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Original article

ENTEROCOCCUS FAECIUM ISOLATED FROM HEALTHY DOGS FOR POTENTIAL USE AS PROBIOTICS

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Summary

Abd El-Razik, K. A., E. S. Ibrahim, A. M. Younes, A. A. Arafa, A. S. M. Abuelnaga & R. H. Hedia, 2020. *Enterococcus faecium* isolated from healthy dogs for potential use as probiotics. *Bulg. J. Vet. Med.,* **23**, No 2, 197–205.

This study aimed to isolate and identify enterococci obtained from fresh faecal swabs of 16 healthy dogs. Following molecular identification, all isolates were screened against the most critical virulence factors as well as enterocin (bacteriocin) determinants to confirm that the isolated enterococcus was safe to be used as host-specific probiotic. *Enterococcus faecium* was isolated and confirmed in 8 out of the 16 samples. Regarding the assessment of the virulence determinants, *E. faecium* strains were negative for tested (*gelE* and *esp*) virulence genes. Furthermore, the genome was evaluated for the incidence of five known enterocin genes by specific PCR amplification. Four strains encoding *entAS-48* gene were found, while only one strain harboured the *entL50A/B* gene. Based on these results, five of the *E. faecium* isolated in this study were considered as promising probiotic candidates for dogs.

Key words: dogs, *Enterococcus faecium*, PCR, probiotic, virulence

INTRODUCTION

Enterococci are lactic acid bacteria of importance in food, public health and medical microbiology. Enterococci are between the most significant commensal bacteria found in the intestinal microbiota of both humans and animals (Fisher & Phillips, 2009; Sukmawinata *et al.*, 2018). The importance of enterococci is controversial, as they are successfully employed in food biopreservation but at the same time they can cause infection and illness. *Enterococcus faecium* obtained from animals are not risky to humans, but can convey genes of antimicrobial resistance for other pathogenic enterococci (Nguyen *et al.*, 2010; Hammerum, 2012). The essential interest to enterococci is related to their potency to yield bacteriocins (Gilmore *et al.*, 2014). Therefore, the application of these bacteria or their natural antimicrobial action to overcome foodborne pathogens and to preserve food has become an issue of valuable significance in

the latest years (Van Heel *et al.*, 2011; Ghrairi *et al.*, 2012).

Kjems (1955) was the first to note the capability of enterococci to produce bacteriocins. Since then, many enterococcal strains producing bacteriocins (frequently known as enterocins) have been identified, some of which have been well characterised at both biochemical and genetic level (Franz *et al.*, 2007).

Enterocins are a family of bacteriocins, similar to other lactic acid bacteriocins, classified into three major classes. Of these classes, the enterocins of class II and class III (mainly enterocin AS-48) are promising due to their possibility to be used as safe food preservatives as they inhibit closely related species of foodborne bacteria. Some bacteriocins are active against Gram-positive food-spoilage pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, and *Clostridium botulinum* (Cotter *et al.*, 2005; Franz *et al.*, 2007). Moreover, several strains capable to stop the Gram-negative bacterial growth were explored in recent studies (Svetoch *et al.*, 2011; Messaoudi *et al.*, 2012).

The aim of this study was to isolate and to identify enterococci from faecal microbiota of healthy dogs to find out the existence of bacteriocin structural genes and lack of virulence determinants. Using PCR, the isolates were screened for virulence genes, as well as for enterocin producing genes as a trial to confirm the safety of the identified enterococci for potential use as safe probiotics.

MATERIALS AND METHODS

Sample collection

Domestic dogs of both sexes and different ages admitted to Faculty of Veterinary Medicine, Cairo, Egypt from November 2016 to January 2017, for medical check-up or for vaccination. Fresh faecal swabs were collected from 16 of these healthy dogs using sterile swabs. The samples were transported in an icebox to the laboratory of Microbiology and Immunology at National Research Centre and immediately processed.

Bacterial isolation and identification

Bacterial isolation and identification was done according to Iseppi *et al.* (2015). Enterococci were isolated by streaking with a 10 µL loop serially diluted faeces samples on Kennel Fecal (KF) – Streptococcus agar (Difco Laboratories, Detroit, MI, USA). Plates were aerobically incubated for 48 h at 37 °C. For each sample, a red colony with the typical enterococcal morphology was randomly selected. All isolates obtained were characterised based on the morphological characteristics and biochemical activities. *E. faecium* laboratory identification was performed by Gram staining, colonial morphology on blood agar, growth and blackening of bile esculin agar, absence of catalase production, resistance to 6.5% sodium chloride. All presumptive enterococci were identified by biochemical characteristics using Api20 Strep system (Biomérieux, France).

Genotypic characterisation of enterococci using PCR

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) following the manufacturer's recommendations. Briefly, 200 µL of the sample suspension was incubated with 20 µL of proteinase K and 200 µL of lysis buffer at 56 °C for 10 min. After incubation, 200 µL of 100% ethanol was added to the lysate. The sample was then washed and centrifuged. Nucleic acid was eluted with 100 µL of elution buffer.

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions

Target gene	Primers sequences	PCR product	Annealing temperature	Reference
16S rRNA	F: AGAGTTTGATCMTGGCTCAG R: TACGGYTACCTTGTTACGACTT	1485 bp	56 °C	Weisburg *et al.* (1991)
Enterocin AS-48	F: GAGGAGTATCATGGTTA R: ATATTGTTAAATTACCAA	339 bp	53 °C	Yousif *et al.* (2005)
Bacteriocin 31	F: TAT TAC GGA AAT GGT TTATATTGT R: TCTAGG AGC CCA AGG GCC	123 bp	53 °C	Yousif *et al.* (2005)
Enterocin L50 A/B	F: TGG GAG CAATCG CAA AAT TAG R: ATT GCC CAT CCT TCT CCA AT	98 bp	53 °C	Belgacem *et al.* (2010)
Enterocin P	F: TAT GGT AAT GGT GTT TAT TGTAAT R: ATG TCC CATACC TGC CAAAC	120 bp	53 °C	Yousif *et al.* (2005)
Enterocin 1071A/1071B	F: CCTATT GGG GGA GAG TCG GT R: ATA CAT TCT TCC ACT TAT TTT T	343 bp	53 °C	Cancilla *et al.* (1992)
Enterococcal surface protein (*esp)*	F: TTGCTAATGCTAGTCCACGACC R: GCGTCAACACTTGCATTGCCGAA	933 bp	45 °C	Eaton & Gasson (2001)
Gelatinase (*gelE)*	F: ACCCCGTATCATTGGTTT R: ACGCATTGCTTTTCCATC	419 bp	54 °C	Eaton & Gasson (2001)

For identification of *E. faecium*, PCR reactions were performed in a final volume of 25 µL reaction containing 12.5 µL of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µL (20 pmol) of forward and reverse 16S rRNA primer (Table 1) (Metabion, Germany), 4.5 µL of distilled water, and 5 µL of DNA template. The reaction was performed in an Applied Biosystem 2720 thermal cycler according to Weisburg *et al.* (1991). The PCR products were separated by electrophoresis on 1.5% agarose gel then photographed and analysed by gel documentation system (Alpha Innotech, Biometra).

PCR detection of virulence determinants

The gelatinase (*gelE*) and enterococcal surface protein (*esp*) genes of virulence factors were screened in all isolates by PCR according to Eaton & Gasson (2001). The primers were used for the amplification of 419 bp from the *gelE* gene and for amplification of 933 bp from esp gene. The sequences of primers used are listed in Table 1, while PCR reactions and conditions were carried out as described in the previous section.

Screening of enterococcal bacteriocins

The genes of five enterocins of *E. faecium*; enterocin P (*entP*), enterocin L50A/B (*entL50AentL50B*), bacteriocin 31 (*bac31*), enterocin AS-48 (*entAS-48*), enterocin 1071A/1071B (*ent1071Aent 1071B*), and enterocin 96 (*ent96*) were amplified using specific enterocin PCR primers (Table 1). PCR amplification was performed as described in Table 1. The amplification of the five genes was performed at 94 ^{o}C for 5 min, 35× (94 ^{o}C for 1 min, 53 ^{o}C or 1 min, and 72 ^{o}C for 40 s), and 72 ^{o}C for 7 min. The PCR products were analysed on 1.5% agarose gel.

Phylogenetic tree construction

The positive PCR products for 16S rRNA were sequenced in MACROGEN Company (Korea) on 3730_L sequencers (Applied Biosystem, USA). The accuracy of data was confirmed by two-directional sequencing with the forward and reverse primers used in PCR. The nucleotide sequences obtained in this study were analysed using the BioEdit 7.0.4.1 and ClustalW2 (http:// www.clustal.org/) programmes. The resulting sequences were aligned with *16S rRNA* gene of reference sequences of *Enterococcus* spp. using a neighbour-joining analysis of the aligned sequences implemented in the programme CLC Sequence Viewer 6.

RESULTS

Bacterial isolation and identification

The presented data showed that 8 out of 16 samples were characterised as *Enterococci* spp. based on Gram staining, catalase, oxidase, and biochemical activity. Further genotypic identification of these 8 isolates using PCR targeting *16S rRNA* gene followed by DNA sequencing confirmed it as *Enterococcus* (Fig. 1).

Nucleotide sequence and accession numbers

Eight *Enterococcus* sequences obtained in this study were deposited in the GenBank database under accession number KY490544–KY490551. Phylogenetic analysis confirmed that all eight isolates were *E. faecium* (Fig. 2). Regarding the phylogenetic tree, all Egyptian isolates formed a separate cluster and have high homology with *E. faecium* isolate LT593851 (strain= "E.F 500) isolated from human dental cavity and *E. faecium* isolate JX420820 (strain="I4") isolated

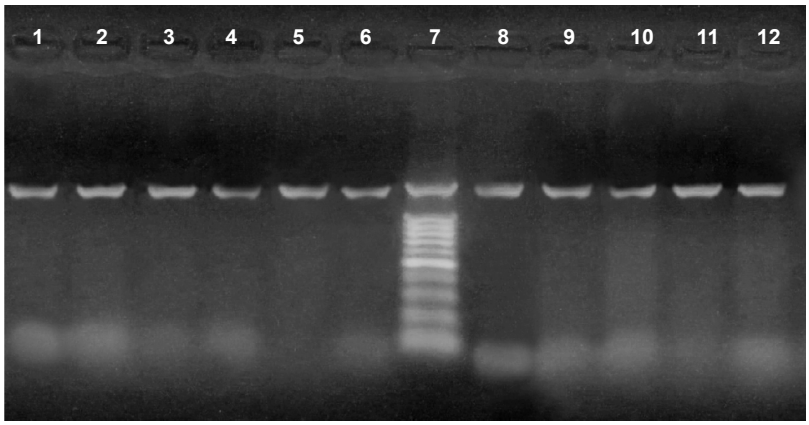

Fig. 1. PCR analysis of *16S rRNA* gene (1485 bp) in *Enterococcus* spp, Lane 1: Positive control; Lanes 2–6 & 8–12: *Enterococus* isolates, Lane 7: 100 bp ladder.

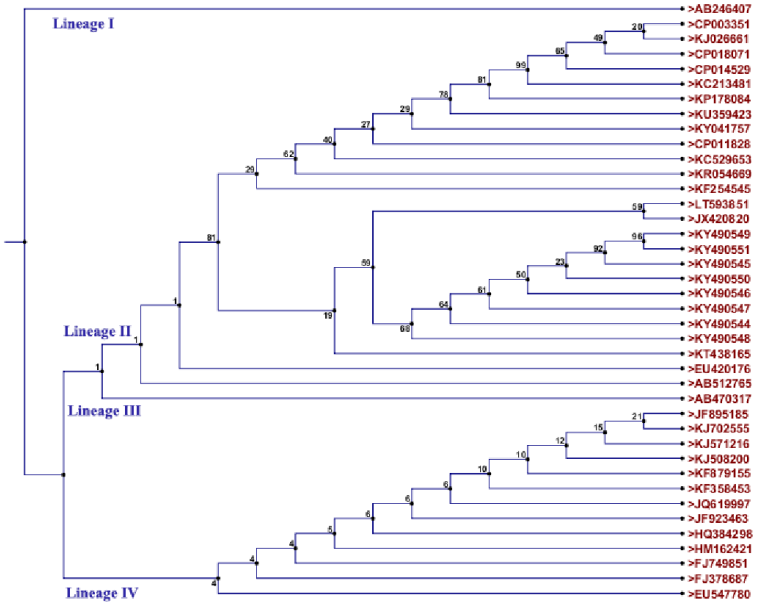

Fig. 2. Phylogenetic relationship of selected strains of *Enterrococcus faecium* from various sources, representing the four distinct lineages, based on the *16S rRNA* gene. The GenBank accession numbers of the isolates used are given.

from municipal sewage waste. The *E. faecium* isolated from milk as well as milk products of different animal species or from canine faeces constructed other clusters.

Distribution of virulence determinants

Enterococci were screened for the most critical virulence determinants, gelatinase *gelE* and enterococcal surface protein *esp*.

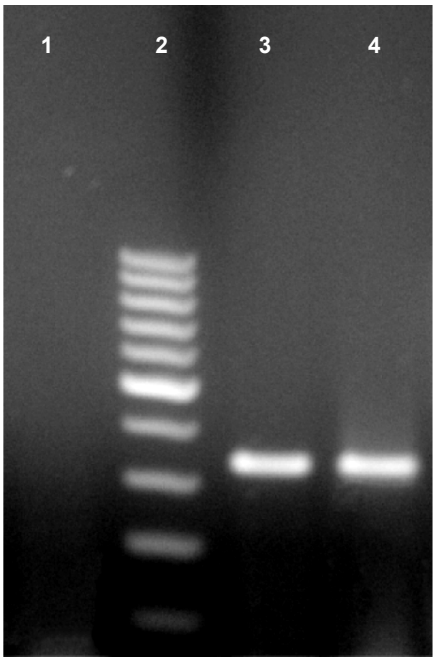

Fig. 3. Analysis of *Enterococcus* isolate for the presence of enterocin AS-48 gene. Lane 1: Negative control; Lane 2: 100 bp ladder; Lane 3: Positive control; Lane 4: *E. faecium* isolate KY490544 (339 bp).

The primers were used for the amplification of 419 bp from the *gelE* gene and for amplification of 933 bp from *esp* gene. None of the isolates was found to harbour neither the *esp* gene nor the *gelE* gene.

Detection of bacteriocins

The distribution of enterocins genes within the tested enterococci showed that two enterococci strains harboured *entAS-48* as shown in Fig. 3, while only one isolate harboured *EntL50A/B* gene (Fig. 4). The other enterocin genes: *bac31*, *entP*, and *ent1071A/1071B*, were absent.

DISCUSSION

E. faecium is the most normally occurring enterococcal species in dairy industry,

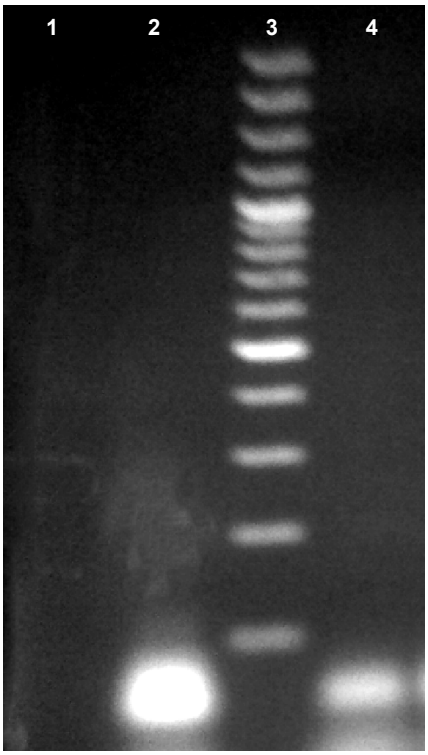

Fig. 4. Analysis of *Enterococcus* isolate for the presence of *Enterocin L50 A/B gene* (98 bp). Lane 1: Negative control; Lane 2: Positive control (98 bp); Lane 3: 100 bp ladder; Lane 4: *E. faecium* isolate KY490544.

fermented vegetable and raw fruits. Lactic acid bacteria, e.g. *E. faecalis* and *E. faecium,* are potential probiotics located within the intestine of healthy animals and human, and intermittently cause opportunistic nosocomial infections in critically ill individuals (Olawale *et al.*, 2011). Enterococci have the ability to survive in gastric juice, bile salts and adhere to the host intestinal cells (Rossi *et al.*, 2003), in addition to secretion of antimicrobial substances as bacteriocins with both bactericidal and bacteriostatic effect against species that are strongly associated to the manufacturer bacterium (Nes *et al.*, 2007).

In this study, *E. faecium* was the only enterococcal species detected in 50% (8/16) of the tested samples. Our results agreed with those of Iseppi *et al.* (2015) and Kubašová *et al.* (2017) who reported that *E. faecium* was the most prevailing species among the selected enterococci strains.

Because of their relationship with several human infections, there are rising concerns about the safety of *Enterococcus* bacteria. The potential pathogenicity of enterococci is related to the occurrence of virulence factors. They include the *esp* gene which is in charge of a cell wall protein involved in immune evasion, support of adhesion, colonisation, and biofilm formation (Kubašová *et al.*, 2017); the *gelE* involved in toxin production that hydrolyses gelatin, elastin, collagen and haemoglobin (Sava *et al.*, 2010). In this study, our isolates were negative for the genes encoding for gelatinase (*gelE*) and enterococcal surface protein (*esp*). Similar to these results, Iseppi *et al.* (2015) showed negative results against *esp* virulence gene among all isolates. Moreover, Enayati *et al.* (2015) found that none of the *E. faecium* isolated from either surface water or wells harboured the *gelE* gene. In addition, the *esp* gene was found in two out of nine canine multidrug-resistant strains enterococci isolates (Abdel-Moein *et al.*, 2017). The lack of these most important virulence determinants in our isolates is a key opportunity for their use as probiotics.

The probiotic action of enterococci is commonly associated with the ability to produce enterocins. Enterococci are recognised to secrete antimicrobial substance like other lactic acid bacteria, making them prospectively valuable for the prevention of bacterial foodborne disease (Franz *et al.*, 2011). Several enterococci generate at least one bacteriocin that is ribosomally synthesised, act against a broad variety of foodborne pathogens such as *Listeria* species. Two *Enterococcus* strains are currently registered as probiotics and available on the market, namely *E. faecium* SF68® and *E. faecalis* Symbioflor 1 for the successful treatment of colibacillosis in animals and gastroenteritis in humans (Vimont *et al.*, 2017).

The PCR technique has previously been used successfully in enterococci and lactobacilli to detect known bacteriocins. In the present study, four isolates (50%) harbouring the *entAS-48* gene and one isolate harbouring *entL50A/B* gene were confirmed using specific enterocin PCR primers. In agreement with previous studies, Shehata *et al.* (2017) recorded that the overall occurrence of *entAS-48*, and *entL50A/B* structural genes in the selected *Enterococcus* spp. isolated from faecal content samples of healthy chickens was 100% (5/5), and 60% (3/5), respectively. Furthermore, El-Ghaish *et al.* (2011) found that bacteriocins produced by *E. faecium* E980 could be identified as enterocins P and L50A structural genes from Egyptian dairy products.

CONCLUSION

The obtained data showed that all *Enterococcus faecium* isolates from dogs lacked the most critical virulence determinants (*esp*, *gelE*); besides, four strains harboured the *entAS-48* gene and one strain harboured the *EntL50A/B* gene. Therefore, characterised bacteriocinogenic enterococci could be used as perspective probiotic candidates for dogs. *Enterococcus* species has some valuable characteristics including tolerance to both gastric juice and bile salts, it can also interfere with the flavour of several food products as a result of production of several com-

ponents including enterocin that could be useful in combating harmful bacteria. For that reason, further investigations are required to confirm its *in vivo* effects.

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