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.

Salma H. Abu Hafsa is with Livestock Research Department, Arid Lands Cultivation Research Institute, City of Scientific Research and Technological Applications, Alexandria, Egypt (phone: 002-01000313649; e-mail: hashim_salma@yahoo.com)

A. Mendonca and B. Brehm-Stecher are with Department of Food Science and Human Nutrition, Iowa State University, Ames, Iowa, USA

A. A. Hassan is with Animal Production Research Institute, Ministry of Agriculture, Dokki, Giza, Egypt

S. A. Ibrahim is with Food Microbiology and Biotechnology Laboratory, North Carolina Agricultural and Technical State University, 173 Carver Hall, Greensboro, NC 27411, USA.

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 (Biomérieux, Lyon, France), and by sequenced 16S rDNA using the Big Dye terminator cycle sequencing kit (Applied BioSystems, DNA Facility, Molecular biolog 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 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 MHCl), 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 of 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 drop plate 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* 10^6 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^{o}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. enteritidis*: In this study, *S. enteritidis* was provided by (Food Science and Human Nutrition Department, College of Agriculture and Life Science, ISU, USA), *S. enteritidis* was grown in Tryptic Soy Agar TSA (Difco, Becton Laboratories, Detroit, MI, USA) at 37 °C for 24 h until reach concentration of 10^9cfu/ml. and then diluted to 10^6cfu/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 10^6 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. enteritidis* 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 10^8cfu ml^{-1} of both *E. faecium* and *S. enteritidis*. The tubes were incubated at 37°C for 24 h and 48 h. 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

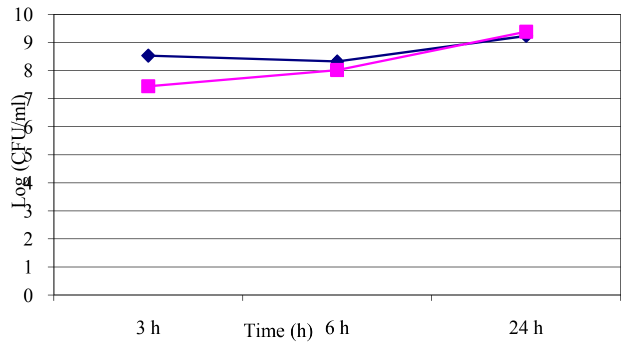

Fig. 1 Tolerance of *E. faecium* to bile salt at 0.2 % bile salts for 3, 6, and 24 h at 37°C. control (♦); *E. faecium* (■)

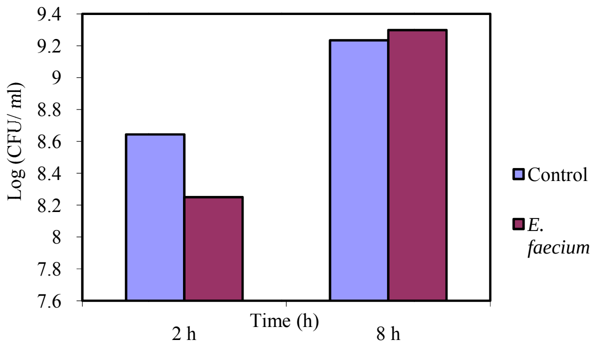

Fig. 2 Acid tolerance (pH 2.5) of *E. faecium* for 2 and 8 at 37°C

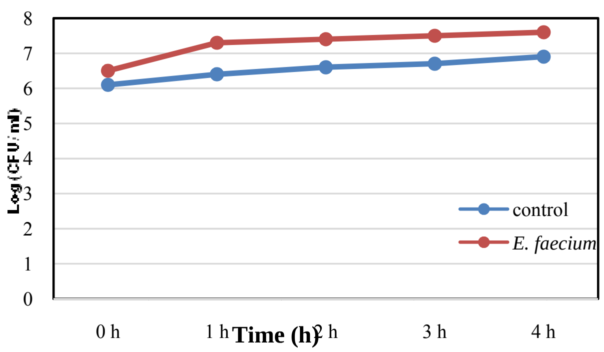

Fig. 3 Resistance to simulated gastric juice of *E. faecium* containing pepsin and acidified at pH 2.5 after 4 h of incubation at 37°C, each point represents the mean ± s.e.m. of two independent experiments

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 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.

2. Co-Culture Growth Curves

Salmonella enteritidis was significantly reduced after co-incubation with the selected *E. faecium* strain for 24 and 48h. However, pH in both coculture and in the pure culture slightly decreased at 24 and 48h after incubation. Data from coculture growth curve study were showed in Table II.

TABLE I
GROWTH INHIBITION OF *S. ENTERITIDIS* BY *E. FAECIUM*

isolate	Inhibition zone (mm) of *S. enteritidis*	
	mini	maxi
E. faecium	9.75	10.30

TABLE II
COCULTURE TEST BETWEEN *E. FAECIUM* AND *S. ENTERITIDIS*

Time (h)	Control				Coculture of *E. F* and *S. E*		
	E. F	pH	*S.E*	pH	*E. F*	*S.E*	pH
0 h	8.362	6.20	8.362	7.31	8.362	8.362	6.86
24h	9.170	3.83	8.648	5.93	9.233	ND	3.98
48h	8.155	3.50	8.732	5.73	7.653	ND	3.84

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. enteritids*

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. *Enteroccoci* 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 preventively 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

[1] H. A. Abdel-Rahman., S. M. Shawky, H. Ouda, A. A. Nafeaa, and S. H. Orabi, "Effect of Two Probiotics and Bioflavonoids Supplementation to the Broilers Diet and Drinking Water on the Growth Performance and Hepatic Antioxidant Parameters". Global Veterinaria 10 (6): 734-741, 2013.
[2] R. Herich, and M. Levkut, "Lactic acid bacteria, probiotics and immune system". Veterinární medicína, 47, 169-180, 2002.
[3] P. Hlivak, J. Odraska, M. Ferencik, L. Ebringer, E. Jahnova, and A. Mikes, "One-year application of probiotic strain *Enterococcus faecium* M-74 decreases serum cholesterol levels". Bratisl. Lek. Listy 106, 67–72, 2005.
[4] Y. Huang, and Y. Zheng, "The probiotic Lactobacillus acidophilus reduces cholesterol absorption through the down-regulation of Niemann–Pick C1-like 1 in Caco-2 cells". Br. J. Nutr. 9, 1–6, 2009.
[5] L. Pascual, F. Ruiz, W. Giordano, and I. L. Barberis, "Vaginal colonization and activity of the probiotic bacterium *Lactobacillus fermentum* L23 in a murine model of vaginal tract infection". Journal of Medical Microbiology 59, 360–364, 2010.
[6] O. Ashayerizadeh, B. Dastar, M. Shams Shargh, E. Rahmatnejad, and A. Ashayerizadeh, "Influence of prebiotic and two herbal additives on interior organs and hematological indices of broilers". Journal of Animal and Veterinary Advances. 8(9), p. 1851-1855, 2009.
[7] A. Bhardwaj, G. Kaur, H. Gupta, S. Vij, and R. K. Malik, "Interspecies diversity, safety and probiotic potential of bacteriocinogenic

Enterococcus faecium isolated from dairy food and human faeces". World Journal of Microbiology and Biotechnology, 27, 591-602, 2011.

[8] J. A. Patterson, and K. M. Burkholder, "Application of prebiotics and probiotics in poultry production. Poultry Sci., 82: 627-631, 2003.

[9] A. Ahmadova, S. D. Todorov, Y. Choiset, H. Rabesona, T. M. Zadi, Kuliyev, A., B.D.G.M. Franco, J. M. Chobert, and T. Haertlé, "Evaluation of antimicrobial activity, probiotic properties and safety of wild strain *Enterococcus faecium* AQ71 isolated from Azerbaijani Motal cheese". Food Control 30, 631–64, 2013.

[10] B. B. Matijašic´, T. Obermajer, and I. Rogelj, "Quantification of *Lactobacillus gasseri, Enterococcus faecium* and *Bifidobacterium infantis* in a probiotic OTC drug by real-time PCR". Food Control 21, 419–425, 2010.

[11] L. Saavedra, M. P. Taranto, F. Sesma, and G. F. de Valdez, "Homemade traditional cheeses for the isolation of probiotic *Enterococcus faecium* strains". Int. J. Food Microbiol. 88, 241–245, 2003.

[12] J. Benyacoub, P. F. Perez, F. Rochat, K. Y. Saudan, G. Reuteler, N. Antille, M. Humen, G. L. De Antoni, C. Cavadini, S. Blum, and E. J. Schiffrin, "*Enterococcus faecium* SF68 enhances the immune response to Giardia intestinalisin mice". J. Nutr. 135: 1171–1176, 2005.

[13] D. G. V. Emmanuel, A. Jafari, K. A. Beauchemin, J. A. Leedle, and B. N. Ametaj, "Feeding live cultures of Enterococcus faecium and *Saccharomyces cerevisiae* induces an inflammatory response in feedlot steers". J. Anim. Sci. 85:233–239, 2007.

[14] U. Lodemann, K. Hubener, N. Jansen, and H. Martens, "Effects of *Enterococcus faecium* NCIMB 10415 as probiotic supplement on intestinal transport and barrier function of piglets". Arch. Anim. Nutr. 60:35–48, 2006.

[15] L. Scharek, J. Guth, K. Reiter, K. D. Weyrauch, D. Taras, P. Schwerk, P. Schierack, M. F. Schmidt, L. H. Wieler, and K. Tedin, "Influence of a probiotic *Enterococcus faecium* strain on development of the immune system of sows and piglets". Vet. Immunol. Immunopathol. 105:151–161, 2005.

[16] W. Theppangna, K. Otsuki, and T. Murase, "Inhibitory effects of *Enterococcus* strains obtained from a probiotic product on *in vitro* growth of *Salmonella* enteric serovar *Enteritidis* strain IFO3313". J. Food Prot. 69: 2258–2262, 2006.

[17] D. Taras, W. Vahjen, M.Macha, and O. Simon, "Performance, diarrhea incidence, and occurrence of Escherichia coli virulence genes during long-term administration of a probiotic *Enterococcus faecium* strain to sows and piglets". J. Anim. Sci. 84:608–617, 2006.

[18] M. Tokumaru, H. Konuma, M. Umesako, S.Konno, and K. Shinagawa, "Rates of detection of Salmonella and Campyobacterin meats in response to the sample size and the infection level of each species". Int. J. Food Microbiol. 13:41–46, 1991.

[19] M. A. Ehrmann, P. Kurzak, J. Bauer, and R. F. Vogel, "Characterization of lactobacilli towards their use as probiotic adjuncts in poultry". J. Appl. Microbiol. 92:966–975, 2002.

[20] L. M. Shin, R. J. McNally, S. M. Kosslyn, W. L. Thompson, S. L. Rauch, and N. M. Alpert, "Regional cerebral blood flow duringscript-driven imagery in childhood sexual abuse-related posttraumatic stress disorder: A positron emission tomographic investigation". Am J Psychiatry 156:575–584, 1999.

[21] K. Perelmuter, M. Fraga, and P. Zunino, "*In vitro* activity of potential probiotic Lactobacillus murinus isolated from the dog". Journal of Applied Microbiology, 104, 1718-1725, 2008.

[22] Y. Huang, and M. C. Adams, "*In vitro* assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria". International Journal of Food Microbiology, 91, 253 -260, 2004.

[23] A. R. Madureira, M. S. Gião, M. E. Pintado, A. M. P. Gomes, C. Freitas, and F. X. Malcata, "Incorporation and survival of probiotic bacteria in whey cheese matrices". J. Food Sci. 70(3): M161-M165, 2005.

[24] M. Succi, P. Tremonte, A. Reale, E. Sorrentino, L. Grazia, and S. Pacifico, "Bile salt and acid tolerance of *Lactobacillus rhamnosus* strains isolated from Parmigiano Reggiano cheese". FEMS Microbiology Letters, 244, 129-137, 2005.

[25] S. Pieniz, R. Andreazza, T. Anghinoni, F. Camargo, and A. Brandelli, "Probiotic potential, antimicrobial and antioxidant activities of *Enterococcus durans* strain LAB18s. Food Control 37, 251-256, 2014.

[26] P. Sun, J. Wang, and Y. Jiang, "Effects of *Enterococcus faecium* (SF68) on immune function in mice". Food Chemistry, 123, 63-68, 2010.

[27] B. Hyronimus, C. L. Marrec, S. A. Hadj, and A. Deschamps, "Acid and bile tolerance of spore-forming lactic acid bacteria". International Journal of Food Microbiology, 61, 193-197, 2000.

[28] J. Gong, R., J. Forster, and H. Yu, "Diversity and phylogenetic analysis of bacteria in the mucosa of chicken ceca and comparison with bacteria in the cecal lumen". FEMS Microbiol Lett 208:1–7, 2002.

[29] J. B. Payne, J. A. Osborne, P. K. Jenkins, and B. W. Sheldon, "Modeling the growth and death kinetics of Salmonella in poultry litter as a function of pH and water activity". Poult. Sci., 86: 191-201, 2007.

[30] EFSA, The community summary report on trends and sources of zoonoses and zoonotic agents in the European Union in 2007. EFSA J 223:1–320, 2009.

[31] H. R. Taheri, H. Moravej, F. Tabandeh, M. Zaghari, and M. Shivazad, "Screening of lactic acid bacteria toward their selection as a source of chicken probiotic". Poult. Sci. 88:1586-1593, 2009.

[32] M. Chichlowski, J. Croom, B. W. McBride, G. Davis, L. Daniel, and M. Koci, "Direct-fed microbial and salinomycin modulate whole body and intestinal oxygen consumption and intestinal enterocytes cytokine production in the broiler chick". Poult. Sci., 86: 1100-1106, 2007.

[33] M. A. Riley, and J. E. Wertz, "Bacteriocins: evolution, ecology, and application". Annual review of microbiology, Vol.56, pp. 117-137, ISSN 0066-4227, 2002.

[34] P. Audisio, A.De Biase, G. Antonini, M. Belfiore, and M. Oliverio, "Morphological, molecular and ecological evidence of a new Euro-Anatolian species of the *Meligethes coracinus* complex (*Coleoptera*: *Nitidulidae*). Insect Systematics and Evolution, Volume 31, Issue 4, p. 361 – 385, 2000.

[35] R. Herich, T. Kokinčáková, A. Lauková, and M. Levkutová, "Effect of preventive application of Enterococcus faecium EF55 on intestinal mucosa during salmonellosis in Chickens". Czech J. Anim. Sci., 55, (1): 42–47, 2010.

[36] V. Strompfová, and A. Lauková, "*In vitro* study on bacteriocin production of enterococci associated with chickens". Anaerobe 13: 228-237, 2007.

[37] M. Ashraf, and N. A. Akram, "Improving salinity tolerance of plants through conventional breeding and genetic engineering: an analytical comparison". Biotechnol. Adv., 27: 744-52, 2009.