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Identification of lactobacilli residing in chicken ceca with antagonism against *Campylobacter*

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Summary. Bacteriocins produced by *Lactobacillus salivarius* have been recently recognized as a natural means to control *Campylobacter* and *Salmonella* in live poultry. This finding is of relevance since *Campylobacter jejuni* and *Campylobacter coli* are the predominant species isolated from poultry that are associated with human campylobacteriosis. In the present work, lactic acid bacteria (LAB) isolated from the cecum of twenty Tunisian chickens were identified and those isolates with antagonism against *Campylobacter* were further characterized. Following their preliminary confirmation as LAB, 150 strains were identified by combining morphological criteria, biochemical tests, and molecular methods, the latter inluding intergenic 16S-23S PCR, specific lactobacilli PCR, and a biphasic approach. Most of the LAB isolated belonged to the genus *Lactobacillus*, among them *Lb. sakei* (33.3%), *Lb. salivarius* (19.4%), *Lb. reuteri* (8.6%), and *Lb. curvatus* (8.6%). The other LAB strains included those of the genus *Weissella* (16.7%), *Enterococcus faecalis* (5.3%), *Leuconostoc mesenteroides* (2.7%), *Lactococcus graviae* (2.7%), and *Streptococcus* sp. (2.7%). The *Lactobacilli* strains were tested for their antagonism against *C. jejuni* and *C. coli*. The activity of three of them, *Lb. salivarius* SMXD51, *Lb. salivarius* MMS122, and *Lb. salivarius* MMS151, against the aforementioned target strains could be ascribed to the production of bacteriocins. [**Int Microbiol** 2011; 14(2):103-110]

Keywords: *Lactobacillus* ssp. · *Campylobacter jejuni* · lactic acid bacteria (LAB) · chicken cecum · bacterial antagonism

Introduction

Campylobacteriosis is the most common cause of gastroenteritis worldwide, with occasionally serious outcomes. The number of human *Campylobacter* infections reported yearly in Europe is between 180,000 and 200,000 cases [16]. *Campylobacter* strains involved in these infections most often include the thermotolerant species *C. jejuni.* Campylobacteriosis generally results from the consumption of contaminated poultry products, with cross-contamination most frequently occurring during processing. Bacterial colonization of the chicken digestive tract begins in the first hours of life and each region of the intestinal tract is colonized by a distinctive microbial population [6,18,48]. Among the species identified in the cecum, *C. jejuni* occurs in more than 90% of commercial chickens [36]. This colonization is asymptomatic and thought to be mediated by the activation of several biological pathways [36].

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Lactic acid bacteria (LAB) constitute important members of the microbial population in chicken intestine, crop, and feces and play an important role in maintaining the ecological equilibrium between the different species of microorganisms inhabiting these environments. Recently, Nazef et al. [31] showed that most LAB found in poultry feces were *Streptococcus* and *Lactobacillus* species, whilst Souza et al. [37] reported the presence of different lactobacilli species in chicken cecum. Attempts to use LAB as a probiotic for poultry have been described. Karimi-Torshizi et al. [25] isolated several LAB strains from the digestive tracts of healthy broilers, among which *Lb. fermentum* TMU121, *Lb. rhamnosus* TMU094, and *Pediococcus pentosaceous* TMU457 were considered as potential probiotics due to their antagonistic effects against *Escherichia coli* and *Salmonella* species. Formulations containing lactobacilli strains are commercially available as probiotics for use in poultry and some have recently been used to reduce *Salmonella enterica* serovar Heidelberg in chicks and turkey poults [30]. The ability of LAB to reduce *Salmonella* species has been successfully demonstrated in assays conducted in broiler chicks [22,40]. Zhang and Doyle [50] developed the concept of exclusive culture based on the use of competitive strains. The authors isolated 41 strains of *Lb. salivarius* with strong antagonism against *Salmonella* and *Campylobacter*; however, they did not elucidate the nature of this antagonism.

Probiotics are administered orally to poultry to help the birds fight illness and disease [17,32]. One of the desired features of probiotic strains is the production of broad-spectrum bacteriocins. Probiotics are not the only approach to the control of *Campylobacter*; others include phage therapy [9], competitive exclusion [33], vaccines [36], and bacteriocin treatment [38,39,42]. However, probiotics are acceptable and cost-effective alternatives to antibiotics that can help in easing public concerns regarding the emergence of antibiotic resistance [34]. The aim of this study was to isolate LAB present in Tunisian poultry ceca and to identify those with antagonism against *Campylobacter* strains.

Materials and methods

Bacterial strains used in this study. *Campylobacter jejuni* NCTC 11168, *C. jejuni* 81176, *C. coli* CIP 702, and *C. coli* CIP7081 (laboratory collection) were stored at –80°C in brain heart infusion (BHI) medium supplemented with 15% glycerol as cryoprotectant. When necessary, the aforementioned strains were recovered on blood agar (SSI) and propagated on Karmali medium with *Campylobacter* selective supplement (Oxoid LTD, Basingstoke, Hants, England). These strains served as indicator organisms for the assessment of bacterial antagonism. *C. jejuni* 11168 and *C. coli* 702 were grown at 41°C for 18 to 24 h in Brucella medium under a modified atmosphere of 85% N_2, 10% CO_2, and 5% O_2. *Lactobacillus salivarius* NRRL B-30514 [38] was used as positive control and was grown anaerobically at 37°C in de Man-Rogosa-Sharpe (MRS) medium [12] for 18 to 24 h. Anoxic experimental conditions were maintained in anoxic jars (Anaerocult A, Merck, Darmstadt, Germany).

Animal sampling and isolation of cecum LAB. The Tunisian chickens (Hubbard: JV, F15, FLEX) used in our study were fed a commercial corn-soy diet (63% corn, 33% soy) containing vitamin-mineral supplementation (CMV, 4%) and vegetable oil (2–3%) devoid of animal protein and growth-promoting antibiotics. Chickens were reared under controlled management conditions (diet, room temperature, cleaning).

Twenty healthy 6-week-old Tunisian chickens were killed by cervical dislocation. The intestines were removed from the carcasses under sterile conditions and transported in isothermic sacks to the laboratory. One gram of each cecal tissue was added to 10 ml of sterile physiological solution (0.85% NaCl) and roughly homogenized in a stomacher. The homogenate was serially diluted to yield dilutions of 10^{-5}, 10^{-6}, and 10^{-7}, with 100 µl of each one plated in duplicate onto MRS agar medium. The plates were incubated in the anoxic jars at 37°C for 48 h. The resulting colonies were first characterized morphologically by microscopy, Gram staining, and the detection of catalase activity. Gram-positive isolates devoid of catalase activity were considered as LAB and used in further studies. All isolates were stored as 40% glycerol stock cultures at –80°C.

DNA extraction and molecular identification of LAB isolates using 16S/2–23S PCR and 16S rDNA sequencing. Total DNA was extracted using the Qiagen DNeasy Tissue Kit (Qiagen, Courtaboeuf, France). Molecular identification was initially carried out by 16S-23S PCR amplification [20] using the primers 16S-p2 (5′-CTTGTACACACCGCCCGTC-3′) and 23S-p7 (5′-GGTACTTAGATGTTTCAGTTC-3′) as previously described [24]. Isolates exhibiting two 16S-23S intergenic spacer region (ISR) fragments, expected to be *Lactobacillus* strains, were subjected to a second round of PCR using the previously designed lactobacilli-specific primers LbLMA1 (5′-CTCAAAACTAAACAAAGTTTC-3′) and R16-1 5′-CTTGTACACACCGCCCGTCA-3′) [14].

Since 16S-23S-PCR amplification allows bacterial identification only at the genus level, the 16S rDNA sequence of each isolate was determined. The respective 16S rDNA genes were first amplified using the primers fD1 (5′-AGAGTTTGATCTGGCTCAG-3′) and rD1 (5′-TAAGGAGGTGATCC AGCC-3′) and a previously described PCR program [49]. The resulting sequences were assembled into a unique contig with BioEdit sequence alignment software and then submitted to the NCBI database. The computer program CLUSTAL [43,45] was used for sequence alignment and the Basic Local Alignment Search Tool 2 program (BLAST) for sequence representation and similarity searches in the GenBank database.

Screening of LAB with antagonism against *Campylobacter* strains. *Lactobacillus* cell-free supernatants obtained by centrifugation (12000 ×*g* for 10 min) from 18-h cultures were adjusted to pH 6.8 with 1 M NaOH, filtered through a 0.22-µm filter (Millipore). Anti-*Campylobacter* bacteriocin activity against *C. jejuni* 11168, *C. jejuni* 81176, C. *coli* 702, and *C. coli* 7081 was evaluated by the agar well diffusion method of Todorov and Dicks [46]. The plates were incubated for 24 h at 37°C in a microaerophilic atmosphere. Antimicrobial activity was then detected by observing the formation of inhibition zones around the supernatant drops. Each supernatant was treated with catalase (5 mg/ml) (Merck, Dijon, France) and proteinase K (0.2 mg/ml) (Boehringer, Mannheim, Germany) to determine the proteinaceous nature of the antimicrobial material. Each supernatant was also heat treated (10 min at 80°C). Neutralization of the supernatant avoided possible erroneous interpretations due to organic acid or hydrogen peroxide production.

Table 1. Antibiotic susceptibilities of *Lactobacillus salivarius* SMXD51 and *Lb. salivarius* NRRL B-30514

Class	Antibiotic tested	Disk load	*Lb. salivarius* SMXD 51	*Lb. salivarius* NRRL B-30514
Penicillins	Penicillin G	10 UI	S*	S
	Ampicillin	10 μg	S	S
	Amoxicillin / Clavulanic acid	20/10 μg	S	S
	Oxacillin	5 μg	S	S
Cephalosporins	Cephalothin	30 μg	R*	R
	Cefoxitin	30 μg	S	S
	Cephalexin	30 μg	S	S
Aminosides	Streptomycin	10 μg	R	R
	Gentamicin	10 UI	S	R
Phenicol	Chloramphenicol	30 μg	S	S
Tetracycline	Tetracycline	30 μg	S	S
Macrolides	Erythromycin	15 μg	S	S
Glycopeptides	Vancomycin	30 μg	R	R
	Spiramycin	100 μg	S	S
Polypeptides	Colistin	50 μg	R	R

*R: resistant; S: sensitive.

Pulsed field gel electrophoresis analysis. Total DNA from *Lb. salivarius* SMXD51, *Lb. salivarius* MMS122, *Lb. salivarius* MMS151, and *Lb. salivarius* NRRL B-30514 was analyzed by pulsed field gel electrophoresis (PFGE). The DNA was prepared in agarose plugs as described previously [44], with the modifications introduced by Jaffres et al. [23].

Antibiotic susceptibility testing. The agar diffusion method was used to determine the antibiotic susceptibility patterns of *Lb. salivarius* SMXD51 and *Lb. salivarius* NRRL B-30514. Eighteen antibiotics from different classes, including penicillins, cephalosporins, aminosides, phenicol, tetracycline, macrolides, glycopeptides, and polypeptides, were tested (Table 1) by suspending the cultures in MRS medium at about 10^7 CFU/ml. These suspensions (1000 μl) were seeded onto MRS agar plates by the flooding technique. The plates were air dried for 15 min, afer which disks impregnated with antibiotics were positioned on the plates. The formation of inhibition zones around the disks was determined after 36 h of incubation at 37°C.

Results

Identification of recovered LAB isolates. Of the 250 colonies obtained on MRS medium, 150 fulfilled the criteria: gram-positive, facultative-anaerobe, and the absence of catalase activity. These were considered LAB isolates and thus subjected to a preliminary molecular identification using the 16S-23S PCR approach, which provides a wealth of information with which to identify microorganisms at the genus level. The different patterns exhibited by our isolates are depicted in Fig. 1A. A nearly identical pattern of two bands (ISRs) of 0.6 and 0.8 kb was seen in 105 of the isolates while in eight of the isolates two bands (ISRs), of 0.65 and 0.7 kb, were detected. Accordingly, the isolates were identified as belonging to *Lactobacillus* and *Enterococcus*, respectively [24]. The 105 isolates were subsequently confirmed by 16S-23S PCR to be lactobacilli strains, based on the use of previously designed primers [14]. These led to the detection of a 0.25-kb fragment (Fig. 1B). Furthermore, in eight LAB isolates, 16S-23S PCR identified a major band of approximately 0.7 kb, considered to be specific for *Leuconostoc* or *Lactococcus* species, whilst 25 LAB isolates with three bands (ISRs) were tentatively identified as *Carnobacterium* or *Weissella* species [24]. This is the first report of a molecular approach to identify LAB isolates from Tunisian chicken ceca. The results were obtained by taking advantage of the robust sequencing of 16S rDNA genes.

Sequencing of the16S rDNA genes of the LAB isolates indicated the presence of *Lb. sakei* (33.3%), *Lb. salivarius* (19.4%), *Lb. reuteri* (8.6 %), *Lb. curvatus* (8.6%), *Weissella* sp. (16.7%), *Leuconostoc mesenteroides* (2.7%), *Lactococcus gravieae* (2.7%), *Enterococcus faecalis* (5.3%), and *Streptococcus* sp. (2.7%). These sequences showed high homology scores (98 and 100%) to sequences available in GenBank.

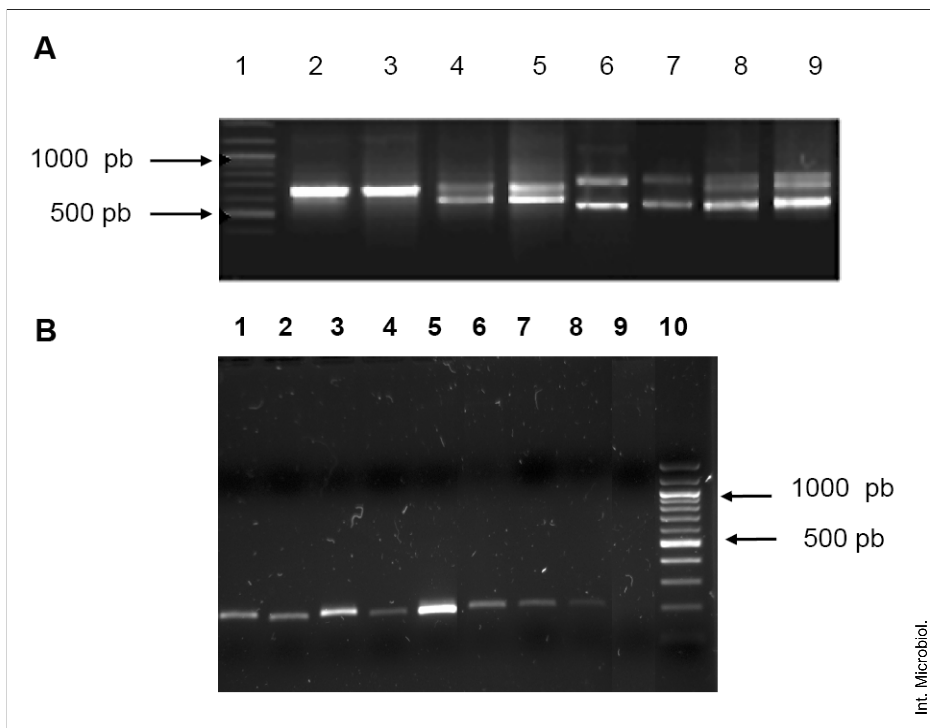

Fig. 1. (**A**) Electrophoresis on a 1.5% agarose gel of PCR-amplified 16S-23S intergenic spacer regions (ISRs) of the lactic acid bacteria (LAB) isolates. Lanes 1 and 9: molecular weight markers (100-bp DNA ladder); lanes 2 and 3: *Leuconostoc* isolates; lanes 4 and 5: *Enterococcus* isolates; lanes 6 and 7: *Lactobacillus* isolates; lanes 8 and 9: *Weissella* isolates. (**B**) PCR amplification of *Lactobacilli* species with primers specifically designed for this genus. Lanes 1 to 8: PCR profiles of LAB isolates belonging to *Lactobacillus* species, as determined based on 16S-23S PCR amplification. Lane 9: positive control (PCR on *Lactobacillus* strain); lane 10: molecular weight markers (100-bp DNA ladder).

Anti-*Campylobacter* assessment. Three LAB isolates, *Lb. salivarius* SMXD51, *Lb. salivarius* MMS122 and *Lb. salivarius* MMS151, that demonstrated anti-*Campylobacter* activity were identified. The activity of each supernatant was not affected by the addition of catalase but was abolished by proteinase treatment. Each supernatant was heat stable, since the activity against *Campylobacter* was still present after 10 min incubation at 80°C (Fig. 2). Thus, the capacities of the LAB isolates to inhibit *C. jejuni* or *C. coli* strains were due to the production of bacteriocins.

Genotype patterns of anti-*Campylobacter* strains. The genotype of *Lb. salivarius* SMXD51, *Lb. salivarius* MMS122, *Lb. salivarius* MMS151, and *Lb. salivarius* NRRL B-30514 was determined by PFGE. Similar genetic patterns were obtained for *Lb. salivarius* SMXD51, *Lb. salivarius* MMS122, and *Lb. salivarius* MMS151 but not for *Lb. salivarius* NRRL B-30514, indicating that our LAB isolates are identical but distinct from *Lb. salivarius* NRRL B-30514 (Fig. 3). Attempts to amplify total DNA isolated from *Lb. salivarius* NRRL B-30514 and *Lb. salivarius* SMXD51 with primers 5′-ACNAAYGGNGTNCAYTGYAC-3′ and 5′-TRTCYTGNAGNCGNCCCCAT-3′, recently described [47], were unsuccessful. It should be noted that the aforementioned primers were designed from bacteriocin OR-7 and not from its codifying DNA.

Antibiotic resistance. *Lactobacillus salivarius* SMXD51 and *Lb. salivarius* NRRL B-30514 were tested against 15 antibiotics of eight classes. These strains were sensitive to the majority of the antibiotics, with resistance observed only in the case of cephalothin, streptomycin, vancomycin, and colistin.

Discussion

The composition and dynamics of the intestinal microbiota contribute positively to host health, growth, and maturation in part by acting as a barrier to colonization by pathogens.

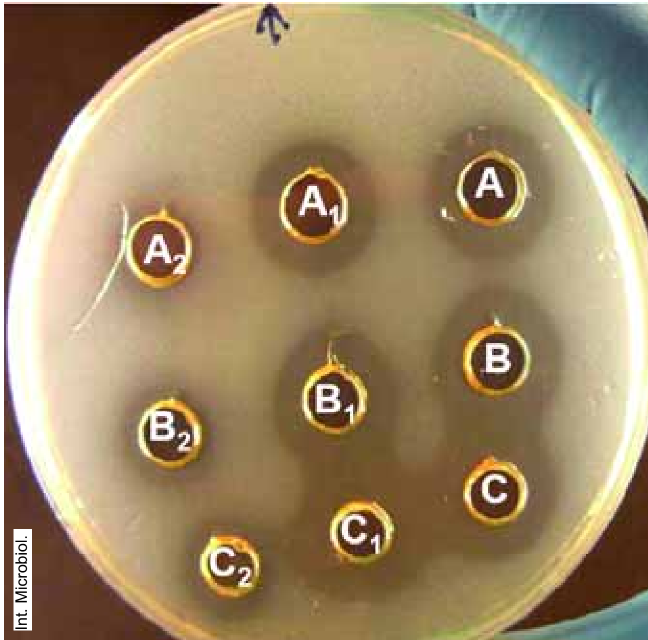

Fig. 2. Agar well diffusion test showing inhibition of *Campylobacter jejuni* 11168 by supernatants prepared from *Lactobacillus salivarius* SMXD51, *Lb. salivarius* MMS122, and *Lb. salivarius* MMS151. **A, B, C**: Supernatants of *Lb. salivarius* SMXD51, *Lb. salivarius* MMS122, and *Lb. salivarius* MMS151. **A1, B1, C1**: Activity of each supernatant upon heat treatment (10 min at 80°C). **A2, B2, C2**: Activity of each supernatant upon treatment by proteinase K (0.2 mg/ml).

The use of antibiotics is an issue of current concern because of the emergence of antibiotic resistance in human and zoonotic pathogens. This, in turn, has led to recommendations for the strict control of antibiotics administered as prophylactic agents. Moreover, the effects of antibiotics on the intestinal microbiota and ultimately on feed conversion as well as on the growth and health of animals of agricultural importance are not completely understood.

The aim of this study was to establish a repertory of LAB present in the ceca of Tunisian chickens and then to investigate the antagonism of these bacteria against *C. jejuni* and *C. coli.* The bacteriocins with anti-*Campylobacter* activity reported in the literature have often been shown to be produced by lactobacilli strains. Among the LAB isolates obtained in this study, *Lb. salivarius* SMXD51, *Lb. salivarius* MMS122, and *Lb. salivarius* MMS151 were able to inhibit *C. jejuni* and *C. coli*. Similar patterns of resistance by *Lb. salivarius* SMXD51 and *Lb. salivarius* NRRL B-30514 to the antibiotics tested were also determined. Although antibiotic resistance has been reported in some lactobacilli strains [35], the nature of the resistance in our strains needs to be further characterized. Natural bacterial resistance to antibiotics is not considered to pose a risk to animal or human health, in contrast to acquired resistance, which is known to be propagated by DNA elements such as plasmids and transposons.

Campylobacter jejuni and *C. coli* are usually isolated from poultry and both species have been frequently associated with human campylobacteriosis [10]. LAB produce a wide range of bacteriocins with antagonism against against gram-negative bacteria, including *Campylobacter*. Stern et al. [39] pioneered research in this field by isolating and characterizing bacteriocin OR-7 from *Lb. salivarius* NRRL B-30514. In

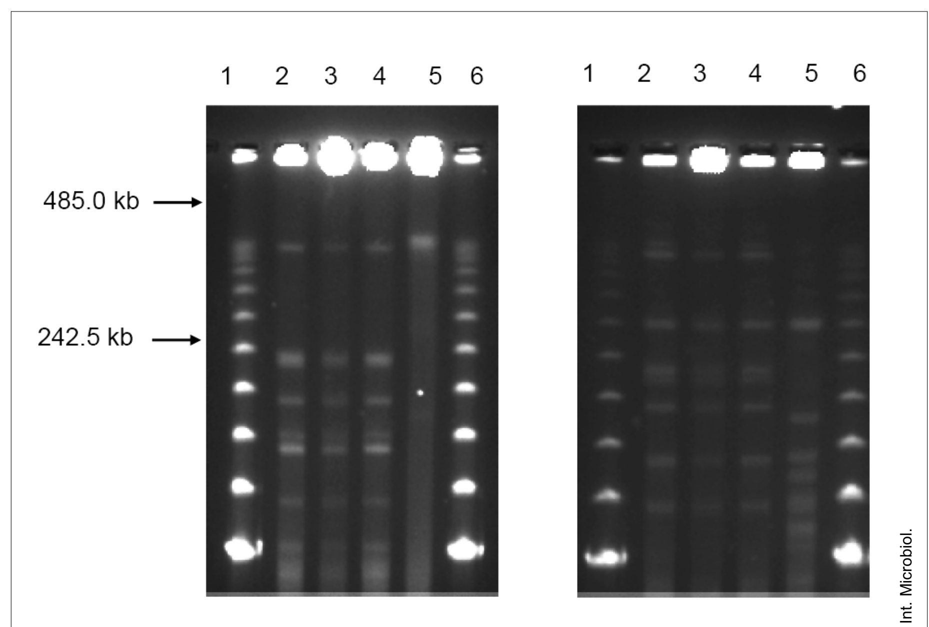

Fig. 3. PFGE patterns of total DNA of *Lactobacillus salivarius* strains after digestion with ApaI (**A**) and SmaI (**B**). Lanes 1 and 6: DNA molecular markers. Lane 2: *Lb. salivarius* MMS 122; lane 3: *Lb. salivarius* SMXD51; lane 4: *Lb. salivarius* MMS 151; lane 5: *Lb. salivarius* NRRL B-3051.

addition to the in vitro antagonism of *C. jejuni*, OR-7 was shown to significantly reduce colonization by this species in chickens by as much as one-million fold. These findings have encouraged further screening of LAB bacteriocins. Recently, bacteriocin E 50-52, produced by *Ent. faecium* NRRL B-30746, was shown to reduce colonization by *C. jejuni* as well as by *Sal. enteritidis* in chicken ceca, liver, and spleen [41].

Natural molecules with antagonism against Gram-negative bacteria, including *C. jejuni,* can provide a cost-effective alternative to the use of antibiotics, especially given current concerns by the scientific community and by the public over the emergence of antibiotic resistance [32,34]. Antimicrobial peptides known to be active against *C. jejuni* thus far include reuterin [2] and bacteriocins B602 [38], OR7 [39], E50-52 [41], and L-1077 [42]. Note that both garlic [29] and an extract from *Eucalyptus occidentalis* [27] are among the non-LAB substances reported to anti-*Campylobacter* activity. The antagonism attributed to garlic is apparently mediated by alterations in the protein, lipid, and polysaccharide content of *Campylobacter* cell membranes [27] while that attributed to the *Eucalyptus* extract, which has very low minimal inhibitory concentration values, remains to be clarified [25].

Understanding the microbial ecology of chicken gut is an important issue in the development of exclusive cultures or probiotics. While Zhang et al. [50] isolated 41 strains of *Lb. salivarius* with strong antagonism against *Salmonella* and *Campylobacter*, they did not demonstrate the nature of this antagonism. In a study by Souza et al. [37] of chickens bred either under intensive or extensive rearing conditions, the LAB isolates were mainly *Lactobacillus* strains and, to a lesser extent, *Enterococcus* spp. Among the former, *Lb. acidophilus* were identified under extensive conditions, and *Lb. reuteri, Lb. crispatus, Lb. vaginalis*, *Lb. agilis*, and *Lb. johnsonii* under intensive conditions.

The complex microbiota of the poultry cecum includes not only *C. jejuni* [7] but also high numbers of gram-positive cocci, which are challenged by *Eubacterium* and *Clostridium* spp. [4,5]. A review dedicated to the bacterial population in the digestive tract of chickens [19] reported a predominance of *Lactobacillus* strains (68.7%) in the ileum and jejunum followed up by strains of *Streptococcus* (6.6%) and *Enterococcus* (6.4%). According to the same review [19] these proportions are dramatically different in the cecum, with *Lactobacillus* strains contributing only 8.2%, *Streptococcus* strains 0.7%, and *Enterococcus* strains 1% of the bacterial population. However, different authors have reported different findings regarding the composition of the microbiota in the chicken digestive tract. According to Bjerrum [6], the lactobacilli comprise only a small proportion (5 to 6%) while Dumonceaux et al. [15] have found a large number of lactobacilli strains (25%) with a high degree of diversity. In addition, the two studies also identified different species, with Bjerrum et al. [6] reporting the presence of *Lb. salivarius, Lb. agilis* and *Lb. kitasatonis* and Dumonceaux et al. [15] *Lb. crispatus*, *Lb. buchneri, Lb. johnsonii, Lb. vaginalis* and *Lb. salivarius* subsp. *salivarius*. In our work, the predominant cecal isolates of LAB were *Lb. sakei, Lb. salivarius, Lb. reuteri*, and *Lb. curvatus*. Other, non-lactobacilli species included *Weissella* sp. (16.6%) and *Enterococcus* sp. (5.5%). The presence of *Weissella* sp. in chicken ceca together with lactobacilli strains such as *Lb. delbrueckii*, *Lb. acidophilus*, *Lb. crispatus*, *Lb. reuteri*, and *Lb. aviarius* has been also reported by Lu et al. [28].

The bacterial diversity characteristic of the cecum is of major importance in mediating interactions, such as cell-cell communication, among the members of the intestinal microbiota and with colonizing pathogens. However, the exact composition of the microbiota differs depending on the age, rearing environment, and diet of chickens [26,28]. Research on the ceca of Tunisian chickens revealed the presence of *Lb. sakei*, one of the predominant food-associated *Lactobacillus* species reported in human feces [8] and in the intestines of salmonids [3] and chicken [13] but not in mammals (besides humans). *Lb. sakei* CTC 494 is a potential probiotic strain because of its high degree of adhesion to chicken intestinal epithelial cells, its antagonistic activity against some food-borne pathogens, including *L. monocytogenes*, and its capacity to decrease biogenic amine accumulation during sausage fermentation [22]. In this study, the antagonism against *Campylobacter* shown by the lactobacilli strains isolated from the chicken cecum could be attributed to the production of bacteriocin-like substances. The purification and characterization of the putative bacteriocin produced by the most active strain, *Lb. salivarius* SMXD51, are currently being pursued in our laboratories.

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Competing interests. None declared.

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