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Characterization of probiotic strains: An application as feed additives in poultry against *Campylobacter jejuni*

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ABSTRACT

Campylobacteriosis is at present the most frequent zoonosis in humans and the main source is poultry meat contaminated by *Campylobacter jejuni*. An alternative and effective approach to antibiotic administration to livestock to reduce bacterial contamination is the use of probiotics, which can help to improve the natural defence of animals against pathogenic bacteria. In this study 55 lactic acid bacteria and bifidobacteria were screened for desirable properties for their application as probiotics against *Campylobacter* in poultry. All bacteria were examined for their antimicrobial activity against three *C. jejuni* strains. Strains exhibiting the highest anti-*Campylobacter* activity were examined for their survival in the gastro intestinal tract (low pH and presence of bile salts) and food/feed processing conditions (high temperature, high NaCl concentration and starvation) and basic safety aspects such as antibiotic susceptibility and hemolytic activity were studied. On the basis of these activities, two strains, namely *Lactobacillus plantarum* PCS 20 and *Bifidobacterium longum* PCB 133, were chosen for an *in vivo* trial in poultry. They were separately administered to healthy chickens in order to evaluate their capability of colonizing the GI tract of poultry and to estimate their effect on *C. jejuni* population. The results evidenced that *L. plantarum* PCS 20 was not present in poultry feces at detectable concentration, whereas *B. longum* PCB 133 significantly increased after two weeks of daily administration and its amount was still high after a wash-out period of 6 days. In the same period, *C. jejuni* concentration in poultry feces was significantly reduced in chickens administered with *B. longum* PCB 133. Therefore, *B. longum* PCB 133, possessing interesting probiotic properties and a marked anti-*Campylobacter* activity both *in vitro* and *in vivo*, is an excellent candidate for being employed as additives to feed for poultry for the reduction of food-borne campylobacteriosis in humans.

1. Introduction

Food safety is of fundamental importance to the consumer, food industry and economy. Despite significant investment in this field, the incidence of food-borne diseases is still on the rise in the European Union (EU) (Hugas et al., 2009; Smulders et al., 2008). Several food-borne diseases are caused by the growth of pathogenic microorganisms in the food; among them, zoonoses are infections which are transmitted from animals to humans either directly or via the food chain. In order to protect human health, it is important to identify which animals and foods are the main sources of these infections and to develop appropriate intervention strategies to prevent zoonotic diseases from occurring.

Campylobacteriosis is the most frequently reported zoonotic disease in humans in the EU in recent years (Hugas et al., 2009; Westrell et al., 2009) and the bacterial species most frequently implicated is *Campylobacter jejuni* (Humphrey et al., 2007; Keener et al., 2004). Epidemiological data obtained by the European Food Safety Authority (EFSA) (EFSA, 2005) have concluded that poultry meat is the major source of sporadic campylobacteriosis. It has been reported that 26% of samples of fresh broiler meat and 25% of broiler flocks tested in the EU in 2007 were found positive for *Campylobacter* (Westrell et al., 2009). *Campylobacter* is considered to be a commensal organism in many avian species, including those grown commercially, and the spread of *Campylobacter* spp. among chickens is very rapid (Keener et al., 2004). Reducing the proportion of *Campylobacter* infected poultry flocks and/or reducing the number of *Campylobacter* in live poultry will lower the risk to consumers considerably (Keener et al., 2004; Westrell et al., 2009). A possible way to reduce *Campylobacter* contamination in poultry is to develop new actions at the primary production level.

The increasing concern about the spreading of antibiotic resistance in humans has determined the elimination of antibiotics as growth promoters in livestock (Feed Additives Regulation 1831/2003/EC; Schwartz et al., 2001). Therefore, feed companies and researchers have been looking for alternative products and strategies that can

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help to maintain animal gut health in order to prevent or reduce the prevalence of pathogens in the food chain. An alternative and effective approach to antibiotic administration to livestock is the use of probiotics, which can help to improve gut microbial balance and therefore the natural defence of the animal against pathogenic bacteria (Modesto et al., 2009; Patterson and Burkholder, 2003).

The use of probiotics in the farm industry dates back to the 1960s (Fuller, 1999), but much of the information on their use on farm animals was initially derived from in-house experiments which had several weak points, considering that the origin of the strain was rarely given and the real ability of the probiotic to colonize the gut was often not assessed (Fuller, 1999; Nahashon et al., 1996). Study performed later devoted more attention to the capability of the administered strains of colonizing the gut and reducing pathogen incidence, as reviewed by Musa et al. (2009). In the last years several efforts have been dedicated to define targeted microbial mixtures that could have preventive activity in poultry against *Salmonella* infections. Different *Lactobacillus* strains have demonstrated to protect chickens from this pathogen (Pascual et al., 1999; Van Coillie et al., 2007; Vicente et al., 2007) and to have a protective effect on raw chicken meat against *Listeria monocytogenes* and *Salmonella enteriditis* (Maragkoudakis et al., 2009). A reduction of necrotic enteritis due to *Clostridium perfringens* was evidenced upon administration of *L. johnsonii* FI9785 (La Ragione et al., 2004). The use of bifidobacteria in poultry feeding is, to our knowledge, less widespread with respect to lactobacilli administration, although bifidobacteria are an important component of the chicken gut microbiota (Amit-Romach et al., 2004) and have shown to exert positive effect when administered to other animals such as piglets (Modesto et al., 2009; Shu et al., 2001). *In vivo* trials have regarded the use of a probiotic mixed preparation also containing a *Bifidobacterium* strain (Montzouris et al., 2007) and the administration of a synbiotic mixture containing galacto-oligosaccharides and *B. lactis* (Jung et al., 2008), evidencing an increase of bifidobacteria in the poultry gut; the only experiment with a selected strain focused on the administration of a commercial *B. bifidum* strain to poultry, resulting in a reduction of cellulitis in broiler chickens (Estrada et al., 2001).

To date, only a few studies have evidenced a possible role of probiotics in preventing the shedding of *C. jejuni* at the level of primary production, although *in vitro* studies reported a strong antimicrobial activity of several probiotic strains towards this pathogen (Chaveerach et al., 2004; Fooks and Gibson, 2002). Morishita et al. (1997) reported a 70% reduction in the frequency of *C. jejuni* in chicks with the use of a commercial probiotic containing *L. acidophilus* and *Enterococcus faecium*. Willis and Reid (2008) showed that *C. jejuni* was present at a lower lever in broiler chickens fed with a standard diet supplemented with a probiotic formulation containing *L. acidophilus*, *L. casei*, *B. thermophilus*, and *E. faecium* (10^8 cfu/g) with respect to the control, although the real colonization of the administered strains was not studied.

Therefore, more research is needed in finding new probiotic strains with inhibiting activity against *Campylobacter* and capable of colonizing the guts of poultry animals with the final aim of reducing the contamination of the intestinal pathogen at the farm level and in the chicken meat.

In this work a number of lactic acid bacteria (LAB) and bifidobacteria were screened for desirable functional properties for their application as probiotics against *Campylobacter* in poultry. In particular, their antimicrobial activity against three *C. jejuni* strains, their survival in the gastro intestinal (GI) tract and food processing conditions were studied in addition to the determination of basic safety aspects such as antibiotic susceptibility and hemolytic activity. The two strains showing the best probiotic performances were administered to poultry in order to evaluate their capability to colonize the GI tract of poultry animals and to estimate their effect on *C. jejuni* population.

2. Materials and methods

2.1. Microorganisms and culture conditions

55 strains of LAB and bifidobacteria were used in this study. They were obtained by the collections of the Agricultural University of Athens (strains designed with PCA), Danisco A/S (strains designed with PCD), the Max Rubner Institute in Kalsruhe, Germany (strains designed with PCK), the University of Maribor-Faculty of Agriculture and life sciences (strains designed with PCS), and the Bologna University Scardovi collection of bifidobacteria (BUSCoB) (strains designed with PCB) (Table 1). The PCB strains were all belonging to the genus *Bifidobacterium* and have been previously isolated and characterized (Scardovi et al., 1979). LAB strains were cultivated in de Man, Rogosa and Sharpe Broth (MRS, Merck, Darmstadt, Germany) at 37 °C. *Bifidobacterium* strains were cultivated in Tryptone, Peptone, Yeast Extract medium (TPY, prepared according to Biavati and Mattarelli (2006)). All strains were stored at −80 °C.

Three *C. jejuni* strains were used as target strains: the type strain *C. jejuni* CIP 70.2 ^{T} from the Collection de l'Institut Pasteur, Paris, France, *C. jejuni* LMG 8842 from BCCM™ and *C. jejunii* 221/05 isolated from poultry (kindly provided by the Department of Veterinary Public Health and Animal Pathology, Ozzano dell'Emilia, Bologna, Italy). They were stored at −80 °C. One loop of each solution was streaked on Columbia Agar Base plates (Oxoid, Ltd., Basingstoke, Hampshire, England), which were incubated at 42 °C under microaerophilic atmosphere (5% O_2, 10% CO_2, 85% N_2) generated by using CampyGen Atmosphere Generation System (Oxoid) in anaerobic jars for 24–48 h. One typical colony of each strain was transferred into Meuller–Hinton broth (Oxoid) and kept under microaerophilic atmosphere for 24–48 h.

2.2. Assessment of the antimicrobial activity against Campylobacter strains

2.2.1. Agar spot test using living cells

A modification of the protocol of Kizerwetter-Swida and Binek (2005) was employed. Briefly, agar plates of MRS and TPY were used for LAB and bifidobacteria, respectively. 10 µl of LAB or bifidobacteria cultures (A_{600} of about 0.1) were spotted on the plates, which were incubated anaerobically for 24 h at 37 °C. After strain growth, the plates were overlaid with 10 ml of Nutrient Broth (Oxoid) additioned with 0,7% agar inoculated with each *Campylobacter* strain at 10^7 CFU/ml. Plates were incubated under microaerophilic atmosphere at 37 °C, after 24–48 h the presence of inhibition zones was evaluated. Each assay was performed in duplicate.

2.2.2. Well diffusion agar assay using pH neutralized cell free supernatants (NCSs)

A modification of the protocol of Collado et al. (2005) was used. Overnight cultures of LAB and bifidobacteria were harvested by centrifugation at 15,000×*g* at 4 °C for 15 min; the supernatants were centrifuged again in the same conditions in order to completely remove bacterial cells. The supernatants were adjusted to pH 6.5 with 10 N NaOH to obtain the NCSs. 500 µl of a *Campylobacter* culture at the concentration of 10^7 CFU/ml was added to 20 ml of Nutrient Agar (1.5% agar, Oxoid) maintained at 40 °C, poured into petri dishes, and allowed to solidify. Wells of 5 mm in diameter were made on the agar layer with a sterile metal cylinder, and filled with 50 µl of NCSs from each strain. After 48 h of incubation in microaerophilic conditions at 42 °C, the inhibition zones were observed. Each assay was performed in duplicate.

2.3. Preliminary characterization of the antimicrobial activity

To characterize the nature of any observed inhibitory activity, the well diffusion agar assay was repeated by adding to NCSs different enzymes such as trypsin, proteinase k and pronase (all purchased at