Inhibitory effects of *Lactobacillus salivarius* and *Lactobacillus crispatus* isolated from chicken gastrointestinal tract on *Salmonella enteritidis* and *Escherichia coli* growth

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Abstract
Probiotics are live cultures of microbes; often lactic acid bacteria, but also some other species, which when fed to animals, improve their health and growth through altering the intestinal microbial balance. In the present research, healthy chickens' gastrointestinal (GI) tracts were screened for the presence of lactic acid bacteria with probiotic properties. The probiotic properties of the isolates taken from different parts of the GI tract were evaluated. They were examined for resistance to 2% (w/v) bile salts and acidic pH, capability to adhere to the intestinal epithelium and inhibitory effects on the growth of *Salmonella enteritidis* and *Escherichia coli*. Fermentation profile analyses and sequencing data of the conserved 16S rRNA genes showed that from a total of five selected clones, four clones isolated from the duodenum and caeca were *Lactobacillus salivarius* and the fifth clone, isolated from the duodenum, was *Lactobacillus crispatus*. All the selected clones were able to adhere to the chicken's epithelial cells. The lactobacilli isolated from different parts of the GI tract had probiotic properties suitable for use in animal feed. Due to the inhibitory effects of the isolated lactic acid bacteria on the growth of pathogenic microbiota, it can be concluded that these bacteria are good candidates for treatment of chicken GI infectious diseases.

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INTRODUCTION

The change in the bacterial population of the chicken's digestive tract depends on the its age and diet. In poultry, the lack of intestinal microbiota, as observed in newly hatched chicks, has been considered as a major factor in the susceptibility of chicks to bacterial infections (Netherwood *et al*., 1999; Patterson and Burkholder, 2003; Tierney *et al*., 2004). Chicks, reared under traditional animal husbandry methods, when exposed to faecal bacteria from healthy adult chickens, acquire early protection against enteropathogens (Nurmi and Rantala, 1973). Digestive microbiota have been shown, by the use of media-based culture techniques, to develop from simple populations in young chicks to more diverse mixtures in mature birds. The sterile gut of chicks will be colonized as early as 3 to 6 h after hatching as anaerobic bacteria break down uric acid. Streptococci and enterobacteria are the next species to colonize the small intestine and caeca within 2 to 4 days, post-hatch. *Lactobacillus* spp. have been found to predominate the small intestine and anaerobes, such as *Escherichia coli* and *Bacteroides* are the main bacteria that colonize the caeca after the first week (Mead, 1989).

In the early years of the 20th century, Elie Metchnikoff had recorded, amongst his wide-ranging observations of factors affecting longevity, that Bulgarians consumed large amounts of fermented milk as part of their diet and that they were known to reach advanced ages by the standards of the day (Metchnikoff, 1908). In a time of relatively unsophisticated knowledge of the large bowel ecosystem, it was a small leap of faith to assume that the consumption of fermented milk would "implant" lactic acid-producing bacteria in gut and that these bacteria would proliferate and suppress the growth of putrefactive bacteria, just as had been observed with milk in the laboratory (Tannock, 2003).

Following these observations, big efforts were made with regard to identifying pathogenic and food fermenting bacteria. These culminated in discovering the impact of commensal gut microbiota on health, leading to the concept of probiotic bacteria. Probiotics are live micro-organisms, generally regarded as safe (GRAS) for human and animal use. Administration of probiotics in human and animal models has proven to be beneficial in the treatment and prevention of intestinal infections and in the reduction of mucosal inflammation (Fuller and Gibson, 1997; Chen *et al*., 2009). Improved weight gain, increased egg production and decreased incidences of infections are amongst the several claims that have been made in association with these products (Netherwood *et al*., 1999).

Dietary supplements for farm animals started and scaled-up rapidly in the 1960s and in the following years, lactic acid-producing bacteria were included as health-promoting agents. The *Lactobacillus* spp, members of Gram-positive lactic acid bacteria, are also GRAS. They are potentially food-grade, a status corroborating their particular suitability for oral therapeutic applications. The effectiveness of selected *Lactobacillus* strains to prevent and treat infectious bacterial and viral diarrhea, *Helicobacter pylori* derived gastroenteritis, and urovaginal infections has been demonstrated in well-designed *in vitro* and *in vivo* experimental studies (Isolauri *et al*., 1991; Hume *et al*., 2003).

In chickens, *Campylobacter*, *E. coli* and *Salmonella* are the main pathogenic microorganisms that colonize the intestinal tract. These colonizers do not cause serious illness in birds, but *Salmonella*-infected chickens are pathogenic to humans, causing illness and even death through contaminated egg yolks or meat (Mishu *et al*., 1991; Saelinger *et al*., 2006). The objective of this study was to isolate and characterize lactic acid bacteria with inhibitory effects on the growth of *E. coli* and *S. enteritidis*, from the chicken gut.

MATERIALS AND METHODS

Isolation of bacteria: Two healthy 14-21 days old line 7_2 chickens were selected. These chickens were fed traditionally and housed at an ambient temperature of between 21 to 22°C. Different parts of the gastrointestinal (GI) tract of were isolated. Each part was separately washed in sterile phosphate buffer saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). The entire tract was cut into smaller sections, transferred to 50 ml sterile tubes containing sterile PBS and vortexed vigorously for 1 min. The sections of tissue were then removed and the supernatants were centrifuged at 4°C for 20 min at $6000 \times g$. The supernatant was removed and the precipitates was diluted (1:100), cultured on Rogosa agar plates (Merck, Germany) and incubated for 48 h in an anaerobic jar at 35 ± 2°C. This procedure was separately performed for each part of the GI tract (gizzard, intestine and caeca). The cultured plates were checked after 48 h and seventy colonies representing different parts of the GI tract were randomly selected and sub-cultured onto new Rogosa agar plates. Each colony was then cultured in de Man, Rogosa and Sharpe (MRS) broth (Merck) under anaerobic condition at 37°C. The resulting cultures (in MRS broth) were then stored at -70°C for further usage.

Assessment of probiotic characteristics: Criteria for screening of *Lactobacillus* strains for use as probiotics include functional characteristics such as the ability to resist environmental conditions found in the GI tract (low gastric pH and bile salts), ability to adhere to host intestinal mucosa and antagonize or competitively exclude pathogens by secretion of antimicrobial substances or to compete for nutrients and adhesion sites (Blom and Mørtvedt, 1991; Abee *et al*., 1994; Jacobsen *et al*., 1999).

Sensitivity to bile salts and acidic pH: Resistance to bile salts was checked by the ability of isolates to grow on Rogosa agar containing 2% (w/v) bile salt (Merck). The cultures were checked after 48 h of anaerobic incubation at 37ºC. Resistance to acidic pH (pH 3.0) was checked by centrifuging the overnight cultures of the isolated bacteria at 6000 ×g for 15 min at 4°C and

re-suspending the pellets in the same volume of 0.9% (w/v) NaCl, pH 3.0. The suspensions were then incubated at 37°C for 3 h under anaerobic conditions. After centrifuging the cultures, the resulting pellets were plated onto Rogosa agar and incubated anaerobically at 37°C for 48 h.

Inhibitory activity: The overnight cultures of the isolated bacteria in MRS broth were centrifuged at 4°C as described above and the pellets were streak-cultured on brain heart infusion (BHI, Merck) agar plates cultivated with *E*scherichia *coli* and *Salmonella enteritidis* and were incubated overnight at 37°C. The growth inhibition zones were checked after 48 h. In an alternative test, the inhibitory effects of the isolated supernatants on the growth of *E. coli* and *S. enteritidis* in BHI broth were also evaluated.

In vitro adherence of selected isolates to epithelial cells: The ileum segments from the 2-10 days old chickens were opened and washed five times with sterile PBS (pH 7.2). The epithelial cells of ileum segments were scrapped into sterilized PBS by a cell scraper. The cell suspensions were examined by microscopy to ensure that the epithelial cell concentration was adjusted to 5×10^5 cells/ml and that there were no adherent bacteria on the surface of the epithelial cells For this purpose, a human squamous cell line (A431) was used as a negative control. The adherence of the putative lactic acid bacteria strain to the epithelial cells was assayed as follows: the overnight cultures of lacteic acid bacteria were centrifuged and the cell pellets were resuspended in PBS (pH 7.2) leading to approximately 0.5×10^9 CFU/ml. One ml of each of the selected lactic acid bacteria suspensions was mixed with 1ml of the eukaryotic cell suspension. The mixtures were shaken gently in a glass plate at 37°C for 1 h. Adhesion was observed under a 200x magnification by phase contrast microscopy, after staining by 0.5% (w/v) crystal violet for 5 min.

Strain identification

Gram staining: Selected clones were stained by Gram staining reagents (Merck) using standard procedures (Sambrook and Russell, 2001).

Molecular characterization: I) Preparation of DNA template: the selected isolates were cultured in MRS broth for 16 h at 37°C. After centrifugation at 6000 ×g at 4°C for 15 min, the pellets were washed twice in PBS. Genomic DNA was extracted using the DNA iso-

lation kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. II) Polymerase chain reaction (PCR) procedure for 16S ribosomal RNA: The 16srRNA gene is used for phylogenetic studies as it is highly conserved between different species of bacteria and archaea. 16S rRNA was partially amplified using *Lactobacillus* 16S rRNA specific primers by PCR. The primers, For-mehr-lac (5´-AGAGTTTGATC-CTGGCTCAG-3´) and Rev-mehr-lac (5´-AAGGAGGT-GATCCAGCCGCA-3´), were designed in our lab using published sequence data available in sequence databases. The online Internet tools including ClustalW, Fasta3 and MEGA4 were used for identifying the primers with desired specificity towards a conserved domain of the *Lactobacillus* 16S rRNA sequence (Woo *et al*., 2002). After gel electrophoresis (Sambrook and Russell, 2001) and extraction of the PCR products using the QIAquick gel extraction kit (Qiagen, Germany) they were sequenced. The results were finally checked with Genbank reports (NCBI).

Fermentation profile: The selected colonies of the isolates were cultured on Rogosa agar plates separately. After 24 h of incubation at 37°C, the cultured cells were suspended in API50CHL medium, prepared according to the manufacturer's instructions. The carbohydrate fermentation profiles of the bacterial colonies were checked by the API50CH kit (BioMérieux, France). A positive test was indicated by a color change from purple to yellow due to anaerobic production of acid. For the esculin test, a color change from purple to black was considered as a positive case. The homogenized suspension of each isolate was transferred into a tube of kit and covered with sterile mineral oil (Sigma, USA) to maintain anaerobic conditions. All inoculated trays were incubated at 35 ± 2°C and checked after 24, 48 and 72 h for changes in color.

RESULTS

Isolation of bacteria: After 48 h of anaerobic incubation at 37°C on Rogosa agar plates, over 1000 colonies of putative lactic acid bacteria were isolated from chickens' GI tract including gizzard, intestine and caeca. A total of 70 colonies from each section of the GI tract were randomly selected for further investigation.

Inhibitory characteristics: The selected isolates

could all grow in MRS medium containing 2% w/v bile salt and were viable at pH 3.0. Upon screening of the colonies for inhibitory effects on the growth of *E. coli* and *S. enteritidis*, the bacterial cultures (*S. enteritidis* and *E. coli*) with 0.5 and 4 McFarland turbidities were produced in liquid medium supplemented with 50% supernatant of the selected clones and without supplementation, respectively. In these inhibitory assays, 31 colonies inhibited the growth of *S. enteritidis* and 27 colonies inhibited that of *E. coli*. Among these isolates, 5 colonies with the strongest inhibitory activity on the growth of both *S. enteritidis* and *E. coli* were chosen for further investigations (Table 1). No isolates from the gizzard or crop showed any inhibitory effects on the growth of these two pathogenic strains. In evaluation of the adhesiveness properties of the isolates with regard to the ileum segments, those bacteria showed an adhesion efficiency of at least 20 bacterial cells per epithelial cell.

Strain identification: Gram staining results showed that all of the selected colonies (C2, C3, C5, C14, C15) were Gram-positive bacilli. Genomic DNA extracted from each selected colony was used as template in the PCR procedure. The PCR products showed a 670 bp band on 1% (w/v) agarose gel (Fig. 1). The 670 bp bands extracted from the gel were sequenced. According to the results of 16S rRNA sequencing, four out of the five selected colonies were genetically identified as *Lactobacillus salivarius* (C15, C3, C2 and C5), of which two (C15 and C3) originated from the small intestine and the other two (C2 and C5) from the caeca and duodenum, respectively. The fifth colony (C14), isolated from the duodenum was identified as *Lactobacillus crispatus*.

Fermentation profile for these isolates was obtained by the API50CH fermentation assay. There were some differences in the fermentation patterns of the selected isolates. The fermentation profiles confirmed the identity of the genetically characterized strains. Interestingly, the strains of *L. salivarius* isolated from caeca showed different fermentation patterns when compared to those isolated from the small intestine. (fermentation pattern of C15 was D-Xylose[+], Amygdalin[+], Arbutin[+], Salicin[+], D-Maltose[+], D-Celiobiose[+], Tagatos[+], L-Rhamnose[-], D-Melezitose[-], Xylitol[-], Potassium Gluconate[-], and C5 showed the converse results for these).

DISCUSSION

Feeding farm animals with sub-therapeutic levels of

Table 1. The probiotic characteristics of the selected isolates.

Isolates	Probiotic characteristics			
	Resistance to		Inhibitory activity	
	Bile salts	Acidic pH	*E. coli*	*S. enteritidis*
C15	+	+	4+	3+
C3	+	+	4+	4+
C14	+	+	4+	4+
C5	+	+	3+	4+
C2	+	+	4+	4+

Resistance to bile salts and acidic pH are shown by +. Intensity of inhibitory activity to *E. coli* and *S. enteritidis* is shown from + to 4+. C15 and C3, *L. salivarius* and *L. salivarius* originating from the small intestine, respectively; C14 and C5, *L. crispatus* and, *L. salivarius* originating from the duodenum, respectively and C2, *L. salivarius* originating from caeca.

antibiotics has been documented to increase weight gain and improve feed efficiency. The exact mechanism has not been completely understood. Since feed antibiotics provide a relatively greater improvement in farms with poor hygiene, their effectiveness seem to be at least partially due to the suppression of some pathogenic bacteria in livestock. Some scientists believe that this effect on pathogens is the primary or the only relevant effect of the antibiotics affecting growth promotion (Elwinger *et al.*, 1998; Gusils *et al.*, 1999a; Waldenstedt *et al.*, 1999).

Evaluation of the possible alternatives to sub-therapeutic use of antibiotics, one must consider not only their relative short- and long-term costs but also their ability to produce the same positive effects as the antibiotics. Interest in the use of probiotics to improve the growth and health of livestock has recently been rekindled by current proposals to curtail sub-therapeutic doses of antibiotics in animal feed. The results of experimental trials with probiotics, initiated 20-30 years ago, have been shown to be inconsistent due to
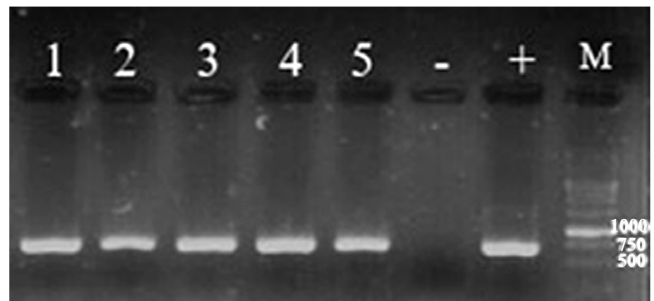

Figure 1. PCR results of 16S rRNA. 1: C15, 2: C3, 3: C14, 4: C5, 5: C2, -: negative control and +: positive controls (*Lactobacillus casei*) and M: molecular weight marker (Fermentas,Germany).

variations in bacterial cultures used, age of pigs treated and other factors related to feed composition and husbandry practices (Bergonzelli *et al*., 2005).

Probiotics, like antibiotics, appear to have a more pronounced effect on farms where housing and hygiene are not optimal (Audisio *et al*., 1999; Gusils *et al*., 1999b; Walter, 2008). Probiotic microorganisms added to the feed may protect farm animals from intestinal pathogens by several possible mechanisms, sometimes referred to as competitive exclusion (CE), which include: adherence to intestinal mucosa, thereby preventing the attachment of pathogens; production of antimicrobial compounds such as bacteriocins and organic acids; competition with pathogens for nutrients, and stimulation of intestinal immune responses. In addition, probiotics may affect the permeability of the gut and increase uptake of nutrients (Salminen *et al*., 1998; Maxwell *et al*., 2004; Collado *et al*., 2006). The following criteria are accepted as indicators for probiotics selection: resistance to acid and bile; attachment to epithelial cells; colonization of the intestine; production of an antimicrobial substance; good growth characteristics and beneficial effects on health (Madsen *et al*., 2001; Ghosh *et al*., 2004; Servin, 2004; Shin *et al*., 2008).

During the recent years, researchers have focused on the investigation of *Lactobacillus* species, such as. *L. plantarum*, *L. reuteri* and *L. salivarius* to protect chickens against *Salmonella*. The growth inhibitory effects of these bacteria have been associated mainly with the production of antimicrobial metabolites (de Vos *et al*., 2005; Olivares *et al*., 2006). PREEMPT is the only probiotic product approved by the food and drug administration (FDA) of the United States of America for use in poultry. It is a mixture of 29 bacterial strains maintained in continuous cultures, freeze dried for administration to one day-old chickens. This probiotic is sprayed onto the birds and the bacterial inocula are then introduced into the gut when they preen their feathers. This mixture of bacteria colonizes the gut of birds and subsequently prevents the establishment of *Salmonella* species, commonly present as a contaminant of poultry feed (Saelinger *et al*., 2006).

In this study, 5 isolates resistant to acidic pH and bile were selected. The results showed that they were part of the microbiota of the GI tract. The results of this study confirmed their ability to adhere to host intestinal mucosa, which is considered as an important selection criterion for probiotic bacteria. Their isolation from the GI tract of healthy chicks, plus their inhibitory effects on the growth of *S. enteritidis* and *E. coli* and good growth characteristics suggested that

they can be used as probiotics in poultry (Vicente *et al*., 2008).

Fuller and Gibson (1997) pointed out that host specificity of the adherence of lactobacilli is closely connected with the presence of some specific receptor molecules in the host cells, which can be recognized by specific molecules in the bacterial cells. The mechanisms behind the antibacterial activity of *Lactobacillus* probiotic strains appear to be multi-factorial (Servin, 2004). This effect probably results from the ability of probiotics to adhere to mucosal surfaces and inhibit the attachment of other pathogenic bacteria, to secrete factors with antimicrobial activity and to modulate cells of the immune system. In particular, by producing metabolites such as acetic and lactic acids, which result in a low pH and antibacterial compounds including bacteriocins, non-bacteriocins and non-lactic acid molecules, *Lactobacillus* strains inhibit the growth of bacterial pathogens and are sometimes able to kill them (Ghosh *et al*., 2004).

Based on the sequence of 16s rRNA and fermentation profiles, the isolates were identified as *Lactobacillus* spp. – regarded as safe for probioics use (Rehman *et al*., 2008). By using preselected "good" microbes, it is hoped that inexpensive and identified bacterial cultures with the ability to reduce or exclude specific pathogens, such as *S. enteritidis* and *E. coli* and improve the health and growth of poultry, can be produced. This study has also presented a selection technique, which makes probiotics production economical.

At present, ongoing studies are being carried out to use these biochemically and genetically identified lactobacilli as antigen delivery vehicles for immunization of poultry against different pathogens.

Acknowledgements

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