Probiotic Properties of *Enterococcus faecium* EF9296 Strain Isolated from Silage

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Abstract

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Ensilage means preserving forage fed by livestock when fresh material is less available. When the silo is opened, the silage is exposed to air and this leads to aerobic deterioration whereby aerobic microorganisms degrade lactic acid. Therefore, it is necessary to use some additives to control microbes in silage fermentation. The strain EF9296, isolate from silage was allotted to the species *Enterococcus faecium* by genotyping. This strain is sensitive to ampicillin (10 μg), erythromycin (15 μg), tetracycline, rifampicin, vancomycin (30 μg) and it is kanamycin-resistant (30 μg). EF9296 strain possess good adhesive capability to human as well as to canine mucus (human 5.5 log 10 cfu/ml, canine 4.7 log 10 cfu/ml). In addition, EF9296 is bile tolerant, lactic acid producing (0.997 ± 0.29 mmol/l) with ureolytic activity (16.9 ± 1.2 nkat/ml).The strain EF9296 produces a bacteriocin-like substance with the inhibitory activity against Gram-positive bacteria including enterococci and *L. monocytogenes* with activity from 100 to 800 AU/ml. Amplified fragment of bacteriocin produced by 9296 strain corresponds with PCR signal for a structural gene of well-known enterocin A. *Ent. faecium* 9296 strain reached the stationary phase at 8 h in Trypticase-soy broth (11.57 ± 0.07 log 10 cfu/ml) and at 10 h in rumen fluid (9.25 ± 0.48 log 10 cfu/ml). It means, EF9296 strain grew in both media, although its growth in rumen fluid was slower in comparison with counts in TSY broth. In addition, the antilisterial effect of *Enterococcus faecium* EF9296 in rumen fluid was detected in experimental sample after 6 h from EF9296 strain application in comparison to the *Listeria* control sample (0.4 log). This effect was prolonged up to the end of the experiment (8 h - 0.44 log, 10 h – 0.65 log, 12 h – 0.62 log). *Enterococcus faecium* EF9296 strain might be used as potential probiotic to protect silage against microbial contaminants e.g. *Listeria* spp.

Bacteria, silage, Listeria, Enterococci

Silage is the feedstuff produced by fermentation of forage crops of variable but often high moisture content. Ensilaging means to preserve forage (when abundant) for feeding livestock when fresh material is less available. In general, silage fermentation is a natural process whereby epiphytic lactic acid bacteria ferment water soluble carbohydrates in the crop to a number of products, primarily lactic acid, thereby reducing the pH as rapidly as possible, inhibiting spoilage microbes and preserving the maximum amount of nutrients in the product (Merry and Davies 1999). In Europe, an estimated 160 million tonnes of forage dry matter are ensiled annually (Wilkinson et al. 1996).

The ensilage process is divided into the initial aerobic phase, the fermentation phase and second aerobic phase when the silo is opened. The last phase has consequences for the quality of the product fed by livestock (Merry and Davies 1999). When the silo is opened and/or sealed inadequately, the silage is exposed to air. This may lead to aerobic deterioration. Aerobic microorganisms in silage degrade lactic acid and residual water-soluble carbohydrate to CO_2 and protein and amino acids to amines, amides, and ammonia (Seale 1986). Aerobic deterioration generates considerable heat, increases pH, and decreases digestibility (Woolford 1990). The main contaminants associated with aerobic spoilage activity in silage are yeasts, moulds, *Bacillus* spp. and *Listeria* spp. (McDonald et al. 1991). When lactic acid bacteria fail

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to produce sufficient lactic acid during fermentation to reduce pH and inhibit the growth of butyric acid-producing bacteria, i.e. clostridia, the resulting silage will be of poor quality (McDonald et al. 1991). That is why to control silage microflora is important. There are many bacteria among lactic acid microorganisms, probiotic strains including, which are able to control the growth of undesirable flora in silage. Probiotics represent live microorganisms or microbial preparations or metabolites of stabilized autochtonous microorganisms which asses benefit effect on hosts organisms and affect microbial composition with stimulating effect on digestion and immunity of macro-organism (Nemcová 1997). Probiotic lactobacilli are also the most commonly species used as silage inoculants. However, among enterococci that belong to lactic acid bacteria (LAB) has been found bacteria with probiotic character too. It is believed that the inoculated populations of LAB genotypes become dominant in silage, thereby increasing the lactic acid concentration and decreasing pH values, gas, and protein decomposition (Inglis et al. 1999). Probiotic LAB are also fed by livestock to improve intestinal microbial balance, including elimination or reduction of undesirable microorganisms (Zhao et al. 1998). Viable microbial preparations, principally including lactic acid bacteria, have been proposed as a supplement in animal fodder (Cavazzoni and Adami 1991).

The use of silage for animal feeding has sometimes been associated with pathological problems, including listeriosis (Wiedemann et al. 1996). Because *Listeria* can grow at low temperatures (Tienungoon et al. 2000), hay crop silage stored in large plastic bags has frequently been contaminated (Wilkinson 1999).

The aim of this study was to characterize the strain of *Enterococcus faecium* EF9296 and to test it as potential silage probiotic (resulting from the selection criteria most typical of probiotics, i.e. adhesion ability of potential probiotic strain, antimicrobial activity, production of organic acid, tolerance to bile as well as from metabolic properties such as urease activity) to control, especially listerial contamination in silages.

Materials and Methods

Isolation and identification of the strain

Parallel samples of grass silages (10g) were added into 90 ml of saline solution (0.85%, pH 7) and vortexed. Then the samples were treated by the standard microbiological method; 100 µl from the appropriate dilutions were plated onto M-*Enterococcus* agar (Becton & Dickinson, Cockeysville, USA). Single colonies were picked up, checked for purity and used for genotypization. Genotypization of 9296 strain was provided by tDNA-PCR method after DNA extraction. For DNA preparation the strain was cultivated on Slanetz-Bartley agar (Oxoid, Basingstoke, Hampshire, England) at 37 ºC for 24 h and checked for purity. One bacterial colony was suspended in 20 µl lysis buffer (0.25% sodiumdodecylsulphate, 0.05 NaOH) and heated at 95 ºC for 5 min. Lyzed cells were centrifuged briefly at 16 000 × g to remove cell debris. Supernatant was used as template for tDNA-intergenic PCR (tRNA-intergenic length polymorphism analysis, Baele et al. 2000) followed by capillary electrophoresis (Welsh and McClelland 1991). Following tRNA-gene consensus primers 5A(5'AGTCCGGTGCTCTAACCAACTGAG), T3B(5´AGGTCGCGGGTTGGAATCC) were used in order to amplify the intergenic regions between tRNA genes. The amplicons were separated by means of capillary electrophoresis using an ABI Prism 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA) at 60 ºC with a constant voltage of 1.5 kV (10 mA). The fragment lenghts were derived from the peak positions after intrapolation with the peak position of the size standard fragments. Electropherograms were interpreted visually and with a software program developed and described by Baele et al. (2000).

Intestinal mucus preparation and *in vitro* adhesion assay

Human intestinal mucus was isolated from the healthy part of resected colonic tissue as described Ouwehand et al. (2002). The use of human intestinal material was approved by the joint ethical commitee of the University of Turku and Turku University Central Hospital. Canine mucus was prepared according to Kirjavainen et al. (1998) and Ouwehand et al. (2002).

Adhesion to mucus was studied as described Ouwehand et al. (1999) using scintillation liquid. Results are expressed as the average of at least three independent experiments in four parallels (log 10 value of the number of bacteria adhered per microtitre plate).

Antimicrobial susceptibility testing

Antibiotic sensitivity or resistance was tested by agar disc diffusion method using Columbia agar (Becton & Dickinson) enriched with 10% of defibrinated sheep blood. The following antibiotic discs (Becton & Dickinson)

were used: ampicillin (10 µg), erythromycin (15 µg), tetracycline, rifampicin, kanamycin, vancomycin and chloramphenicol (30 µg). The strain was classified as resistant or sensitive according to the manufacturer´s instructions. *Ent. faecium* CCM4231 was used as a positive control.

Testing of metabolic properties

To test tolerance of *Enterococcus faecium* EF9296 strain to bile, the strain was cultivated in Brain Heart broth enriched with 5% of oxgall (Becton & Dickinson) at 37 ºC for 24 h. The growth of strain was checked by measuring optical density ($O.D._{600}$) as well as by spreading of 100 µl of cultures or appropriate dilutions onto Slanetz-Bartley agar (Oxoid, Basingstoke, Hampshire, England). Optical density and cells counts were compared with those after 24 h. The growth of the strain was expressed in colony forming units per milliliter (cfu/ml) ± SD.

Production of lactic acid was measured by precipitate method according to Pryce (1969) and expressed in mmol/l after four replicates ± SD.

Urease activity was measured by the spectrophotometric method according to Cook (1976) and expressed in nkat/ml after three replicates ± SD.

Bacteriocin production

Bacteriocin activity was detected by the agar spot technique according to De Vuyst et al. (1996) using Trypticase-soy agar as well as 0.7% Brian heart agar (Becton & Dickinson) with overnight culture of indicator organism. Supernatant of potential producer (10 µl) was spotted onto plates overlaid with 4 ml of 0.7% agar containing 200 µl of indicator organism. The plates were incubated at 37 °C for 24 h. Inhibitory activity was demonstrated by a clear zone around concentrated spots as well as diluted spots and expressed in arbitrary units per ml of culture medium (AU/ml). Arbitrary unit is defined as the reciprocal of the highest twofold dilution demonstrating complete inhibitory activity of the indicator strain.

Bacteriocin specification using PCR

The primer (5'- GGT ACC ACT CAT AGT GG AAA-3', 5'- CCC TGG AAT TGC TCC ACC TAA-3') and PCR reaction for bacteriocin-enterocin specification produced by *Ent. faecium* EF9296 strain was used according to Aymerich et al. (1996). Total genomic DNA used as a template for PCR was isolated as is described in the part Isolation and identification of the strain. DNA from PCR reaction was separated by 2% agarose gel electrophoresis and gel was stained with 0.1 µg/ml of ethidium bromide and visualised by UV light. As positive control was used enterocin A produced by *Enterococcus faecium* EK13 (Mareková et al. 2003).

Growth of EF9296 strain in TSY broth and rumen fluid

The rumen fluid was taken from ruminaly fistulated sheep (Merino). In brief, the rumen fluid was centrifuged at 10 000 g for 30 min and boiled to reach sterile fluid. Its "sterility" was checked on Columbia agar and TSY agar (Becton & Dickinson) with 10 % of sheep blood after overnight incubation at 37 ºC.

The EF9296 strain was cultivated in Trypticase-soy broth (Becton & Dickinson) overnight at 37 ºC. Strain EF9296 (0.1%) was added into TSY broth and rumen fluid. The samples were collected at 0 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h and 24 h after the inoculation. The growth of EF9296 strain was checked by using the plate counts of appropriate dilutions of the samples in saline solution (0.85%, pH 7) on Slanetz-Bartley agar (fy *E.coli*, Slovakia). The plates were checked for colonies after incubation at 37 ºC for 24 h and the counts were expressed in log 10 cfu/ml ± SD.

Competitive exclusion

Rumen fluid was divided into three equal volumes of 200 ml, which were used as the experimental sample (ES), EF9296 control sample (ECS) and *Listeria* control sample (LCS). Experimental sample was inoculated with 1% of overnight culture of *Listeria innocua* LMG 13568 strain (Universiteit Gent, Laboratorium voor Microbiologie, Belgium) and *Enterococcus faecium* EF9296 strain. *Listeria* control sample was inoculated with *Listeria innocua* LMG 13568. ECS was inoculated with *Enterococcus faecium* EF9296. Samples were cultivated by shaking in the water thermobath-incubator at 37 °C for 24 h using 100-120 U/min. The growth of strains and the inhibitory effect of *Ent. faecium* EF9296 strain against *Listeria innocua* LMG 13658 was checked during 24 h by spreading of 100 µl of the appropriate dilutions onto selective media. *Listeria innocua* LMG 13568 was counted on Oxford agar (Becton & Dickinson) and the count of *Ent. faecium* EF9296 was determined using TSY agar (Becton & Dickinson).

The mean differences (± SD) are presented and expressed conventionally.

Results

The strain EF9296 was allotted to the species *Enterococcus faecium*. It possess good adhesive capability to human as well as to canine mucus (human 5.5 log 10 cfu/ml, canine 4.7 log 10 cfu/ml). Although EF9296 strain is sensitive to all selected antibiotics (ampicillin, vancomycin, rifampicin, tetracycline, chloramphenicol, erythromycin), this strain is kanamycin resistant. In addition, EF9296 is bile tolerant (growing even in the presence of 5% of oxgall), lactic acid producing (0.997 $\pm$ 0.29 mmol/l) with ureolytic activity

(16.9 ± 1.2 nkat/ml). The strain produces a bacteriocin–like substance with the inhibitory activity against Gram–positive bacteria including enterococci (11 isolates from dog feed) with activity from 100 to 800 AU/ml. *Listeria monocytogenes* CCM 4699 (Czech Collection of Microorganisms, Brno, Czech Republic) was inhibited by activity 400 AU/ml. Structural gene of well-known enterocin A was screened for using PCR and agarose gel electrophoresis revealed amplified fragment which corresponds with PCR signal for enterocin A.

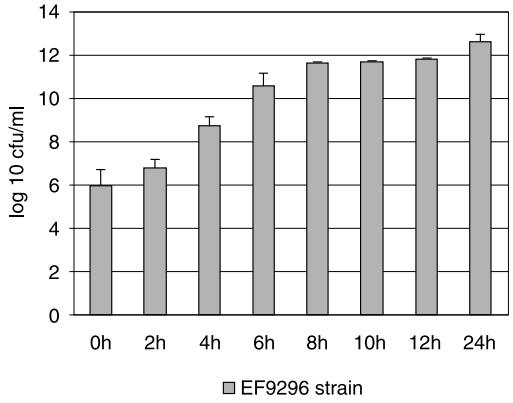

Fig. 1. Growth of *Enterococcus feacium* in TSY broth.

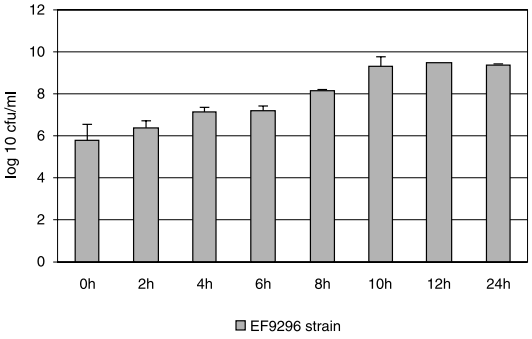

Fig. 2. Growth of *Enterococcus feacium* in rumen fluid.

Enterococcus faecium EF9296 grew very well in TSY broth (Becton & Dickinson) as well as in the rumen fluid (Figs 1, 2). At the start of cultivation (0 h) the counts of EF9296 strain reached 5.96 ±0.73 log 10 cfu/ml in TSY broth and 5.75 ± 0.75 log 10 cfu/ml in rumen fluid. Stationary growth phase in TSY broth was reached after 8 h (11.57 ± 0.07 log 10 cfu/ml). Stationary phase in rumen fluid was detected at 10 h (9.25 ± 0.48 log 10 cfu/ml).

Slight inhibition of *Listeria innocua* LMG 13568 due to EF9296 strain with difference 0.4 log cycles was noted in ES after 6 h from EF9296 strain application in comparison to LCS. This effect was prolonged up to the end of the experiment (at 8 h-differences 0.44 log cycles, at 10 h – 0.65 log cycles, at 12 h – 0.62 log cycles, Fig. 3). *Enterococcus faecium* EF9296 grew very well in the rumen fluid with final count 9.17 ± 0.03 log 10 cfu/ml in ES and 10.12 ± 0.82 log 10 cfu/ml in ECS.

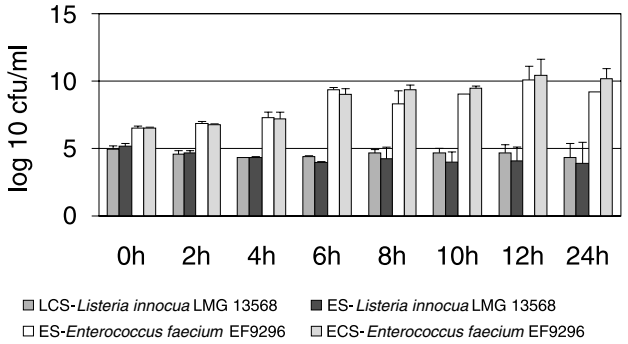

Fig. 3. Inhibition of *Listeria innocua* LMG 13568 due to *Enterococcus faecium* EF9296 in rumen fluid.

Discussion

The mechanism of effectiveness of a probiotic (although it is not fully clucidated) is closely associated with the properties of the production strains. *Enterococcus feacium* EF9296 used in this study is kanamycin resistant. Enterococci are intrinsically resistant against kanamycin (Franz et al. 1999). EF9296 strain binds to human intestinal mucus in a similar manner to that observed for canine mucus. This observation correlated with the adhesive capability of *Lactobacillus* sp. AD1 strain – potential canine probiotic (Strompfová et al. 2004). This suggests that often mentioned species specificity of probiotics is not interfering with the *in vitro* adhesion to intestinal mucus (Lauková et al. 2004). Tolerance to bile salts is considered to be a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host (Havenaar et al. 1992). It is mentioned, that the resistance to bile salts varies a lot among the lactic acid bacteria species and even between strains themselves (Xanthopoulos 1997). EF9296 strain presented here as well as the other probiotic strain *Lactobacillus* sp. AD1 reported by Strompfová et al. (2004) were bile tolerant. The inhibitory components of probiotic strains comprise the production of bacteriocins or organic acid (e.g. lactic acid). EF9296 strain possess bacteriocin activity as well as it produces lactic acid; it can be supposed, that its inhibitory activity is due to actions of both substances. Enterocin A is a frequently detected bacteriocin among bacteriocin-producing enterococci originated from different sources e.g. environmental isolate *Enterococcus faecium* EK13, food isolate *Ent. faecium* CTC 492 (Mareková et al. 2003, Aymerich et al. 1996).

Enterococcus faecium EF9296 grew in both, in TSY broth as well as in the rumen fluid. Kalmokoff et al. (1996) proposed the idea that lactic acid producing as well as bacteriocin-producing species; thus is, also enterococci, might be useful in silage fermentation. The addition of enterocin CCM 4231 to rumen fluid contaminated with *Listeria monocytogenes* OHIO strain (in early log phase) inhibited the growth of *Listeria monocytogenes* after 1 h of enterocin application by prolonged effect mainly at 24 h of cultivation (Lauková and Czikková 1998). Moreover, antilisterial effect of enterocin CCM 4231 was also reported for the other environments e.g. cattle slurry (Lauková et al. 1998). Addition of 200 AU/ml bacteriocin of *Enterococcus faecium* RZS C13 (isolate from silage) to a cell-suspension of *Listeria monocytogenes* resulted in an initial drop in number of viable cells (Vlaemynck et al. 1994). Slight inhibition of *Listeria innocua* LMG 13568 due to EF9296 strain in rumen fluid reported in this study confirmed antilisterial effect of enterococci and their bacteriocins previously refferred. In addition, the others bacteriocins of *Enterococcus faecium* e.g. enterocin A produced by *Enterococcus faecium* EK13 strain with probiotic character or

enterocin M produced by *Enterococcus faecium* AL41 were active against *Listeria innocua* (Lauková and Mareková 2002; Lauková et al. 2003).

Although bacteriocin-producing bacteria are successfull in protection of silage against pathogens, Pauly et al. (1999) reported the effect of mechanical forage treatments on the growth of *Listeria monocytogenes* in grass silage. Precision chopping increased the concentration of lactic acid, reduced pH and counts of *Listeria monocytogenes* dropped on day 35 in the silage compared with unchopped grass.

It can be concluded, *Enterococcus faecium* EF9296 strain might be tested as potential probiotic and used to protect silage against microbial pathogens such as *Listeria* spp. However, additional experiments in silage ecosystem are required (e.g. adherence capability of the strain in silage, storing ability in silage, application and detection of enterocin production in silage, effect of lactic acid and enterocin in silage) and are in processing.

Charakterizácia kmeňa *Enterococcus faecium* EF9296 – potenciálneho silážneho probiotika

Silážovanie t.j. konzervovanie plodín v období, kedy je nedostatok čerstvých krmív, vhodných na kŕmenie hospodárskych zvierat je bežným biotechnologickým procesom v poľnohospodárstve. Po otvorení sila za prístupu vzduchu dochádza k degradácii kyseliny mliečnej aeróbnymi mikroorganizmami. Kvôli týmto faktorom je potrebné používať silážne aditíva, ktoré by kontrolovali bakteriálny ekosystém počas fermentačnej fázy silážneho procesu. Kmeň EF9296 bol izolovaný zo siláže a genotypizovaný ako *Enterococcus faecium*. Tento kmeň je citlivý na ampicilin (10 μg), erytromycin (15 μg), tetracyklin, rifampicin a vankomycin (30 μg), avšak je rezistentný na kanamycin (30 μg). Adheruje rovnako dobre ako na ľudskú, tak aj na psiu mukózu (ľudská 5.5 log 10 cfu/ml, psia 4.7 log 10 cfu/ml). Okrem toho je kmeň EF9296 rezistentný na žlčové soli, produkuje kyselinu mliečnu (0.997 ± 0.29 mmol/l) a je ureolytický (16.9 ± 1.2 nkat/ml). Kmeň EF9296 dosiahol stacionárnu fázu po 8. hodinách rastu v Tryptikáza-sójovom bujóne (Becton & Dickinson; (11.57 ± 0.07 log 10 cfu/ml) a po 10. hodinách rastu v bachorovej tekutine (9.25 ± 0.48 log 10 cfu/ml). Kmeň *Enterococcus faecium* EF9296 zredukoval počty listérií v experimentálnej vzorke bachorovej tekutiny po 6 hodinách od podania kmeňa EF9296 pri porovnaní s kontrolnou vzorkou, ktorá obsahovala len listérie (rozdiel 0.4 log cyklu). Tento efekt pretrvával až do konca experimentu (8h – 0.44 log cyklu, 10h – 0.65 log cyklu, 12h – 0.62 log cyklu). Kmeň *Enterococcus faecium* EF9296 predstavuje potenciálne silážne aditívum, avšak ďalšie testovania *in vitro* ako aj *in vivo* sú nevyhnutné.

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