Publication information

Beneficial Microbes: ISSN 1876-2883 (paper edition); ISSN 1876-2891 (online edition)

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Beneficial Microbes, December 2012; 3(4): 299-308

Wageningen Academic Publishers

Screening for lactic acid bacteria capable of inhibiting *Campylobacter jejuni* in *in vitro* simulations of the broiler chicken caecal environment

J. Robyn[1], G. Rasschaert[1], W. Messens[1], F. Pasmans[2] and M. Heyndrickx[1,2]

[1]Institute for Agricultural and Fisheries Research (ILVO), Technology and Food Science Unit, Brusselsesteenweg 370, 9090 Melle, Belgium; [2]Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium; joris.robyn@ilvo.vlaanderen.be

Received: 7 May 2012 / Accepted: 19 August 2012

Abstract

Thermotolerant *Campylobacter* spp., specifically *Campylobacter jejuni* and *Campylobacter coli,* are the most common bacterial causes of human gastroenteritis in developed countries. Consumption of improperly prepared poultry products and cross contamination are among the main causes of human campylobacteriosis. The aim of this study was to identify lactic acid bacterial (LAB) strains capable of inhibiting *C. jejuni* growth in initial *in vitro* trials ('spot-on-lawn' method), as well as in batch fermentation studies mimicking the broiler caecal environment. These experiments served as an indication for using these strains to decrease the capability of *Campylobacter* to colonise and grow in the chicken caeca during primary production, with the aim of reducing the number of human campylobacteriosis cases. A total of 1,150 LAB strains were screened for anti-*Campylobacter* activity. Six strains were selected: members of the species *Lactobacillus reuteri*, *Lactobacillus agilis*, *Lactobacillus helveticus*, *Lactobacillus salivarius*, *Enterococcus faecalis* and *Enterococcus faecium*. After treatment with catalase, proteinase K and α-chymotrypsin, anti-*Campylobacter* activity of cell-free culture supernatant fluid (CSF) for all six strains was retained, which indicated that activity was probably not exerted by bacteriocin production. Based on the activity found in CSF, the compounds produced by the selected strains are secreted and do not require presence of live bacterial producer cells for activity. During initial *in vitro* fermentation experiments, the *E. faecalis* strain exhibited the highest inhibitory activity for *C. jejuni* and was selected for further fermentation experiments. In these experiments we tested for therapeutic or protective effects of the *E. faecalis* strain against *C. jejuni* MB 4185 infection under simulated broiler caecal growth conditions. The best inhibition results were obtained when *E. faecalis* was inoculated before the *C. jejuni* strain, lowering *C. jejuni* counts at least one log compared to a positive control. This effect was already observed 6 h after *C. jejuni* inoculation.

Keywords: *in vitro*, fermentation experiments, *Enterococcus faecalis, Campylobacter jejuni*

1. Introduction

Thermotolerant *Campylobacter* spp., specifically *Campylobacter jejuni* and *Campylobacter coli* (Tauxe, 2002), are the most common bacterial causes of human gastroenteritis in many developed countries (Allos, 2001). Apart from causing enteritis, *Campylobacter* may also cause sequelae ranging from Guillain-Barré syndrome, reactive arthritis, irritable bowel syndrome and inflammatory bowel disease (Gradel *et al.*, 2009; Havelaar *et al.*, 2000; Keenan *et al.*, 2011; Mann and Saeed, 2012). In Belgium in 2009, campylobacteriosis had a notification rate of 53.41 per 100,000 inhabitants (EFSA, 2011). The overall campylobacteriosis notification rate in the European Union (EU) in 2009 was 45.6 per 100,000 inhabitants (EFSA, 2011). In Belgium, the costs of this disease and its sequelae have been estimated at 27 million Euros per year (Gellynck *et al.*, 2008). Extrapolation of these costs for the EU member

states results in a disease burden of 0.1-1 million disability-adjusted life years per year and a total cost between 0.5 and 5 billion Euros per year (EFSA, 2010a).

Several studies have identified the food chain, with poultry meat in particular, as the primary transmission route for pathogenic *Campylobacter* species (Effler *et al.*, 2001; Wilson *et al.*, 2008; Wingstrand *et al.*, 2006). Poultry is a reservoir for *Campylobacter* spp. (Altekruse *et al.*, 1999; Fields and Swerdlow, 1999), but the gastrointestinal tract of poultry can be infected without detectable deleterious effects for the avian host (Stern *et al.*, 1988). In 2008, most broiler chicken carcasses in EU member states were contaminated (on average 76% of carcasses) (EFSA, 2010b; Hermans *et al.*, 2012). Twenty to thirty percent of human campylobacteriosis cases may be attributed to handling, preparation and consumption of broiler meat, while 50 to 80% may be attributed to the chicken reservoir as a whole (broilers as well as laying hens) (EFSA 2010a).

Quantitative risk assessment models have indicated that lowering the *Campylobacter* shedding on the farm by 1, 2 or 3 log units could result in 55, 84 or 94% reduction in the number of campylobacteriosis cases, respectively (Messens *et al.*, 2007). Therefore, reduction of *Campylobacter* in the poultry reservoir is an essential step to control this food safety problem. Although *Campylobacter* contamination can be targeted at multiple levels and several control measures can be implemented (Ganan *et al.*, 2012), on-farm control of *Campylobacter* would have the greatest impact because the intestine of living poultry is the only amplification point for *Campylobacter* in the entire food chain. Additionally, on-farm control is the strategy with the highest consumer approval (Wagenaar *et al.*, 2006, 2008).

The wide-spread use of antibiotics has generated an evolutionary selection of resistant bacteria. Other antimicrobials, such as bacteriocins, bacteriophages, probiotics and antimicrobial peptides or acids are now being considered as alternatives. As lactic acid bacteria (LAB) display a wide range of antimicrobial activities, it might be interesting to use them as an alternative to antibiotics or a supplement to antibiotics. Their most important antibacterial activity is the production of lactic acid and acetic acid. Certain strains are also known to produce bioactive molecules such as ethanol, formic acid, hydrogen peroxide and diacetyl, among others (Lindgren and Dobrogosz, 1990). Many LAB strains also produce bacteriocins, bacteriocin-like molecules or other non-bacteriocin antimicrobial peptides that display antibacterial activity (De Vuyst *et al.*, 1994; Ryan *et al.*, 2008, 2009).

The aim of this study was to identify LAB strains that may lower *C. jejuni* counts in broilers when used as living probiotic additives, either by production of bacteriocins or other antimicrobial compounds. This was done using *in vitro* trials as an initial screening, as well as using batch fermentation studies to mimic the broiler chicken caecal environment to predict possible future use as living probiotic additive in chicken feed.

2. Materials and methods

Bacterial strains and culture media

A total of 1,128 *Lactobacillus, Lactococcus* and *Enterococcus* strains were tested for anti-*C. jejuni* activity (Table 1). They were streaked directly onto De Man, Rogosa and Sharpe agar (MRS; Oxoid, Basingstoke, UK) and incubated under anaerobic atmospheric conditions overnight at 37 °C. Most *Lactobacillus* strains were from poultry sources, either from caecal droppings or vaginal swabs of laying hens and broilers (Van Coillie *et al.*, 2007). All *Enterococcus* strains were isolated from broilers, except for the *Enterococcus faecalis* MB 5259 strain from dairy origin. Most *Lactococcus* strains were from unknown sources, except for the strain MB 32 isolated from a cat's tonsil and MB 3791 of dairy origin.

C. jejuni (Table 1) strains were grown on *Campylobacter* Blood-Free Selective Agar base (CCDA; Oxoid) + CCDA selective supplement (Oxoid) (mCCDA) plates incubated at 41.5 °C for approximately 24 h under microaerobic atmospheric conditions (5% O_2, 10% CO_2, 85% N_2) in a Forma Series II 3110 Water-Jacketed CO_2 incubator (Thermo Scientific, Waltham, MA, USA). These strains were collected from broiler houses and their surroundings (Herman *et al.*, 2003; Messens *et al.*, 2009).

Screening for anti-*Campylobacter* activity on plates

Strains were evaluated for antimicrobial activity against various genetically different *C. jejuni* strains using a pricking method (Method A), the agar block method (Method B) (Stern *et al.*, 2006) or the 'spot-on-lawn' method (Method C) (De Vuyst *et al.*, 1996). To select the LAB strains with the best *C. jejuni* inhibitory ability, we used the three screening methods in succession. In Method A each LAB strain was grown on MRS agar and incubated anaerobically for 24 h at 37 °C. One colony of each LAB strain was picked from the MRS agar plate and pricked into the surface of a *Brucella* soft agar (BSA) plate. BSA plates consisted of *Brucella* broth (BD Bioscience, Sparks, MD, USA) and 0.7% (w/v) bacteriological agar (Oxoid) buffered at pH 6.0 using a phosphate buffer to compensate for acid production by LAB strains in the soft agar surface. Each BSA plate contained approximately 10^6 colony forming units (cfu) of one of the 10 *C. jejuni* strains. These *C. jejuni* strains were freshly grown on CCDA under a microaerobic atmosphere at 41.5 °C for 24 h. After pricking the LAB into the BSA agar surface, plates were incubated microaerobically for 16 h at 41.5 °C.

Table 1. Bacterial strains used in the study.

Genus	Species	Number of strains	Isolated from (no)[5]
Lactobacillus[1]	*bulgaricus*	21	dairy (1)
	casei	6	pig (1), feed (1)
	helveticus	1	dairy(1)
	vaginalis	25	laying hen (8), pig (5)
	agilis	5	laying hen (3)
	salivarius	82	laying hen (28), dairy (1), pig (2)
	reuteri	122	laying hen (95), pig (23), feed (2)
	plantarum	15	feed (8), pig (2), food (4)
	johnsonii	24	laying hen (5), pig (18), feed (1)
	crispatus	12	laying hen (6)
	gallinarum	15	laying hen (4)
	kitasatonis	49	laying hen (9), pig (35), feed (5)
	brevis	16	feed (15), pig (1)
	acidophilus	49	laying hen (26), pig (2)
	mucosae	22	feed (12)
Lacotococcus[1]	*lactis*	22	cat (1), dairy (1)
Enterococcus	*faecalis*[1]	4	dairy (1)
	faecium[1,3]	420	broiler (419)
	faecalis or *faecium*[2]	240	broiler, pig
Campylobacter[1]	*jejuni*[4]	10	broiler (10)

[1] These strains originate from the Institute for Agricultural and Fisheries Research, Melle, Belgium.
[2] These strains originate from the Veterinary and Agrochemical Research Centre, Brussels, Belgium.
[3] These strains originate from the Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium.
[4] *Campylobacter jejuni* strains: MB 4185, MB 4188, MB 4189, MB 4194, MB 4196, MB 4201, MB 4206, KC 100.1, KC 59.1 and KC 67.2.
[5] Non-designated strains were isolated from unknown sources.

In method B, approximately 10^6 cfu of each LAB strain were individually suspended into MRS soft agar plates. The plates consisted of MRS broth (Oxoid) + 0.7% bacteriological agar. After suspension, they were incubated anaerobically for 24 h at 37 °C. Round agar blocks (6 mm in diameter) containing bacterial growth were aseptically excised from the MRS soft agar and placed onto the surface of plates filled with buffered BSA agar containing approximately 10^6 cfu of a freshly grown *C. jejuni* strain. Subsequently, they were incubated under microaerobic atmosphere for 16 h at 41.5 °C.

In method C, 20 µl of cell-free culture supernatant fluid (CSF) adjusted to pH 6.0 was spotted onto the surface of buffered BSA plates containing a *C. jejuni* strain. The cell-free CSF was derived from a LAB strain culture incubated for 16 h at 37 °C in MRS broth under anaerobic conditions. Then the culture was centrifuged at 13,000×*g* for 10 min and the supernatant adjusted to pH 6.0 by adding 5 M NaOH to rule out the influence of acids on the *C. jejuni* strains. The supernatant was subsequently filter sterilised using a 0.22 µm Millex filter (Millipore, Bedford, MA, USA).

In all the methods described, inhibition by the isolates was evaluated by measuring the diameters of the resulting *C. jejuni* growth-free zones around the block, growing colony or spot, respectively. Inhibition was tested against 10 (Method A) genetically diverse *C. jejuni* strains. These *C. jejuni* strains were selected from 92 *Campylobacter* isolates belonging to 22 genotypes (+ 2 variants) that were genotyped using fluorescent amplified fragment length polymorphisms, pulsed field gel electrophoresis and *Fla*A-restriction fragment length polymorphisms. These *Campylobacter* strains were isolated from commercial broiler flocks and the environment of broiler farms in Belgium (Herman *et al.*, 2003; Messens *et al.*, 2009). Subsequently, 2 (MB 4185 and KC 100.1) out of 10 genetically diverse *C. jejuni* strains were used as reference strains in Methods B and C.

Screening for bacteriocin production

Selected LAB were screened for possible bacteriocin production using a method for crude bacteriocin purification. Each LAB was incubated for 16 h at 37 °C in 1 l of MRS broth under anaerobic conditions. The culture was centrifuged at 12,000×*g* for 10 min. The supernatant

was adjusted to pH 6.0 by adding 5 M NaOH and 100 U/ml catalase to either compensate for organic acids or remove hydrogen peroxide, respectively. Soluble peptides were then isolated from the supernatant by a 40% saturated (Anonymous, 2012) ammonium sulphate precipitation (Abo-Amer *et al.*, 2006; Deraz *et al.*, 2005). This crude preparation sample was filtered through 0.22 µm Millex filters.

The influence of proteolysis on the anti-*Campylobacter* activity of the crude preparation sample was determined by transferring the following enzymes to tubes containing 1 ml of the filtered crude preparation: α-chymotrypsin (final concentration: 0.5 mg/ml; Sigma, St. Louis, MO, USA) and proteinase K (final concentration 0.1 mg/ml; Sigma). After 3 h of incubation at 37 °C, the mixture of crude preparation and enzymes was analysed for antimicrobial activity using Method C. The control was untreated filtered crude preparation. To evaluate thermal stability, 1 ml crude preparation solution was heated at 90 °C for 15 min and antimicrobial activity was tested again as described above.

Anti-*Campylobacter* activity in batch cultures

LAB that exhibited *C. jejuni* inhibition *in vitro* on plates using the aforementioned methods were tested in batch culture experiments. These controlled conditions reproduced the same pH (6.5), temperature (41.5 °C) and atmospheric conditions (microaerobic) favourable for *C. jejuni* growth in the broiler caecum. The highly colonising *C. jejuni* MB 4185 (= KC40) strain used was isolated from broilers (Herman *et al.*, 2003). LAB strains were grown overnight under anaerobic conditions on MRS agar at 37 °C and subsequently diluted in Ringers solution (Oxoid) to a final concentration of 10^5-10^6 cfu/ml. *C. jejuni* strain MB 4185 was grown overnight on mCCDA under microaerobic conditions at 41.5 °C and subsequently diluted in Ringers solution to a concentration of 10^5-10^6 cfu/ml. The New Brunswick Scientific BioFlo110 fermentor (New Brunswick Scientific, Enfield, CT, USA) contained 500 ml of *Brucella* broth + *Campylobacter* growth supplement (Oxoid) plus 0.05% (w/v) mucin from porcine stomach type II, 0.05% (w/v) mucin from porcine stomach type III (both from Sigma) and 20 g/l D(+)-glucose monohydrate (Merck, Darmstadt, Germany). The fermentor was autoclaved for 10 min at 121 °C. The sterile fermentor vessels were inoculated to obtain a final concentration of ~10^3 cfu/ml for the *C. jejuni* strain (control vessel) as well as ~10^3 cfu/ml for both the *C. jejuni* strain and the LAB strain (experimental vessel). Incubation in the fermentor vessel was performed at 41.5 °C, while the pH was kept stable at 6.5 using 5 M NaOH. The atmosphere was kept microaerobic by blowing a gas mixture of 5% O_2, 10% CO_2 and 85% N_2 (Air Liquide, Paris, France) directly into the growth medium.

Further batch cultures studies of the selected *E. faecalis* strain against *C. jejuni* MB 4185 were carried out under simulated broiler caecal growth conditions. In the first experimental design, one of the plate-screened LAB strains exhibiting anti-*Campylobacter* activity was inoculated 6 h after the initial *C. jejuni* MB 4185 incubation, i.e. in the middle of the exponential growth phase of the *C. jejuni* strain. For these experiments, both reactor vessels contained the growth medium described above without D(+)-glucose monohydrate. These fermentation experiments were performed until all plate-screened LAB strains exhibiting anti-*Campylobacter* activity were tested. During fermentation experiments, samples (~10 ml) were taken aseptically from both reactor vessels at 0 h (inoculation of *C. jejuni* MB 4185), 6 h (inoculation of LAB), 12, 24, 28 and 48 h of fermentation. Based on the results obtained during these initial experiments, one LAB strain was selected for use in two subsequent experimental designs during which D(+)-glucose monohydrate was continually added to the growth medium to improve LAB growth.

In the second experimental design, *C. jejuni* MB 4185 and the LAB strain selected during the first experimental design were inoculated at the same time. Samples were taken from both reactor vessels at 0, 2, 4, 6, 12, 24 and 48 h of fermentation. In the third experimental design, the same LAB strain was inoculated 24 h before addition of *C. jejuni* MB 4185. Samples were taken at 0 h (inoculation of LAB), 24 h (inoculation of *C. jejuni* MB 4185), 30, 48 and 72 h of fermentation.

In all three experiments, 10-fold dilution series in Ringers solution were made of the samples taken from both reactor vessels and streaked onto MRS agar for enumeration of LAB or mCCDA for enumeration of *C. jejuni*. These plates were incubated for 24 to 48 h under anaerobic conditions at 37 °C (MRS agar plates) or under microaerobic conditions at 41.5 °C (mCCDA plates).

3. Results

Screening for anti-*Campylobacter* activity

A preliminary screening of 1,150 strains using Method A resulted in a selection of 91 LAB strains that caused growth inhibition of at least 8 of the 10 *C. jejuni* strains tested. Of these strains, *C. jejuni* MB 4206 was inhibited by the lowest number of LAB strains and *C. jejuni* KC 59.1 was inhibited by the highest number of LAB strains. Most of the 91 LAB strains selected originated from poultry, except for three *Lactobacillus vaginalis* strains (pig), one *Lactobacillus helveticus* strain (dairy), one *Lactobacillus salivarius* strain (dairy), one *Lactobacillus sakei* strain (unknown origin) and one *E. faecalis* strain (dairy).

Next, these 91 LAB strains were screened again using Method B for inhibition of two *C. jejuni* strains (MB 4185 and KC 100.1). Nineteen LAB strains showed inhibition of both strains. In a third round of experiments using Method C, the supernatant of the 19 LAB strains was tested for activity against *C. jejuni* MB 4185 and KC 100.1. Based on the size of the *C. jejuni* growth free zone (>4 mm), 6 strains were selected. The size of this selection criterion was arbitrarily chosen to select a manageable number of strains. These strains were *Lactobacillus reuteri* MB 2928 (poultry), *Lactobacillus agilis* MB 2924 (poultry), *L. helveticus* MB 52561 (dairy), *L. salivarius* MB 5262 (dairy), *E. faecalis* MB 5259 (dairy) and *Enterococcus faecium* MB 5260 (poultry) (Table 2). Activity in the supernatant was retained for these six strains after treatment of the supernatant with catalase, proteinase K and α-chymotrypsin (data not shown).

Anti-*Campylobacter* activity in batch cultures

A first series of batch fermentation experiments was carried out to specify which of the six selected LAB strains best inhibited *C. jejuni* MB 4185. The reactor vessels contained medium without D(+)-glucose monohydrate and each LAB strain was inoculated 6 h after the initial *C. jejuni* MB 4185 incubation. The results showed a reduced growth of *C. jejuni* MB 4185 of 0.46 and 1.22 $\log_{10}$ by *E. faecalis* MB 5259 during the 48 h of fermentation (Figure 1, Figure 2A). When one of the other five strains was co-inoculated in the fermentation vessel, only *L. agilis* MB 2924 and *L. salivarius* MB 5262 were able to reduce *C. jejuni* MB 4185 growth but only up to 0.86 $\log_{10}$. The other three strains did not exhibit notable *C. jejuni* reduction (Figure 1).

Table 2. Lactic acid bacterial strains selected by successive Methods A, B and C for culture screening against *Campylobacter jejuni*.

Genus	Species	Strains selected with Method A (n)[1]	Strains selected with Method B (n)[2]	Strains selected with Method C (n)[3]
Lactobacillus	*vaginalis*	MB 3058, 3059, 3064 (3)	-[4]	-[4]
	helveticus	MB 5261 (1)	MB 5261 (1)	MB 5261 (1)
	agilis	MB 2882, 2924 (2)	MB 2882, 2924 (2)	MB 2924 (1)
	salivarius	MB 2828, 2834, 2835, 2836, 2858, 2893, 2909, 2910, 2920, 2932, 2934, 2964, 3014, 3016, 3024, 5262 (16)	MB 2828, 2834, 2920, 2924, 3024, 5262 (6)	MB 5262 (1)
	sakei	MB 3035 (1)	-[4]	-[4]
	reuteri	MB 2856, 2874, 2921, 2923, 2927, 2928, 2973, 2997 (8)	MB 2927, 2928 (2)	MB 2928 (1)
	crispatus	MB 3008 (1)	-[4]	-[4]
	acidophilus	MB 2974 (1)	-[4]	-[4]
	mucosae	MB 2922 (1))	-[4]	-[4]
Lactococcus	*lactis*	-[4]	-[4]	-[4]
Enterococcus	*faecium*	B2 6a, B2 97d, B7 7, B8 31, B2 63e, B2 70d, B2 74d, B2 59d, B2 59e, B2 97e, B2 62e, B2 100d, B14 1, B2 72C, B2' 26, B2 99a, B7' 6, B8 6, B2 62C, B2 66e, B2 72b, B7' 14, B7' 15, B2 66c, B2' 7, B2 74C (=MB 5260), B2 63a, B2' 25, B12 20, S25 3, iuM VT 86 (31)	MB 5260=B2 74C, B2' 26, B2 99a, B8 6, B2 72B, B2 66C, B2' 7 (7)	MB 5260 (1)
	faecalis	IsPT 32A, IsPT 29, IsPT 59b, IsPT 5, IsPT 3112a, IsPT 33a, IsPT 292117, IsPT 26, IsPT 25, IsPT 30a, IsPT 31121b, IsPT 1, IsPT 150a, IsPT 32B, IsPT 10B, IsPT 43B, IsPT 37C, IsPT 37B, IsPT 20a, IsPT 10A, IsPT 7B, IsPT 137a, IsPT 36A, IsPT 138B, IsPT 139A (26)	MB 5259 (1)	MB 5259 (1)

[1] LAB strains selected based on growth inhibition of at least 8 out of 10 genetically different *C. jejuni* strains using the pricking method.
[2] LAB strains selected based on the growth inhibition of two genetically different *C. jejuni* strains (MB 4185 and KC 100.1) using the agar block method.
[3] LAB strains selected based on the size of zone of growth inhibition of two genetically different *C. jejuni* strains (MB 4185 and KC 100.1) using the spot-on-lawn method.
[4] No LAB strains selected.

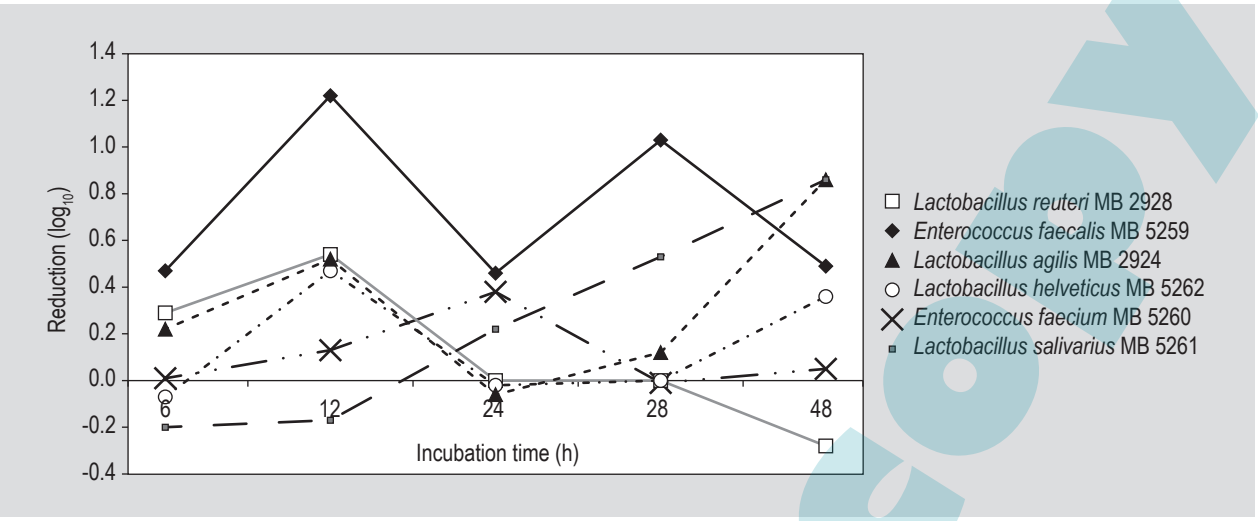

Figure 1. Log_{10} reduction in number of *Campylobacter jejuni* MB 4185 in a fermentation vessel containing lactic acid bacteria compared to a control vessel containing only *C. jejuni* MB 4185. Lactic acid bacterial strains were inoculated 6 h after initial *C. jejuni* MB 4185 incubation in reactor vessels containing growth medium without D(+)-glucose monohydrate.

Next, post-inoculation of the *E. faecalis* MB 5259 strain was done in sugar supplemented growth medium (10^9 cfu/ml after 24 to 48 h of incubation), leading to *E. faecalis* MB 5259 counts that were around 3 log higher than in the absence of sugar (10^6 cfu/ml after 24 to 48 h of incubation) (Figure 2A,B). *C. jejuni* MB 4185 growth reduction was also higher when sugar was added (Figure 2B). In the presence of sugar, reduction of *C. jejuni* MB 4185 amounted to 1.40 and

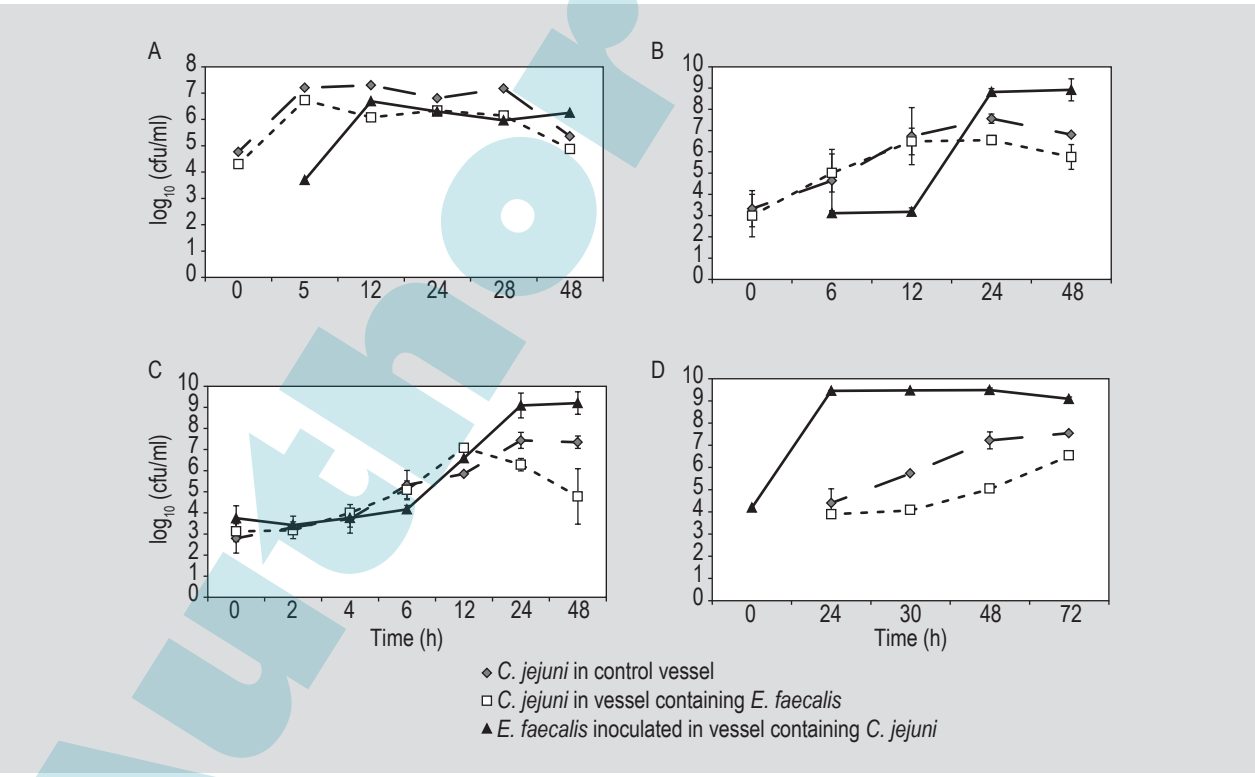

Figure 2. Fermentation study in a bioreactor to screen for therapeutic or protective effects of *Enterococcus faecalis* MB 5259 on *Campylobacter jejuni* MB 4185 numbers. The control bioreactor only contained *C. jejuni*. (A) Pre-inoculation of *C. jejuni* followed 6 h later by *E. faecalis* in the absence of D(+)-glucose monohydrate. (B) Pre-inoculation of *C. jejuni* followed 6 h later by *E. faecalis* in the presence of D(+)-glucose monohydrate. (C) Simultaneous inoculation of *C. jejuni* and *E. faecalis* in the presence of D(+)-glucose monohydrate. (D) Pre-inoculation of *E. faecalis* followed 24 h later by *C. jejuni* in the presence of D(+)-glucose monohydrate.

1.60 $\log_{10}$ after 24 to 48 h of incubation, respectively, while in the absence of sugar only 1.02 and 0.49 $\log_{10}$ reduction was obtained after the same incubation time.

Based on the results above, we used the *E. faecalis* MB 5259 strain and added sugar to the growth medium to screen for therapeutic or protective effects on *C. jejuni* MB4185 in batch experiments. Figure 2C shows the results obtained when *E. faecalis* MB 5259 strain and *C. jejuni* MB 4185 were inoculated at the same time. After 24 to 48 h of incubation, approximately one $\log_{10}$ of *C. jejuni* MB 4185 growth reduction was obtained. Reduction was best when the *E. faecalis* MB5259 strain was inoculated 24 h before addition of *C. jejuni* MB 4185. Figure 2D shows reduction of *C. jejuni* MB 4185, which already amounted to 1.66 $\log_{10}$ after only 6 h of incubation and mounted up to 1.69 $\log_{10}$ after 24 h of incubation.

4. Discussion

During broiler production and processing, faecal material containing pathogens such as *C. jejuni* may be transferred onto meat (Hilbert *et al.*, 2010), which can lead to campylobacteriosis in humans. Ensuring a lower *C. jejuni* load in broiler caeca and on broiler carcasses and derived food products will cause a lower number of human campylobacteriosis cases. In order to combat *C. jejuni* colonisation in broilers during primary production, it has been suggested to identify antimicrobial compounds to which *C. jejuni* is susceptible or to which resistance is low and disappears after selective pressure is removed. This is true for bacteriocins and some other compounds (Hoang *et al.*, 2011). As intervention against *C. jejuni* infection in humans may be hindered by the increasing widespread antibiotic resistance among pathogenic bacteria like *C. jejuni*, antibacterial compounds should be alternatives to antibiotics. The aim of our study was to identify LAB that are able to produce bacteriocins or other antimicrobial compounds that reduce *Campylobacter* growth in *in vitro* studies of the broiler caecal environment. Such results indicate the ability to influence colonisation and/or survival of *C. jejuni* in the broiler caecum during primary production.

In total 1,150 LAB strains, mostly of chicken origin, were tested for their ability to inhibit *C. jejuni* growth *in vitro*. Most tested strains belonged to bacterial species which have been known to produce metabolites capable of inhibiting growth of different pathogens including *Campylobacter* (Contreras *et al.*, 1997; Line *et al.*, 2008; Messaoudi *et al.*, 2011; Ryan *et al.*, 2008; Stern *et al.*, 2006). Van Coillie *et al.* (2007) demonstrated that some of the strains we examined could inhibit *Salmonella* growth.

Six non-bacteriocinogenic strains that cause *in vitro* inhibition of *C. jejuni* were identified. This raises the question which metabolites or compounds that are produced by these strains might be involved in growth inhibition of *C. jejuni*. Inhibition may be caused by (1) a variety of compounds exhibiting antibacterial activity such as volatile fatty acids and hydrogen peroxide, which are documented to be produced by the selected isolates (Garneau *et al.*, 2002; Gilliland and Speck, 1977; Riley *et al.*, 2002); (2) production of bacteriocins with a mechanism of action similar to that of ionophore antibiotics (Tagg *et al.*, 1976); or (3) non-bacteriocin (Nazef *et al.*, 2008), non-fatty acid compounds (Ryan *et al.*, 2008). Our whole bacterial cell-derived antibacterial activity did not meet the criteria for being considered a bacteriocin (Hechard and Sahl, 2002; Tagg *et al.*, 1976), because it was insensitive to proteases (proteinase K and α-chymotrypsin) for all six selected strains of LAB. Protease treatment was not done on the 91 LAB strains initially selected, thus it remained unknown if any of the 85 other strains were capable of producing bacteriocins. There was also no indication of activity being caused by heat labile bacteriocins, hydrogen peroxide or volatile fatty acids. Moreover, the neat cell-free CSF from all six tested strains inhibited *C. jejuni* growth, thus the antibacterial compounds are probably secreted. The cell-free CSF contained no live bacterial producer cells, which indicates that they are not required for activity. As described in literature (Ryan *et al.*, 2008), antibacterial activity can also be caused by non-bacteriocin, non-fatty-acid compounds.

In vitro fermentation experiments under controlled temperature, pH and atmosphere were carried out to further elucidate the ability of live LAB cells to inhibit *C. jejuni* growth under conditions simulating the growth conditions of the broiler caeca. Initial fermentation experiments indicated that *C. jejuni* growth was best inhibited by *E. faecalis* MB 5259. Literature studies describing *in vitro* and *in vivo* experiments have reported that *Enterococcus* spp. strains could inhibit *C. jejuni* growth. They were supplemented as viable strains *in vitro* in plating methods, but not in fermentation experiments (Line *et al.*, 2008; Nazef *et al.*, 2008). *In vivo*, purified bacteriocin from an *Enterococcus* spp. was added to feed as an inhibiting agent (Line *et al.*, 2008). Combining the results of these studies with those obtained in the present study, it was decided to use the *E. faecalis* MB 5259 strain in all following batch fermentation experiments.

Three batch fermentation experiments were developed to screen for a therapeutic or protective effect caused by *E. faecalis* MB 5259, i.e. pre-, post- or simultaneous inoculation of *E. faecalis* MB 5259 with respect to *C. jejuni* MB 4185 inoculation. Fermentation designs using pre- and simultaneous inoculation were intended to screen for a protective effect of the *Enterococcus* strain against *C. jejuni* infection. These experiments mimic natural rearing situations in which the inoculation of broiler chickens

with the *Enterococcus* strain is done daily, starting either from birth or at two weeks of age (pre- and simultaneous inoculation, respectively). As broiler chickens are expected to be *Campylobacter*-free during the first two weeks following hatching, the *E. faecalis* strain might be used to exert a protective effect against *C. jejuni* infection. Batch fermentation experiments inoculating *C. jejuni* before the *E. faecalis* strain mimic rearing situations in which broiler chickens would be inoculated with the *Enterococcus* strain prior to chicken processing by weeks, days or even immediately prior to slaughter. In most cases, *C. jejuni* colonisation is already established. This batch fermentation experiment therefore screens for a possible therapeutic effect of the *E. faecalis* strain against *C. jejuni*. In all experiments, a reduction in *C. jejuni* growth was seen of at least one log after 24 to 48 h of incubation, indicating a possible protective as well as a therapeutic effect exerted by the *E. faecalis* strain. Recently, it was agreed that a 1 log reduction is considered to be the minimum for a beneficiary effect (Alexander, 2012). If comparable results would be obtained *in vivo* over longer time periods, a 1 log reduction in *C. jejuni* colonisation of broilers would theoretically lead to a 55% reduction in the number of human campylobacteriosis cases (Messens *et al.*, 2007).

When *E. faecalis* MB 5259 was inoculated before *C. jejuni* MB 4185, there was an immediate influence on the growth of *C. jejuni*. Even 6 h after initial inoculation of *C. jejuni* MB 4185, the difference between *C. jejuni* numbers in the control reactor and those in the vessel containing *E. faecalis* MB 5259 amounted to 1 to 2 log. This effect in reduction was not obtained when *C. jejuni* MB 4185 was inoculated before *E. faecalis* MB 5259 or when both strains were inoculated at the same time. Analogous results on the influence of inoculation time were obtained by *in vitro* experiments using a mixed culture of *Lactobacillus crispatus* and *Clostridium lactatifermentans* (Van der Wielen *et al.*, 2002). These experiments show higher reduction in the number of *Salmonella* serovar Enteritidis present when *Salmonella* was administered to a reactor under caecal growth conditions 48 h after the mixed culture compared to when *Salmonella* was administered before the mixed culture.

To our knowledge, this paper is the first report of corresponding *in vitro* experiments testing for either therapeutic or preventive effects of a probiotic strain, *E. faecalis*, against caecal *C. jejuni* infection under controlled growth conditions. Based on our results and *in vivo* studies that show the possibility of lowering and controlling *C. jejuni* colonisation of broilers by administering different bacterial strains with or without carbohydrate supplements (Morishita *et al.*, 1997; Schoeni and Wong, 1994), an *in vivo* experiment would be needed to ascertain if the selected strain is capable of inhibiting or lowering *C. jejuni* colonisation or growth in the broiler intestinal environment to the same extent. In such an experiment, the *E. faecalis* strain should be inoculated daily in broiler chickens before subsequent *C. jejuni* infection using a seeder model to determine if the strain exerts a protective effect against *C. jejuni* colonisation or the spread of *C. jejuni* in the broiler flock.

Acknowledgements

This work was financed by a grant of the Federal Public Service of Health, Food Chain Safety and Environment (FOD, Brussels, Belgium), project RT08/8-Campoul. *Enterococcus* strains were kindly provided by Davy Persoons (Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium) and Patrick Butaye (Veterinary and Agrochemical Research Centre, Brussels, Belgium).

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