Probiotic Potential and Antimicrobial Activity of Enterococcus faecium Isolated from Chicken Caecal and Fecal Samples

Salma H. Abu Hafsa, A. Mendonca, B. Brehm-Stecher, A. A. Hassan, S. A. Ibrahim

Abstract—Enterococci are important inhabitants of the animal intestine and are widely used in probiotic products. A probiotic strain is expected to possess several desirable properties in order to exert beneficial effects. Therefore, the objective of this study was to isolate, characterize and identify Enterococcus sp. from chicken cecal and fecal samples to determine potential probiotic properties. Enterococci were isolated from chicken ceca and feces of thirty three clinically healthy chickens from a local farm. In vitro studies were performed to assess antibacterial activity of the isolated LAB (using agar well diffusion and cell free supernatant broth technique against Salmonella enterica serotype Enteritidis), survival in acidic conditions, resistance to bile salts, and their survival during simulated gastric juice conditions at pH 2.5. Isolates were identified by biochemical carbohydrate fermentation patterns using an API 50 CHL kit and API ZYM kits and by sequenced 16S rDNA. An isolate belonging to E. faecium species exhibited inhibitory effect against S. enteritidis. This isolate producing a clear zone as large as 10.30 mm or greater and was able to grow in the coculture medium and at the same time, inhibited the growth S. enteritidis. In addition, E. faecium exhibited significant resistance under highly acidic conditions at pH 2.5 for 8 h and survived well in bile salt at 0.2% for 24 h and showing ability to survive in the presence of simulated gastric juice at pH 2.5. Based on these results, E. faecium isolate fulfills some of the criteria to be considered as a probiotic strain and therefore, could be used as a feed additive with good potential for controlling S. enteritidis in chickens. However, in vivo studies are needed to determine the safety of the strain.

Keywords—Acid tolerance, antimicrobial activity, Enterococcus faecium, probiotic.

I. INTRODUCTION

The poultry industry is relatively more efficient than red meat in providing a cheap protein source to fulfill Egyptian population requirements. Investment in this industry has risen to high levels in the last two decades [1]. Probiotics defined as cultures of potentially beneficial bacteria that positively affect the host by regulating the microbial balance and by restoring the normal intestinal permeability and gut micro-ecology [2]. The consumption of probiotics has beneficial effects, including balancing of colonic microbiota, protection of the normal intestinal microbiota and prevention of gastrointestinal disorders, reduction of serum cholesterol, antagonism against food-borne pathogens and improvement in the nutritional value of foods [3]-[5]. In broiler nutrition, probiotic species belonging to Lactobacillus, Bacillus, Bifidobacterium, Enterococcus, have a beneficial effect on broiler performance [6]. Among Enterococcus species, E. faecium is the most widely used in commercial probiotics. E. faecium are lactic acid bacteria (LAB) that have an important role in the environment, food and clinical microbiology. Furthermore, they are regular habitants of the gastrointestinal tract of humans, animals and birds [7]. Addition of probiotics to feed is an interesting alternative to the use of antibiotics, which have created great public concerns due to emergence of antimicrobial resistance [8]. Several studies have mentioned the use of E. faecium as probiotic cultures [9]-[11]. Their ability to survive and compete in the gastrointestinal tract allows their successful use. E. faecium shows effects against enteropathogens [12] and may be useful in animal health [13].

A recent interdisciplinary research study of the modes of action of probiotics in swine showed that E. faecium NCIMB 10415 reduced the pathogenic bacterial load of healthy piglets [14], [15]. In vitro studies further demonstrated that this E. faecium probiotic strain decreased the rate of invasion of a porcine intestinal epithelial cell line by Salmonella enteric serovar Typhimurium, as well as have inhibitory effects on the growth of S. enteric serovar Enteritidis and these effects were explained by both enterotoxin and nonenterotoxin factors [16]. E. faecium has also been shown to influence the composition of the bacterial community in the avian, swine, and canine gastrointestinal tracts [17]. Infections with S. enteric are some of the most important sources of human gastroenteritis [18]. Thus, the aim of this study was to characterize the probiotic potential of the E. faecium LAB isolated from chickens ceca and feces through acid and bile salts resistance, their survival during simulated gastric juice conditions, and their antimicrobial activity against Salmonella enteritidis for use as probiotic in poultry.

II. MATERIALS AND METHODS

A. Origin of Isolate, Identification and Storage Conditions

An isolate belonging to E. faecium species, isolated from thirty three clinically healthy chicken cecal and fecal samples...
collected from a local farm located in Ames, Iowa state, USA, were selected. The isolate was identified by biochemical carbohydrate fermentation patterns using an API 50 CHL kit and API ZYM kits (Biomerieux, Lyon, France), and by sequenced 16S rDNA using the Big Dye terminator cycle sequencing kit (Applied BioSystems, DNA Facility, Molecular biolig Building, ISU, Ames, Iowa, USA), and sequences were resolved on an automated DNA sequencing system (Applied BioSystems model 3730 DNA analyzer). The 16S rDNA sequence of each strain was aligned to the 16S rDNA gene sequence of LAB and other related taxa in order to compare the levels of similarity. They presented some intermediate antibiotic susceptibilities determined by antibiotic discs method. The isolate used in this work were grown on de Man Rogosa and Sharpe (MRS) agar medium at 37°C for 24 h and stored at -70°C in MRS broth containing 20% (v/v) glycerol and sub-cultured twice before use in assays.

B. Acid Tolerance

The resistance under acid conditions was carried out according to [19] with some modifications. E. faecium cells were grown in MRS broth at 37°C for 24 h, then were centrifuged at 4500 rpm at 4°C for 15 min. The pellet was collected in a sterile tube and was washed twice with phosphate buffered saline (PBS); one milliliter of culture was resuspended in 10 ml of sterile MRS broth, pH 7.0 before inoculation in MRS broth adjusted to pH 2.5 (by addition of 1 M HCI), in which pH 7.0 was used as a control. Viable cell counts were determined after exposure to acidic condition for 2 and 8 h at 37°C. The experiment was performed in duplicate. The surviving cells were counted by plating on MRS agar. Survival cell counts were expressed as log values of colony-forming units per ml (CFU/ml). The survival percentage was calculated as follows: % survival = final (CFU/ml) /control (CFU/ml) ×100.

C. Resistance to Bile Salts

Bile salt tolerance was performed as described by [20]. Initially, overnight cultures in MRS broth of E. faecium were harvested at 4500 rpm at 4°C for 15 min and washed twice with PBS. One milliliter of cells was added into tubes containing 10 ml of sterile MRS broth supplemented with 0.2% bile salts (Sigma). Total viable counts were determined after exposure to bile salts at 3, 6 and 24 h incubation at 37°C; by pour plate method after serial dilutions of the sample and incubation at 37°C for 24 h. Values were expressed as log CFU/ml [21].

D. Survival in Simulated Gastric Juice

Survival in simulated gastric juice on the growth of E. faecium was performed according to [22]. After 24 h of incubation in MRS medium, bacterial cells were harvested by centrifugation (4500 rpm for 15 min at 4°C), washed twice with 0.1 M PBS and suspended in 0.5% NaCl solution. Then 0.5 ml aliquot of bacterial suspension was inoculated into 1.0 ml of simulated gastric and incubated at 37°C for 4 h. Also an aliquot of 0.5 ml of inocula was placed into a glass flask with 49.5 ml of buffered peptone water (without pH adjustment or pepsin) as control. Survival cell counts were determined at initial time (0 h) and 1, 2, 3 and 4 h for the gastric tolerance. Simulated gastric juice was prepared fresh daily containing 3 mg of pepsin (Sigma), 1 ml of NaCl solution (0.5%) and acidified with HCl to pH 2.5. The solution was sterilized by filtration through 0.45 mm membranes (Millipore, Bedford, USA). The viable counts were determined by the plate drop method on MRS agar (modified from [23]) values were expressed as log CFU/ml.

E. Inhibitory Effects of Isolated LAB Against S. enteritidis

1. Agar Well Diffusion

The surface of a plate containing MRS agar was swabbed with TSB containing S. enteritidis 106 cfu ml-1. Four wells each 6 mm in diameter were made in the agar plate and (25, 50, 75 and 100 µL) of the culture supernatant of E. faecium LAB were transferred into each well. The plates were incubated at 37°C for 24 h. Each plate was examined for clear inhibition zones around the wells. Diameter of the clear zone was measured by using a vernier caliper.

Preparation of culture supernatant: E. faecium strain from the stock was grown in the tube containing MRS broth twice, incubated at 37°C for 24h and then centrifuged at 4500 rpm for 15 min at 4°C. The supernatant of E. faecium strain was filtered through filter paper (pore size, 0.45 micron), then pH of two tubes for each tubes were measured, the first tube was adjusted up to pH = 6 but the second was measured without adjusting pH.

Preparation of S. enteriditis: In this study, S. enteriditis was provided by (Food Science and Human Nutrition Department, College of Agriculture and Life Science, ISU, USA), S. enteriditis was grown in Tryptic Soy Agar TSA (Difco, Becton Laboratories, Detroit, MI, USA) at 37 °C for 24 h until reach concentration of 109cfu/ml. and then diluted to 106cfu/ml. (equivalent to MacFarland standard No. 0.5) for further use.

The supernatant of E. faecium strain for each tube (pH adjusted and not pH adjusted) were inoculated with 106 cfu ml. of S. enteritidis. The tubes were incubated at 37°C for 2, 4 and 8 h. Then, the serial 10-fold dilutions were plated on TSA to evaluate the S. enteriditis growth. The TSA plates were incubated at 37°C for 24h. Pure cultures of each strain were also subjected to the same conditions and used as controls.

2. Co-Culture Growth Curves

A bottle containing 5 ml of MRS broth and 5 ml of TSB was inoculated with 106cfu ml1 of both E. faecium and S. enteritidis. The tubes were incubated at 37°C for 24 h and then, the serial dilutions were plated on MRS agar to evaluate the E. faecium growth or on XLD agar (Difco, Becton Dickinson, Sparks, MD, USA) to evaluate the S. enteritidis growth. Both MRS and XLD agar plates were incubated at 37°C for 24h. Pure cultures of each strain were also subjected to the same conditions and used as controls. Additionally, pH of the culture solution was measured at 0, 24 and 48 h after coincubation.
### III. RESULTS AND DISCUSSION

One strain of the thirty three isolates, belonging to *E. faecium* species, was selected as probiotic bacteria candidate, and it was Gram-positive, catalase-negative and facultative anaerobic cocci with pair or tetrad cell organization and did not produce gas. Based on comparison of its characteristic with Bergey’s manual and the results of the API test (carbohydrate fermentation test), and the result of 16S rDNA sequences of the *E. faecium* LAP revealed 97% similarity with Ent. faecium ATCC 19434. The isolate was classified as *E. faecium* LAP.

**A. Tolerance to pH, Bile Salts and Gastric Juices**

![Fig. 1 Tolerance of *E. faecium* to bile salt at 0.2 % bile salts for 3, 6, and 24 h at 37°C. control (●); *E. faecium* (■)](image)

The isolate exhibited high tolerance to acidic conditions (Fig. 1). The resistance of the isolate was observed after exposition to acidified media where the presence of colonies was observed in the initial time (>8 log CFU/ml). No significant differences between *E. faecium* LAB (pH 2.5) when compared to the control (pH 7) during the incubation time, ranging between 2 and 8 h. The tolerance of *E. faecium* LAB in the presence of 0.2 % concentration of bile salts was analyzed (Fig. 2). The results showed that *E. faecium* LAB was able to survive at bile salts concentration tested (0.2%) to give an exponential growth from the inoculation (0 h) until 24 h of incubation. There were no significant differences between control (MRS, pH 7, without the addition of bile salts) and with bile salts treatment. The ability of *E. faecium* LAB to survive in the presence of simulated gastric juice was tested by incubation for 4 h at 37°C (Fig. 3). It was observed that simulated gastric juice at pH 2.5 caused no significant differences in cell viability when compared to the control in any of the evaluated times. Similar values of viable cell counts of *E. faecium* LAB were observed during 4 h incubation in simulated juice and control.

The major factors determining the survival of probiotic bacteria include particular characteristics of the strains (e.g., acid and bile tolerance, and resistance to gastric juice), composition of food ingested, and competition of microbiota in the intestine [24]. In order to select isolates with probiotic characteristics, the resistance to pH and bile salts is an importance factor in survival and growth of bacteria in the gastrointestinal tract. Our results showed that *E. faecium* LAB has acid and bile tolerance, surviving to exposure in pH 2.5 and in 0.2 % of bile salts tested. Similarly, [25] showed that *E. durans* LAB18s had acid and bile tolerance, surviving in pH 3.0 and 4.0, and similarly, in all concentrations of bile salts tested (from 0.1% to 1.5%). The strain *E. faecium* SF68 retained viability and increased in number between 30 and 60 min of exposure to bovine bile, exhibiting an intrinsic tolerance towards bovine bile [26]. When exposed to simulated gastric juice for 20 and 60 min, *E. faecium* SF68 exhibited a survival rate (62 and 56%, respectively) that would allow it to pass through the stomach. High acidity in the stomach and the high concentration of bile components in the proximal intestine of the host, influence the selection of potential probiotic strains [27]. *E. faecium* demonstrated high ability to survive in the presence of simulated gastric juice containing pepsin (pH 3.0) this study in agreement with [24]. Thus, *E. faecium* survived to gastrointestinal conditions and this tolerance to low acidity in the stomach, bile components and simulated gastric juice is very important to this strain be considered as an alternative source for future probiotic development in poultry industry.

**B. Inhibitory Effects of Isolated LAB against S. enteritidis**

1. Agar Well Diffusion

Antimicrobial activity of *E. faecium* against *S. enteritidis* was measured as the zone of inhibition in agar well diffusion assay and the values are presented in Table I. Culture supernatant from strain *E. faecium* showed the best inhibition
The microbiota of the chicken’s GIT has received increased attention as the focus of efforts to minimise foodborne illness in humans, to improve animal nutrition and to reduce dependence non-therapeutic antibiotic growth promotions [28]. Environment of GIT is suitable for growing of pathogenic bacteria if pH of GIT goes toward the basic [29]. In the present study, _E. faecium_ showed not only strongly inhibited _S. enteritidis_ in various _in vitro_ tests but also capably survived at pH 2.5 for at least 8 h. On the other hand, Salmonella infections are one of the most important public health concerns worldwide. In general, _Salmonella_ species are widely distributed in the environment and cause a diverse spectrum of diseases in human and animals. Poultry products, eggs and meat are considered to be one of the main sources of human foodborne infections caused by _Salmonella_ [30]. Although among the potential probiotics, LAB are reported to have important effects in poultry [31], there are several factors associated with the success of probiotics used in animals [32]. However in this study, we focused specifically on the selection of LAB for anti _S. enteritidis_ which is an importantly zoonotic pathogen causing salmonellosis in humans and animals. _Enterococci_ are used as probiotic bacteria mainly because of their abilities to produce anti-bacterial substances. Bacteriocins differ from traditional antibiotics in one critical way: they have a relatively narrow killing spectrum and are only toxic to bacteria closely related to the producing strain [33]. _E. faecium_ J96, isolated from a healthy free-range chicken, inhibited _S. pullorum in vitro_, due to its lactic acid and bacteriocin production [34]. In this study although, we did not know the exact mechanisms how LAB inhibited _S. enteritidis in vitro_, decrease pH of the supernatant of LAB was associated with increase diameter of inhibition zone. Thus, this result indicated that lowering pH of the supernatant (probably due to lactic acid) might play a role in inhibiting _S. enteritidis_. However, we cannot rule out other mechanisms; for example, _E. faecium_ can produce antimicrobial compounds called bacteriocin (called enterocins). In birds preventedly treated with strain of _E. faecium_ EF 55 reduced presence of the pathogen was observed in caecum, liver and spleen [35]. It is probably due to competitive exclusion of _Salmonella_ in the gut microenvironment by the applied _Enterococcus_ strain. Another mechanism responsible for the lower multiplication of salmonella in birds treated with _E. faecium_ EF55 is production of bacteriocins by this selected strain as shown in the study of [36]. In this study, we also found that _E. faecium_ did survive and was able to inhibit the growth of _S. enteritidis_ in co-culture medium. _Enterococci_ are part of the normal flora of humans, animals and birds, and some of their strains are used for the manufacturing of foods or as probiotics, whereas others are known to cause serious diseases in humans.

For acidic pH tolerance tests, the previous studies normally incubated the bacterial dilutions with acid solution for a few hours [37], [30] but in this study we allowed the incubation time lasting for 8 h in order to keep the number of the selected LAB strains at minimum.

### IV. Conclusion

This study showed that _E. faecium_, one of the LAB isolated from chicken ceca and feaces their ability to survive exposure to acidic conditions, and resistance to bile salts, survive through gastric juice passage, and strongly inhibited _S. enteritidis_ in various _in vitro_ tests indicating its potential for further investigations toward its selection as a source of chicken probiotics.

### References

7. A. Bhardwaj, G. Kaur, H. Gupta, S. Vij, and R. K. Malik, "Interspecies diversity, safety and probiotic potential of bacteriocinogenic properties and the minimum diameter zone of growth inhibition for _S. enteritidis_ was (9.75 mm), while the maximum zone of inhibition was obtained for _E. faecium_ (10.30 mm), thus _E. faecium_ exhibited high antimicrobial activity.

### TABLE I

<table>
<thead>
<tr>
<th>isolate</th>
<th>inhibition zone (mm) of <em>S. enteritidis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>E. faecium</td>
<td>9.75</td>
</tr>
<tr>
<td></td>
<td>10.30</td>
</tr>
</tbody>
</table>

**TABLE II**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>E. F.</th>
<th>pH</th>
<th>S. E.</th>
<th>pH</th>
<th>E. F.</th>
<th>S. E.</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>8.362</td>
<td>6.20</td>
<td>8.362</td>
<td>7.31</td>
<td>8.362</td>
<td>8.362</td>
<td>6.86</td>
</tr>
<tr>
<td>24h</td>
<td>9.170</td>
<td>3.83</td>
<td>8.648</td>
<td>5.93</td>
<td>9.233</td>
<td>ND</td>
<td>3.98</td>
</tr>
<tr>
<td>48h</td>
<td>8.155</td>
<td>3.50</td>
<td>8.732</td>
<td>5.73</td>
<td>7.653</td>
<td>ND</td>
<td>3.84</td>
</tr>
</tbody>
</table>

Values of log cfu mL⁻¹ and pH are the average values from two replicates, ND – Not Determined. _E. F. E. faecium_ and _S. E. S. enteritidis_.

Another mechanism responsible for the lower multiplication of salmonella in birds treated with _E. faecium_ EF55 is production of bacteriocins by this selected strain as shown in the study of [36]. In this study, we also found that _E. faecium_ did survive and was able to inhibit the growth of _S. enteritidis_ in co-culture medium. _Enterococci_ are part of the normal flora of humans, animals and birds, and some of their strains are used for the manufacturing of foods or as probiotics, whereas others are known to cause serious diseases in humans.

For acidic pH tolerance tests, the previous studies normally incubated the bacterial dilutions with acid solution for a few hours [37], [30] but in this study we allowed the incubation time lasting for 8 h in order to keep the number of the selected LAB strains at minimum.


