Scientific Papers. Series D. Animal Science. Vol. LXV, No. 1, 2022
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

IN VITRO EVALUATION OF *ENTEROCOCCUS FAECIUM* AS PROBIOTIC POTENTIAL IN POULTRY PRODUCTION

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Abstract

Probiotics are important bacteria species due to their benefits to animal health. This study aimed to evaluate some characteristics of Enterococcus faecium (NCIMB 10415) and evaluated its survivability and capacity as a probiotic product. Gram-positive, catalase, antibiotics and haemolysis tests were screened using selective media. The strain was phenotypically characterized and biochemical profile using API 20STREP and identification by apiwebTM (Biomerieux (France) software were done (99.2% very good identification). After 24 h of incubation at 37°C, in aerobic conditions, E. faecium exhibits 11.88 Log_{10} with an optical density (OD 600 nm) yield reaching a maximum from 0.2 at the beginning of the exponential growth phase to 1.7 value. The safety of the strain was confirmed by non-haemolytic activity on TSA agar medium. The impact of 16 antibiotics on our strain ranged from intermediate (75%) to susceptible (12.5%), with resistance to colistin sulphate and erythromycin. Regarding the importance of antibiotics such as vancomycin, the analysis of the E. faecium profile revealed intermediate activity. These data suggested that these bacteria do not create a risk to animal health and may be considered a reliable candidate as probiotic source for application in poultry nutrition.

Key words*: Enterococcus, poultry, probiotics.*

INTRODUCTION

Enterococci represent commensal microorganisms from intestinal microflora of animals and humans (Holzapfel et al., 2018; Lee et al., 2019), but they can also survive in several ecosystems such as soil, vegetables, water surfaces, food and feed (Zommiti et al., 2018).
Belong to the lactic acid bacteria (LAB) group, Enterococci occur important place due to their involvement in feed spoilage and fermentation processes (Zhang et al., 2016), as well for their utilization as probiotics in animal nutrition and also with successful utilization in human health (Franz et al., 2011).
Enterococci probiotics consumption was considered advantageous due to their capacity to produce multiple beneficial metabolites that also contribute to the stability of microorganisms in the gastrointestinal tract (GIT), considering that they are natural gut commensals (Fugaban et al., 2021). Some species of *Enterococcus* were used as probiotics in many countries due to their high ability to produce bacteriocins (Franz et al., 2011).
The application of enterococci in animal nutrition is still under discussion. A proposed classification is necessary to be feasible to divide pathogenic strains from well-evaluated beneficial *Enterococcus* strains (Fugaband et al., 2021). A part from enterococcal strains is successfully used as probiotic to improve animal health; on the other hand, other enterococcal types are associated with nosocomial infections (Franz et al., 2011).
Further, *Enterococcus* species have the capacity to resist in extreme temperatures fluctuation (10-45°C), wide pH gradients (4.5-10.0), high NaCl levels (6.5%), survive heat exposure (up to 80°C, more than 33 min) and grow in the presence of bile salts (Wieland et al., 2017).
It is known that some species from the *Enterococcus* genus ensure a significant place as probiotic bacteria in the host with a strong contribution to protection against pathogens and infectious diseases (Revajová et al., 2022).

Generally, probiotics in animal feed improve performance and health. In addition, the administration of enterococci in animal feed can prevent diseases, inducing the gastrointestinal micro populations or stimulating the immune system (Franz et al., 2011). Further, some representative strains from the genus *Enterococcus* have been studied regarding their application as starter cultures for exploring and obtaining the status of probiotics (Holzapfel et al., 2018) with significant results in poultry digestive function (Zhang et al., 2021). Usually, *E. faecium, E. faecalis and E. durans* are species from the genus *Enterococcus* used as veterinary feed supplements (Foulquié Moreno et al., 2006).

E. faecium has many biological traits (Mao et al., 2020), such as the capacity to survive in body conditions (low pH, bile salts concentration, inhibition and control of the growth of pathogenic bacteria), which makes it to displaying a potential source in feed preservation and in the prevention or treatment of other diseases in hosts (Saelim et al., 2012; Mao et al., 2020).

Therefore, the aim of the present study was conducted to assess some properties of *E. faecium* NCIMB 10415 based on its phenotypical profile as a probiotic candidate culture in poultry nutrition.

MATERIALS AND METHODS

Materials, reagents and strain

To conduct this study, the strain *Enterococcus faecium* NCIMB 10415 (cultivated from Cylactin, DSM, Heerlen, Netherlands) was subjected to several tests; culture media and antibiotics disks were provided from Oxoid Basingstoke (Hampshire, UK).

Culture media, growth conditions and morphological traits

E. faecium NCIMB 10415 was cultivated in Brain Heart Infusion (BHI) broth medium (g/L) containing: beef heart (5.0), calf brains (12.5), D (+) glucose (2.0), sodium chloride (5.0), disodium phosphate (2.5), and peptone (10.0) with a final pH 7.4 ± 0.2.

After 24 h of incubation at 37°C, in aerobic conditions, the colonies of culture strain (broth and agar media) were evaluated for physiological characteristics (colonies morphology, Gram staining, catalase test) according to Bergey's Manual of Systematic Bacteriology (Hammes and Hertel, 2009).

Colony-forming unit (CFU) and optical density

The growth rate of strain was evaluated after cultivation in BHI broth at 37°C, for 24 h, aerobically.

The optical density (**OD**) was measured at 600 nm to estimate the *E. faecium* cells concentration at 4, 8, 12 and 24 h.

API 20 STREP- biochemical characterization

Biochemical characterization was done by analysing carbohydrate fermentation profiles using API 20STREP tests, a bacterial identification system (Bio Merieux, S.A., France). According to manufacturer instructions, the interpretation was done at 4 and 24 h, at 37°C using online software API 20 V 5.1.

Haemolytic observation

Blood agar plates [Trypticase soy agar (TSA, Sanimed) containing 5% (w/v) sheep blood] were used to test haemolysis activity. The strain was performed for the presence of clear zones surrounding the colonies. The interpretation was noted after incubation at 37°C, for 24 h as follows: clear zones corresponding to β-haemolysis, greenish zones as α-haemolysis and the absence of zones indicating no haemolysis which is known as gamma haemolysis (Dumitru et al., 2018; Bazireh et al., 2020).

Antibiotic test

The antibiotic susceptibility of *E. faecium* NCIMB 10415 to different antibiotics including amoxicillin (AMX 25 µg), gentamicin (GN, 10 µg), kanamycin (K, 30 µg), lincomycin (MY, 10 µg), tetracycline (TE, 30 µg), penicillin (P, 10 µg), vancomycin (VA, 5 µg), colistin sulphate (CT, 10 µg), clindamycin (DA, 2 µg), erythromycin (E, 15 µg), amikacin (AK, 30 µg), chloramphenicol (C, 30 µg), oxytetracycline (OT, 30 µg), enrofloxacin (ENR, 5 µg), streptomycin (S, 10 µg), and tilmicosin (TIL, 15 µg) was determined by agar disc diffusion method on BHI plates BHI.

Statistical analysis

The results are presented as mean values of three determinations. The graphics for strain viability

during 24 h of incubation was performed with SigmaPlot V.11 software (San Jose, CA, USA).

RESULTS AND DISCUSSIONS

Culture media, growth conditions and morphological traits

The present strain has the origin from faeces of a healthy breast-fed newborn baby (Asplund, 1991).

The basic parameters examined in terms of effectiveness of probiotic candidates in chicken broiler are some phenotypical traits (micro- and macroscopically analysis, Gram staining, catalase).

It was observed that *E. faecium* NCIMB 10415 in broth medium, on agitation, involves homogeneous turbidity, with an abundant deposit, without surface formations. Regarding the agar BHI medium, the strain presented S-type colonies, whitish, with regular edges and a diameter of 1.5-2.0 mm.

Also, the colonies developed in broth and agar medium were verified by performing smears stained by Gram method, and the characteristic aspects (shape, type of Gram staining, grouping mode, ability of sporulation) allowed the differentiation of the genus *Enterococcus* from other bacterial species (Figure 1).

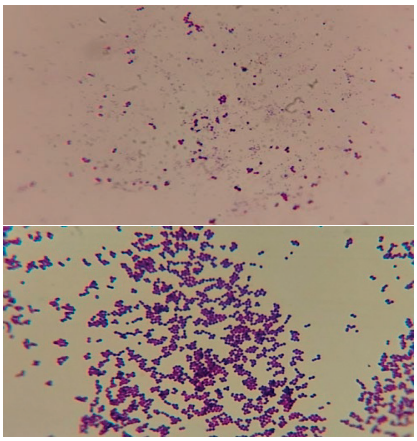

Figure 1. Cultural aspects of *E. faecium* in BHI medium (a: broth, b: agar) by Gram staining x 1000

Following the microscopical examination, the strain was observed as Gram-positive cocci which is a characteristic belonging to the family *Enterococcaceae*, diplo, in short chains and in small staph groups (on solid media).

Regarding the catalase test of *E. faecium* NCIMB 10415, the result was noted as negative, without effervescent at the addition of 3% H_2O_2.

Over the last decades, probiotics occur a higher interest due to their multiple health benefits (Bazireh et al., 2020).

E. faecium is an important opportunistic pathogen easily transmitted between sick and healthy animals. It is known that the present analysed strain belongs to a pioneer type of bacteria, i.e. the first lactic acid bacteria transmitted from the mother's milk to the newborn, allowing the evolution of a healthy microbiome in the gastrointestinal tract (Wopereis et al., 2014). Further, Enterococci are among the most common human intestinal LAB, which harbours numerous useful biotechnological properties, such as the secretion of bacteriocins (enterocins) (Franz et al., 2011).

As a result, *E. faecium* is known to be a significant etiological agent of acute and chronic infections with a high degree of spread in hospital-acquired infections (Bazireh et al., 2020).

Colony-forming unit (CFU) and optical density

The growth of *E. faecium* NCIMB 10415 was drawn under the optimal conditions (pH 7.0, 37°C, 24 h, aerobic) and exhibited a good viability rate, around 11.88 Log_{10} corresponding to a load of 7.6 x 10^{11} CFU/ml.

Regarding the optical density, after washing with SFS, the biomass of *E. faecium* NCIMB 10415 resulting from the fermentative medium was well homogenized with SFS, and the OD was read at a wavelength of 600 nm at 4, 8, 12 and 24 hours (Figure 2).

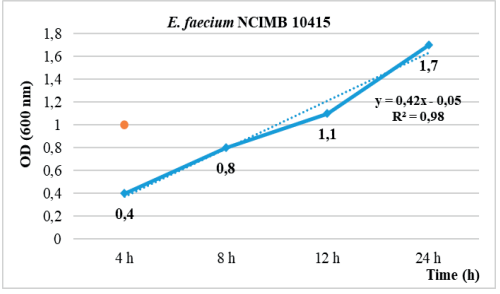

Figure 2. The optical density of *E. faecium* NCIMB during 24 h

E. faecium is one of the most important bacteria producing lactic acid belonging to the native

microbiota of the human and animal gastrointestinal tract (Wu et al., 2019).

Exploring the growth characteristics and optimal growth conditions of probiotic strains is mandatory before they can be used in large-scale industrial production (Mao et al., 2020).

Comparatively to Mao et al. (2020) study, our strain involves a significant OD at 600 nm for 24 h, at 37°C, exhibiting a 1.7 value in the stationary phase (12-24 h). Further, the growth of *E. faecium* evaluated in the author's research did not exceed the 1.0 value of OD during the stationary or ageing phase (after 24 h) comparatively with *E. faecium* NCIMB 10415 strain which involves a best proliferation and a higher concentration in the condition of pH value (7.0) of the selective medium tested.

Generally, *E. faecium* has a considerable environment adaptability, with a strong proliferation and colonization of the animal GIT when it is added as probiotic. Moreover, feed supplementation with *E. faecium* has also been shown to offer a number of benefits, especially against the pathogen *E. coli* (Lodemann et al., 2017) and could provide an alternative source to enterococci for future probiotic development (Saelim et al., 2012).

API 20 STREP- biochemical characterization

The biochemical characteristics of *E. faecium* NCIMB 10415 is shown in Table 1. The capacity of fermentation was based on the strain's ability to ferment the substrates from API 20STREP (Figure 3). Sugar fermentation patterns confirmed that the presence of cocci belonged to *Enterococcus* genus.

E. faecium is the most common species of *Enterococcus* found in animal intestines and has similar properties and biochemical traits, with the capacity to use arabinose as carbon source (Mao et al., 2020). In addition, the strain was able to hydrolyse sodium pyruvate, bile esculin, pyrrolidinyl aryl amidase (at 24 h), α and β galactosidase, leucine aminopeptidase, L-arginine, D-ribose, D-arabinose, D-mannitol, D-lactose, D-trehalose, starch (at 24 h), but negative for hippuric acid, β-glucuronidase, D-sorbitol, inulin, raffinose, and glycogen.

Table 1. Assessment of biochemical characterization of *E. faecium* NCIMB 10415 by API 20STREP kit

Test	Fermentation Substrates	Strain 4 h	Strain 24 h
VP	Sodium pyruvate	+	+
HIP	Hippuric acid	-	-
ESC	Esculin	+	+
PYRA	Pyrrolidinyl arylamidase	-	+
αGAL	α-galactosidase	+	+
βGUR	β-glucuronidase	-	-
βGAL	β-galactosidase	+	+
PAL	2-naphthyl phosphate	-	-
LAP	Leucine aminopeptidase	-	+
ADH	L-arginine	+	+
RIB	D-ribose	+	+
ARA	D-arabinose	+	+
MAN	D-mannitol	+	+
SOR	D-sorbitol	-	-
LAC	D-lactose	+	+
TRE	D-trehalose	+	+
INU	Inulin	-	-
RAF	Raffinose	-	-
AMD	Starch	-	+
GLYG	Glycogen	-	-

- = negative; += positive; ?= doubtful, weakly positive;

Figure 3. API 20 STREP inoculated with *E. faecium* NCIMB 10415

The percentage of identification (ID) was obtained according to the manufacture protocol API Biomerieux (France) and was registered as 99.2% that corresponding to a very good identification (Figure 4).

According to the manufacturer's instructions, the identification of *Enterococcus* strains at the species level was divided into four subgroups: (a) excellent species identification, ID ≥ 99.9%; (b) very good species identification, ID ≥ 99.0%; (c) good species identification, ID ≥ 90.0% and (d) acceptable species identification, ID ≥80.0%. In the present case, the enterococcal strain showed an ID ≥ 99.2%.

Based on the literature data, the present strain is in accordance with the results obtained by (Sanlibaba et al., 2018).

VERY GOOD IDENTIFICATION	
Strip	API 20 STREP V7.0
Profile	5 3 5 7 5 1 1
Note	POSSIBILITY OF Ent.gallinarum OR Ent.casseliflavus IF VancoR

Significant taxa	% ID	T	Tests against			
Enterococcus faecium	99.2	0.98				

Next taxon	% ID	T	Tests against			
Enterococcus durans	0.4	0.54	ARA 15%	MAN 2%		

Complementary test(s)	YELLOW	IRHAMNOSE	GLYCEROL	
Enterococcus casseliflavus	+	+	+	
Enterococcus faecium	-	v	-	
Enterococcus gallinarum	-	-	-	

Figure 4. Percentage of *Enterococcus* identification by software Biomerieux using version API 20 V 5.1 (http://apiweb.mediclim.ro/)

The identification percentage generated by the Biomerieux online program for the analysed strain is similar to the ID of the *E. faecium* strain of IBNA 10 which was isolated according to the data presented by Sorescu et al. (2019) of the ileal contents of a turkey (age 73 days).

Hemolytic activity

Regarding the strain *E. faecium* NCIMB 10415, in 2011, the European Union (EU) approved their utilization as feed additive for different animals. An essential property until to use a strain as probiotic product is based on hemolytic activity. Our strain was found to be α-hemolytic without any clear area around colonies on TSA agar plates (Figure 5).

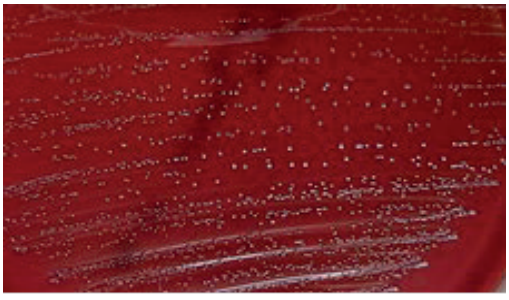

Figure 5. Hemolysis test of *E. faecium* NCIMB 10415

The assay is based on the ability of strain to cleave blood cells from the composition of culture medium, due to the presence of sheep blood (Dumitru et al., 2019). More, if the strain exhibited a transparent zone around the developed colonies which correspond to a hemolytic type (β with a clear hemolysis or γ with colonies that involve a green halo), the respective strain must be eliminated. Only strain which showed non-hemolytic activity were used for further experiments (Bazireh et al., 2020).

The main concern for *Enterococcus* spp. as a probiotic source is their pathogenicity based on the horizontal transfer of virulence factors, which is why hemolytic evaluation should not be neglected (Ben Braïek & Smaoui, 2019). Hemolytic activities α and β are considered a disadvantage for the probiotic potential (Halder et al., 2017). Thus, the hemolysis test is an extremely important safety parameter to develop new probiotic strains for use as supplements in animal feed.

In general, the absence of hemolytic activity is an advantage, which is why the present strain of *Enterococcus* proved to be non-hemolytic and thus can be used safely as a source of probiotic product in poultry nutrition.

It is known that many studies present a positive influence of enterococcal probiotics in poultry diets. For example, Vahjen et al. (2002) showed that a probiotic based on *E. faecium* SF68 in turkey feed led to increase the level of lactic acid bacteria from intestinal tract, respectively from ileum area. Later, in 2010, Samli et al. investigated the effect of the current strain, *E. faecium* NCIMB 10415 on broiler chickens' performance, where were observed significant results (weight gain and feed conversion ratio).

Antibiotic test

The results of the antibiotics resistance are reported in Table 2. The strain exhibited resistance to colistin sulphate and erythromycin, sensitivity for chloramphenicol and enrofloxacin, and intermediate resistance for the rest of antibiotics analysed.

Table 2. Antibiotics resistance of *E. faecium* NCIMB 10415

Strain	Antibiotics discs															
	AMX	**GN**	**K**	**MY**	**TE**	**P**	**VA**	**CT**	**DA**	**E**	**AK**	**C**	**OT**	**ENR**	**S**	**TIL**
	I	I	I	I	I	I	I	R	I	R	I	S	I	S	I	I

amoxicillin (AMX) 25 µg; gentamicin (GN) 10 µg; kanamycin (K) 30 µg; lincomycin (MY) 10 µg; tetracycline (TE) 30 µg; penicillin (P) 10 µg; vancomycin (VA) 5 µg; colistin sulphate (CT) 10 µg; clindamycin (DA) 2 µg; erythromycin (E) 15 µg; amikacin (AK) 30 µg; chloramphenicol (C) 30 µg; oxytetracycline (OT) 30 µg; enrofloxacin (ENR) 5 µg; streptomycin (S) 10 µg; tilmicostin (TIL) 15 µg. Resistant (R): 0–5 mm; Intermediate (I): 6-25 mm; Sensitive (S): 26–35 mm; *E. faecium* NCIMB 10415.

Regarding the vancomycin resistance, the strain showed an inhibition spectrum below 1 cm, thus being characterized by a variation from resistant to intermediate. However, enterococcal resistance to vancomycin has been reported in several studies (Kolář et al., 2002; Vignaroli et al., 2011). For safety reasons, the sensitivity of commercially exploitable strains to commonly used antibiotics is desirable for use as coculture or early crops (Vignaroli et al., 2011). Also, in many cases, antibiotic treatment is indispensable, having a devastating effect on the balance of the intestinal flora (Stoica & Stoica, 2001). Instead, antibiotics, such as tetracycline, are responsible for gastrointestinal imbalances.

The trait of antibiotic resistance is an important property associated with probiotics because it helps selected microorganisms for probiotic purposes to survive in the gastrointestinal tract of the host organism during antibiotic treatment (Mishra and Ghosh, 2018; Choeisoongnern et al., 2021).

These findings were similar to previous studies in which bacterial isolates demonstrated a typical pattern of antibiotic susceptibility to *Enterococcus*, especially colistin (Mishra and Ghosh, 2018). In addition, enterococcal bacteria are part of the natural microflora of humans and animals (Golob et al., 2019), being considered natural commensal microorganisms that contribute to the stability of the host bacterial population in GIT (Fugaban et al., 2021).

CONCLUSIONS

The results obtained in present study showed that. *E. faecium* NCIMB 10415 present relative probiotic properties to be used as an effective probiotic. In addition, the safety characteristics, high survivability, capacity to ferment sugars, absence on hemolysis activity, and antibiotic resistance suggested that, *E. faecium* NCIMB 10415 is a potent probiotic candidate with the ability to proliferate, survive and colonize in the host GIT. Furthermore, as suggested in the EFSA guidelines on the use of *Enterococcus* spp. as feed additives, the present strain may serve as good supplement, starter fermentation culture or probiotic product in poultry feed.

Further investigation is necessary to test and validate the impact of a probiotic based *on E. faecium* NCIMB 10415 on poultry health and growth in farm settings, as well as to find out the optimum level of inclusion.

ACKNOWLEDGEMENTS

This research was supported by funds from the PN 19-09.01.04 granted by Romanian Ministry of Research, Innovation and Digitization and from the National Research Development Project, Projects to Finance Excellence (8 PFE/2022).

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