibition probiotic strains influence C. jejuni growth (Nazef et al., 2008; Ryan et al., 2008).

In conclusion, although we could previously show in vitro inhibitory influence of the E. faecalis MB 5259 strain on C. jejuni MB 4185 in a system mimicking the broiler cecal environmental circumstances, these results were not obtained in a classic in vivo trial. Independent of the size of the inoculum of E. faecalis MB 5259, the strain was not able to reduce cecal numbers of C. jejuni strain MB 5259, despite colonizing the cecum.

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