Research Article

SAFETY EVALUATION OF *ENTEROCOCCUS FAECALIS* FAA025 AND *STREPTOCOCCUS EQUINES* FAA026 FOR HUMAN PROBIOTIC APPLICATION.

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Accepted on: 15-05-2012; **Finalized on:** 30-06-2012.

ABSTRACT

Enterococcus faecalis FAA025 and *Streptococcus equines* FAA026 have been previously selected for their probiotic potentials for human application. This study was carried out to ascertain that the strains are safe for human consumption through screening for tranferable antibiotic resistant and biogenic amines genes. Antibiotic susceptibility pattern of the two bacterial strains were studied by broth microdilution. Polymerase Chain Reactions (PCR) of the isolates were tested to detect tetracycline and neomycin resistant genes. (*tet* W and *aph* 3-II). The presence of 3 biogenic amine genes (tyrosine decarboxylase, (*tdc*), histidine decarboxylase (*hdc)* and agmatine deimininase (*AgDI*) were screened in the 2 bacterial isolates by PCR reactions. The two bacterial strains were sensitive to most tested antibiotics. Tetracycline and neomycin resistant genes could not be detected in the two bacterial strains. The 3 biogenic amines genes were absent in *Streptococcus equines* FAA026 while *tdc* and *AgDI* genes were detected in *Enterococcus faecalis* FAA025. With reference to absence of antibiotic resistant genes and biogenic amines genes, *Streptococcus equines* FAA026 is safe for probiotic use while *Enterococcus faecalis* FAA025 has been screened out of further studies on its potential probiotic properties due to the presence of *tdc* and *AgDI* genes.

Keywords: Probiotics, Genes, Polymerase Chain Reactions, Safety, bacteria.

INTRODUCTION

Enterococci and streptococci are members of Lactic Acid Bacteria (LAB). Both genera have similar properties. Enterococci were formerly classified with Streptococci as group D streptococci but, were later placed in a genus of their own in the 1980s. Enterococci and streptococci have a widespread presence in the environment and have been reported to produce antimicrobial substances inhibitory to spoilage organisms.[1] Moreover, enterococci and streptococci are used as probiotic organisms because of their good growth, adhesive ability, lactic acid production e.t.c.[2] Probiotics are "Live microorganisms which when administered in adequate amounts confer a health benefit on the host."[3]

Probiotic strains must be included into the GRAS (General Regarded as Safe) microorganisms that comprise the genera *Lactobacillus*, *Pedicocci, Bifidobacteria* and some *Streptococcus* strains (Also, other genera that have been proven to be safe can be included). Historical data must indicate that they are safe for human use, based on their occurrence as normal commensals of the human microbiota, and their safe use in different foods and products worldwide. Before using an organism as probiotics, the FAO and WHO guidelines recommend that the probiotic strains must be characterized in series of tests which includes absence of side effects during clinical trials, absence of toxins (including virulent biogenic amines genes) and absence of antibiotic resistance genes.[4]

The probiotic strain must not harbour any virulent antibiotic resistant genes which can be easily transferred to human during consumption. Enterococci has been noted for its resistance to a variety of antimicrobials due to intrinsic or acquired resistance.[5] While intrinsic resistance may be desirable in probiotic strains especially for patients already on antibiotic chemotherapy, acquired resistance is undesirable.

Biogenic amines (BA) are organic bases of low molecular mass that are formed and degraded during the normal metabolism of microorganisms, plants, and animals. They are necessary for several physiological functions in humans. However, they are toxic if high concentrations are ingested or if the detoxification process is inhibited (either genetically or by drugs).[6] Foods likely to contain high levels of BA include fishery products and fermented foods such as cheese, wine, beer, and cured sausages.[7] Biogenic amines form via the decarboxylation of their corresponding amino acids through the action of enzymes produced by microorganisms present in the food (e.g., *Enterobacteriae, Pseudomonas* spp., enterococci, and some LAB.[8] Nonetheless, production is more related to strain than species. Producer strains can appear as contaminants of fermented foods, but they can also form part of starter cultures. It is therefore useful to determine which strains produce undesirable compounds so that these are not part of starter cultures or LAB intended for probiotic use. Histamine and tyramine are the most studied of the BA because they have toxicological effects that derive from their vasoactive and psychoactive properties. These compounds are formed from histidine and tyrosine, respectively, via an enzymatic decarboxylation reaction. The histidine decarboxylase genes (*hdc*) of different Gram-positive bacterial strains have been characterized.[9] The biochemical properties of histidine decarboxylase from *Lactobacillus* have been

studied extensively by Schelp *et al.*[10] However, although tyrosine decarboxylase (*tdc*) enymes have been well characterized in eukaryotes, little is known about their prokaryotic counterparts. Therefore, the aim of this study is to screen for absence of antibiotic resistance genes and biogenic amines genes (tdc, hdc and AgDI) in *Streptococcus equines* FAA026 and *Enterococcus faecalis* FAA025 intended for probiotic use.

MATERIALS AND METHODS

History of strains

The authors have previously isolated *Enterococcus faecalis* FAA025 and *Streptococcus equines* FAA026 from human vagina and selected for further studies on probiotic properties due to their antagonistic activities against uropathogens and enteropathogens, production of organic acids, hydrogen peroxide and different volatile compounds (mainly ethanol from *Streptococcus equines* FAA026) (Unpublished observations).

Determination of Minimum Inhibitory Concentration by broth microdilution

The Minimum Inhibitory Concentration (MIC) of *Enterococcus faecalis* FAA025 and *Streptococcus equines* FAA026 were evaluated by broth microdilution. This method is based on the innoculation of a standardized broth innoculum of the test strain in a dilution series of the antibiotic for which the MIC is determined. The first concentration in the dilution series at which no visual growth can be determined is then considered the MIC. For determination of MIC of different antibiotics to *Enterococcus faecalis* FAA025, VetMic™ Lact-I microdilution tests (SVA, Uppsala, Sweden) were used to determine the MIC of Gentamicin, Kanamycin, Streptomycin, Neomycin, Tetracycline, Erythromycin, Clindamycin and Chloramphenicol while micro plates were used for the following antibiotics: Ampicillin (Apollo Scientific Ltd., Cheshire, UK), Ciprofloxacin (Fluka-Sigma, UK), Trimethoprim – Sulfamethoxazole (TMP-SMX, Celtech Pharma S.A., Madrid, Spain), Fosfomycin, Pharmazam, Barcelona, Spain) and Nitrofurantoin (Laboratorios ERN S.A., Barcelona, Spain).. For determination of MIC of different antibiotics to *Streptococcus equines* FAA026, micro plates were used for the following antibiotics: Kanamycin (Fluka-Sigma, UK), Streptomycin (Fluka-Sigma, UK), Tetracycline (Fluka-Sigma, UK), Erythromycin (Fluka-Sigma, UK), Chloraphenicol (Fluka-Sigma, UK), Ampicillin (Fluka-Sigma, UK), TMP-SMX (Celtech Pharma S.A., Madrid, Spain) and Nitrofurantoin (Laboratorios ERN S.A., Barcelona, Spain).

The two LAB strains were cultured overnight on Lactobacillus Sensitivity Medium (LSM (Oxoid) 90% Isosensitest and 10% Mann Rogosa de Sharpe (MRS) media [11] at 37°C, 5% CO_2. Individual colonies were suspended in a sterile glass tube containing 5 ml sterile saline. The innoculum density was adjusted in a spectrophotometer $OD_{625\ nm}$ until OD between 0.16 - 0.2 was obtained. The innoculated saline suspension was then diluted 1:1000 to obtain a final concentration of about $3x10^5$ cfu/ml. This corresponds to the McFarland (McF) standard 1. Afterwards 100 µl of the diluted suspensions were added to each well and incubated at 37°C for 48 h. Bacterial growth was visually detected as a pellet at the bottom of the well and also by the Microplate Spectrophotometer Benchmark Plus (BioRad, Hercules, CA, USA). The MIC was defined as the lowest antibiotic concentration at which no visual growth was detected or, when it was possible, the breakpoint standard of the FEEDAP panel[12] was used.

Detection of tetracycline and neomycin resistance genes

The bacterial DNA was extracted by by Gen Elute™ Bacteria Genomic DNA kit (Sigma, Aldrich) according to the manufacturer`s instructions. The presence of neomycin resistant gene *aph (`3'')-II* was investigated by using the method of Ouoba *et al.*,[13]. The extracted bacterial DNA was used as template in Polymerase Chain Reactions (PCR) amplification with forward primer (5'-GCT ATT CGG CTA TGA CTC GGC-3') and reverse primer (5'-CCA CCA TGA TAT TCG GCA AGC-3'). Amplification was performed using the following conditions, initial denaturing at 94°C for 3 min, 30 cycles at 94°C for 1 min, annealing temperature of 60°C for 1 min, followed by a final extension step at 72°C for 10 min. The PCR products were analyzed on 1% agarose gel in TAE buffer containing ethidium bromide and visualized under UV light.

The presence of tetracycline resistant gene *tet(W)* was investigated by using the method of Barbosa *et al.*,[14]. The extracted bacterial DNA was used as template in PCR amplification with *tet* 1 primer (5'-GCTCA(T/C) GTTGA(T/C)GCAGGAA-3') and *tet* 2 primer (5'-AGGATTTGGCGG(C/G)ACTTC(G/T)A-3'). The PCR amplification programme was one cycle at 94°C for 5 min, 50°C for 2 min, 72°C for 2 min 29 cycles at 94°C for 1 min, annealing temperature of 50°C for 2 min, 72°C for 2 min and the final cycle of 94°C for 1 min, 50°C for 2 min, 72°C for 10 min. The PCR products were analyzed on 1% agarose gel in TAE buffer containing ethidium bromide and visualized under UV light.

Screening for genes coding for biogenic amines

Polymerase chain reactions analysis

The presence of gene clusters responsible for the formation of the biogenic amines tyramine, histamine and putrescine was evaluated by PCR for *Streptococcus equines* FAA026 and *Enterococcus faecalis* FAA025. To detect the tyrosine decarboxylase gene, (*tdc*), Ten (10 ng) of extracted DNA was diluted in 25 ml of MilliQ water and used directly in the reaction. DNA was amplified in an iCycler thermal cycler (Bio-Rad, Hercules, Calif.). The PCR conditions involved an initial denaturation step (95°C for 5 min), 35 amplification cycles (95^0C for 45 s, 50°C for 1 min, and 72°C for 1 min), and a final extension step at 72°C for 7 min. All amplifications were performed with the use of puRe Taq Ready-To-Go PCR beads following the manufacturer's instructions (Amersham-Biosciences,

Buckinghamshire, UK) and 200 nM of oligonucleotides as primers.

To detect histidine decarboxylase (*hdc*) gene, PCR was performed essentially as described by Le Jeune, *et al.*,[15]. Approximately 1 ng of bacterial DNA or 10^3 colony-forming units treated at 37°C for 1 h with Mutanolysin (Sigma) (15 000 U I-') in TE buffer (10 mmol 1-' Tris-HC1, pH 8, lmmol I-' EDTA) was subjected to PCR in a total volume of 50 p1, with 1 unit of Taq polymerase (Perkin-Elmer Cetus or Promega, Madison, USA), in a reaction mixture recommended by the supplier of Taq polymerase. For amplifications, the samples were heated at 94^0C for 7 min and subsequently 30 cycles were run with the following temperature profile: denaturation at 95°C for 1 min, annealing of the primers at 48°C for 1 min, and primer extension at 72°C for 1 min. The PCR products were separated by gel electrophoresis on a 2 % agarose gel with the Tris-borate EDTA buffer system. The gels were analysed after staining with ethidium bromide. For detection of agmatine deimininase (*AgDI*) gene, PCR conditions were those described by the authors. (Ladero *et al*, personal communication). As positive control, the strain Enterococcus durans 665 was used for detection of *tdc.*[16] *Lactobacillus buchneri* B301 was used for detection of *hdc*[17] while *Lactobacillus brevis* ATCC3810 was used for the detection of *AgDI*. The sequences of the primers are described in table 1.

Table 1: Primers used for detection of biogenic amines genes

Biogenic Amine	Gene	Primers	Sequence 5'-3'	Positiom
Tyramine	*tdc*	*TDC1* *TDC2*	AACTATCGTATGGATATCAACG TAGTCAACCATATTGAAATCTGG	3,791–3,813 4,533–4,510
Histamine	*hdc*	*JV16HC* *JV17HC*	AGATGGTATTGTTTCTTATG AGACCATACACCATAACCTT	169-190 515-536

RESULTS AND DISCUSSION

Antibiotic resistant patterns of *Enterococcus faecalis* FAA025 and *Streptococcus equines* FAA026 were studied. It was observed that *Enterococcus faecalis* FAA025 was generally sensitive to tested antibiotics while noticeable resistance was only observed in tetracycline, neomycin and clindamycin (table 2). *Streptococcus equines* FAA026 had high MIC for TMP-SMX and Nitrofurantoin while sensitive to the remaining tested antibiotics. The resistance gene reservoir hypothesis suggests that beneficial and commensal bacterial population may play a role in the transfer of antibiotic resistance to pathogenic and opportunistic bacteria[18]. This has led to growing concern in the selection of probiotics strains because transfer of resistance genes from animals to human can occur through the food chain. Klare *et al.*,[11] and Aquilanti *et al.*,[19] reported cases of antibiotics resistance in LAB from animals and human isolates. *Enterococcus faecalis* and *Streptococcus* sp. have potential benefit for human health and is use as food starter cultures and probiotics after it has been proven to be safe [20].

The presence of neomycin and tetracycline resistant genes were investigated in *Enterococcus faecalis* FAA025 and *Streptococcus equines* FAA026 through PCR reactions. The genes could not be detected in the two bacterial strains (table 2). The observed high resistance in neomycin and tetracycline is not due to the presence of *aph* and *tet* W genes respectively since they were not detected in the bacterial strain. High resistance to TMP/SMX and Nitrofurantoin by *Streptococcus equines* FAA026 may be intrinsic rather than acquired because some other LAB strains have been reported to be resistant to the two antibiotics[21].

Table 2: Minimum Inhibitory Concentration of different antibiotics to *Enterococcus faecalis* FAA025 and *Streptococcus equines* FAA026

Antimicrobials	Range of concentration studied (µg/ml) with breakpoint	*Entercoccus faecalis* FAA025	*Streptococcus equines* FAA026	Resistant genes investigated
Gentamycin	(0.5 – 256) BP 512	32 S	NT	
Kanamycin	(2 – 1024) BP 1024	128 S	64 S	
Streptomycin	(0.5 – 256) BP 1024	256 S	64 S	
Tetracycline	(0.1 – 64) BP 16	32 R	2 S	*tet* W
Erythromycin	(0.016 – 8) BP 4	1 S	0.5 S	
Clindamycin	(0.03 - 16) BP 4	>10 R	NT	
Neomycin	0.5 – 256) BP 1024	>256 R	NT	aph 3'' –II
Chloraphenicol	(0.12 – 64) BP 8	8 S	8 S	
Ampicillin	(1 – 1024) BP 8	4 S	0.25 S	
Ciprofloxacin	(0.1 – 128)	2 S	NT	
TMP-SMX	(0.25 – 256) 8	8 S	>256	
Fosfomycin	(3.13 – 3200)	100 S	NT	
Nitrofurantoin	(0.25 – 256	64 S	>256	

Note:- The breakpoint has been adapted from the FEEDAP (European Food Safety Authority Panel on additives and products or substances used in animal feed) panel report[12]. No definite breakpoint has been defined for the last four antibiotics. Strains with MIC higher than the breakpoint are considered as resistant. NT- Not Tested

Table 3: Detection of gene clusters responsible for the formation of biogenic amines

	Biogenic amine genes		
	tdc	***hdc***	***AgDI***
Enterococcus faecalis FAA025	+	-	+
Streptococcus equines FAA026	-	-	-

Note: (+) positive amplification, (-) no amplification

It has been reported that resistance of LAB to antibiotics is more pronounced in sub-Sahara Africa than in other parts of the world because of the indiscriminate use of antibiotics both in human and animals[22]. In these circumstances, it is extremely important to know and characterize the resistance to antibiotics in strains potentially intended for food and/or therapeutic applications. However, the two bacterial strains tested in this study were sensitive to most tested antibiotics and the presence of transferable antibiotic resistant genes was not detected in the strains. Therefore, the strains have the potential of being utilized as probiotic strains based on the possible absence of transferable antibiotic resistance genes.

The presence of genes coding for production of virulent biogenic amines was investigated in the two LAB strains used in this study. The 3 investigated genes were absent in *Streptococcus equines* FAA026. However, *tdc* and *AgDI* genes were detected in *Ent. faecalis* FAA025 (Table 3). Lactic acid bacteria are among the Gram-positive bacteria with potential to produce toxic biogenic amines derived from the amino acid catabolism[23]. These compounds can cause several toxicological problems and/or may act as potential precursors of carcinogenic nitrosamines [24]. Their presence is not desirable in probiotic products. The presence of the genes responsible for the production of the three biogenic amines were not detected in *Streptococcus equines* FAA026, therefore the strain has a potential for probiotic application. However, the presence of the genes responsible for the production of tyramine and putrescine were detected in *Enterococcus faecalis* FAA025. Børrensen *et al*,[25] reported the presence of *tdc* enzyme in *Enterococcus faecalis* and this characteristic seems to be strain dependent[26].

CONCLUSION

The presence of genes involved in the synthesis of biogenic amines is not a desirable characteristic in strains intended for food applications; nevertheless, the presence of these genes in *Enterococcus faecalis* FAA025 strains do not guarantee that the biogenic amines will be produced in the food matrix environment. Therefore, further work needs to be done to determine if biogenic amines will be produced by *Enterococcus faecalis* FAA025 when used as starter culture or probiotics. If so, the strain will be screened out of further studies.

Acknowledgements: Special thanks are given to Drs. Maria Fernández and Victor Ladero for the biogenic amine genes detection.

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