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Enterococci Isolated from Japanese Quails Exposed to Microgravity Conditions and Stability of their Properties

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Abstract

Enterococci isolated from the crop and caecum of Japanese quails exposed to 7 day conditions of microgravity were re-vitalized after their dry-freezing long storage. Originally, the strains were isolated from Japanese quails after their landing from flight onboard the orbital station Mir during the experiment in August 1990. Because taxonomy as well as the studies concerning the bacteriocins, especially those produced by enterococci, have been continually developed for years, the aim of this study was to confirm species identification, stability of the properties of enterococci as well as to test new properties after their long storage. Genotyping allotted the strains to the species *E. faecium*. Lactic acid production was detected in similar amounts in the strains before and after their long-storage in dry-frozen form. The strains were vancomycin-sensitive and kanamycin-resistant before as well as after their long-time storage. Variability in sensitivity to different antibiotics was found among the strains tested even before and after long-time storage. Each of the strains possessed at least one structural enterocin gene. The structural genes for enterocin A, P, B, L50B were detected in *E. faecium* EP7. *E. faecium* EP2, EEP4 have the genes for ent A, B, L50B. The gene for ent P was detected only in the strain EP7. The most often detected was ent A gene followed by ent genes B, L50B. All strains inhibited growth of at least 4 out of 15 indicators. The stability of the enterococcal properties determined before as well as after their dry-freezing was not influenced during their long-term storage; moreover, new properties were determined.

Enterococci, storage, enterocin gene, properties, stability

Japanese quails represent suitable animal laboratory model for several reasons: their small size, low husbandry costs, short generation interval and adaptability to a wide range of husbandry conditions. Therefore, in past they were an object of the scientific space research programme (Boďa 1979) called Interkozmos that was finished in 1990s. In this programme many research issues were investigated and solved; e.g. the effect of microgravity on endocrine functions and adaptation processes (Juráni et al. 1988), embryonic development (Boďa et al. 1992) and/or morphological changes in the small intestine under space-flight conditions (Cigánková et al. 2000). Moreover, in the framework of the Interkozmos programme-Incubator 2, firstly also microflora of Japanese quails exposed to microgravity conditions was analysed in comparison to conventional Japanese quails with an impact on lactic acid bacteria such as lactobacilli, enterococci and staphylococci (Lauková et al. 1991, 1993a, 1995). The most studied were enterococci that were also found to produce antimicrobial substances - bacteriocins (Lauková et al. 1993bc). The studied isolates were dry-frozen and stored for a long period for other tests. Because taxonomy as well as the studies concerning bacteriocins, especially those produced by enterococci, were continually widely developed for years and enriched with new knowledge (Aymerich et al. 1996; Casaus et al. 1997; Cintas et al. 1998, 2000; De Vuyst et al. 2003; Mareková et al. 2007; Franz et al. 2007), the aim of this study was to confirm the species identification, stability and properties of enterococci after their long-time storage as well as to genotype them and to test them for the previously not determined properties.

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Materials and Methods

Six strains of enterococci isolated from the crop (EEP4, EP2) and caecum (EFP7, EP7, EFP4, EFP5) of Japanese quails exposed to 7 days of microgravity conditions were re-vitalized after their long-time dry-freezing storage (18 years). Originally, the strains were isolated from Japanese quails after their flight onboard the orbital station Mir during the experiment carried out in August 1990. The samples were treated as described previously (Lauková et al. 1991). Dry-frozen strains were cultivated in the Brian Heart Infusion (Becton & Dickinson, Cockeysville, USA) for 18 h at 37 ºC. Then they were plated onto M-Enterococcus agar as well as Brian Heart agar supplemented with defibrinated sheep blood to check their purity. In the past, the strains were phenotyped by the API 20 Strep identification kit (BioMeriux, l'Etoile, France) and analysed to produce lactic acid (Pryce 1969) have bacteriocin-like activity (Skalka et al. 1983) and tested for sensitivity/resistance to antibiotics (NCCLS 2002). After their re-vitalization, they were genotyped using primer sequences and PCR method (Woodford et al. 1997) to confirm their taxonomy. Beside the above-mentioned tests, they were tested for possession of structural genes for enterocin production, and their antimicrobial activity was tested against additional indicator organisms (Table 2).

To detect the structural genes for enterocin production, 2 µl of template was added to 8.75 µl of the reagent mixture which contained 0.5 µl each of the primers, 1 µl of (10 mM) dNTPs, 1.5 µl of (5 mM) $MgCl_2$, 5 µl of 10 × reaction buffer, 0.25 µl of 1 U Taq polymerase (Invitrogen) and water to the total volume 50 µl. The sequences of the primer pairs used for PCR-amplification of the structural enterocin genes (Ent) A, P, L50B, and B are listed in Table 1. The reaction conditions for EntA detection included 5 min denaturation at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 58° C, 30 s at 72 °C; then by 5 min at 72 °C and a cool down to 4 °C. For EntP, L50B and B, the temperature of 56 °C instead of 58 °C was used as the annealing temperature. PCR products were visualised by 2% agarose electrophoresis, containing 1 µg of ethidium bromide. Positive control strains for PCR reactions were as follows: *E. faecium* EK13 (CCM 7419) strain for EntA (Mareková et al. 2003), *E. faecium* AL41 strain for EntP (Lauková et al. 2003), *E. faecium* L50 strain for EntL50B and B (Cintas et al. 1998; Casaus et al. 1997). DNA extraction from each strain was obtained by a simple procedure: 1 loopful of bacterial colony (10 µl) was resuspended in 30 µl of sterile injection water and vortexed for 10 min. Then the supernatant aliquots were used as template DNA.

Table 1. The sequences of the primer pairs used for PCR-amplification of the structural enterocin genes

EntA	F	5' – GGT ACC ACT CAT AGT GGA AA - 3'	
	R	5' – CCC TGG AAT TGC TCC ACC TAA - 3'	(Aymerich et al. 1996)
EntP	F	5' – GCT ACG CGT TCA TAT GGT AAT-3'	
	R	5' – TCC TGC AAT ATT CTC TTT AGC - 3'	(Cintas et al. 1997)
EntL50B	F	5' – ATG GGA GCA ATC GCA AAA TTA - 3'	
	R	5' – TAG CCA TTT TTC AAT TTG ATC - 3'	(Cintas et al. 1998)
EntB	F	5' – CAA AAT GTA AAA GAA TTA AGA TCG - 3'	
	R	5' –AGA GTA TAC ATT TGC TAA CCC - 3'	(Casaus et al. 1997)

The antimicrobial activity of the strains was originally tested for 4 indicators such as *Streptococcus bovis* - Sb 36 (Dr. Jonecová, Faculty of Medicine, University of PJ Šafárik, Košice, Slovakia); *Enterococcus faecium* EF26/42 - own isolate (from rumen contents), *Staphylococcus aureus* CB40 (University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic), *Corynebacterium renale* CCM 5740 (Czech Culture Collection of Microorganisms in Brno, Czech Republic). Additionally, the following 11 indicator strains were used: *E. avium* EA5, *L. innocua* LMG 13568 (Collection of Microorganisms in Ghent, Belgium), *E. faecium* H_{14a}, *Enterococcus* sp. H_2, H_4, LT_2, H_{12}, H_{14b}, *Staphyloccocus* sp. SH_{14}, SH_9 (isolates from chickens, *Salmonella enterica* serovar Enteritidis PT4 (Dr. Šišák, Brno, Czech Republic). Activity was confirmed by the occurrence of inhibitory zone around indicator organisms.

Results

Before dry-freezing the tested strains were allotted by phenotyping to the species *Enterococcus casseliflavus* (EC7/EFP7), *Enterococcus* sp. EP2, EFP5, *E. avium* EP7 and *E. faecium*, EEP4, EFP4. However, genotyping using PCR method allotted all six strains to the species *E. faecium*.

Lactic acid production was detected in the same or very similar amounts in the strains before as well as after dry-freezing in the range values from 0.54 ± 0.07 up to 2.0 ± 0.10 mmo/l (Table 2).

Table 2. Lactic acid production and antibiotic sensitivity or resistance of enterococci, isolated from Japanese quails under microgravity conditions before and after 18 years dry-freezing storage

Strains	LA	Van	Kan	Gen	Tct	Stm	Ery	Rif	Amp	Ch	Pn
EC 7[1]	0.90 ± 0.05	S	R	R	R	R	S	S	R	nd	R
EFP7*	0.93 ± 0.01	S	R	S	S	S	S	S	S	S	S
EP7[2]	0.93 ± 0.0	S	R	R	S	R	S	R	S	S	R
EP7*	0.93 ± 0.02	S	R	R	S	R	S	R	S	S	R
EFP4[3]	nd	S	R	R	R	S	S	R	R	R	nd
EFP4*	0.86 ± 0.0	S	R	S	S	S	S	S	S	S	S
EFP5[4]	0.54 ± 0.07	S	R	S	S	S	S	S	S	S	S
EFP5*	1.05 ± 0.02	S	R	S	S	S	S	S	S	S	S
EP2[5]	0.98 ± 0.05	S	R	S	S	S	S	S	S	S	S
EP2*	1.24 ± 0.02	S	R	S	S	S	S	S	S	S	S
EEP4[6]	2.00 ± 0.10	S	R	R	R	R	R	R	R	R	R
EEP4*	1.50 ± 0.07	S	R	R	R	R	S	R	S	S	S

LA-lactic acid expressed in mmol/l; Van-vancomycin (30 µg); Kan-kanamycin (30 µg); Gen-gentamicin (10 µg); Tct–tetracycline (30 µg); Stm-streptomycine (30 µg); Ery-erythromycin (15 µg); Rif-rifampicin (30 µg); Amp-ampicillin (10 µg); Ch-chloramphenicol (30 µg); Pn-penicilline (10 IU); S-sensitive; R-resistant; nd-not determined; [1-6]*Enterococcus faecium*–before dry-freezing; *the strains after re-vitalizing after 18 years

The strains were vancomycin-sensitive and kanamycin-resistant before as well as after dry-freezing (Table 2). Variability in sensitivity to gentamicin, tetracycline, streptomycin, ampicillin, rifampicin, penicillin and chloramphenicol was found among the strains tested even when sensitivity or resistance were compared before and after dry-freezing. The strains were also sensitive to erythromycin; only EEP4 strain was originally resistant, and it was found to be polyresistant.

Table 3. The presence of structural genes for enterocins P, A, B, L50B and inhibitory activity of tested enterococci against the additional indicator microorganisms

Strains	Ent P	A	B	L50B	Sb[1]	Sa[2]	EF[3]	EA5[4]	H_{12}[5]	Li[6]	Cr[7]
EFP7	-	-	+	-	+	+	+	-	+	-	+
EP7	+	+	+	+	+	+	+	-	+	-	-
EFP4	-	+	-	-	+	+	+	-	-	-	+
EFP5	-	+	-	-	nd	nd	nd	-	-	-	-
EP2	-	+	+	+	+	+	nd	-	+	-	+
EEP4	-	+	+	+	+	+	nd	-	+	-	+

+: the presence of gene or inhibition of the indicator organism; -: absence of the structural gene or inhibition of the indicator; [1]Sb-*Streptococcus bovis* 36 (Dr. Jonecová, Faculty of Medicine, University of PJ Šafárik, Košice, Slovakia); [2]*Staphylococcus aureus* CB40-Czech Culture Collection of Microorganisms, CCM, Brno; [3]*Enterococcus faecium* EF26/42, [4]*E. avium* EA5 (Dr. Lauková, IAP SAS, Slovakia); [5]*Enterococcus* sp. H_{12}-(Dr. Michlovičová, IAP SAS); [6]*L. innocua* LMG 13568-Ghent, Belgium; Cr[7]-*Corynebacterium renale* CCM 5740; *Enterococcus* sp. H_2, H_4, LT_2, H_{14b}, *E. faecium* H_{14a}, *Staphylococcus* sp. SH_{14}, SH_9 (Dr. Michlovičová, IAP SAS, Košice) *S. enterica* serovar Enteritidis PT4 were not inhibited by the tested strains; besides *E. faecium* EEP4 strain which inhibited the strains indicated in the table as well as enterococci and staphylococci from chickens (together 13 strains among 15 tested).

Each of the six strains possesses at least one structural enterocin gene among those tested (Table 3). The structural genes for ent A, P, B and L50B production was detected in *E. faecium* EP7 strain. *E. faecium* EP2 and EEP4 have the genes for ent A, B, L50B production. The gene for ent P was detected only in the strain EP7. The most detected was ent A gene (in 5 isolates) followed by the gene for ent B production (in 4 isolates) and L50B (in 3 isolates).

All strains inhibited the growth of at least 4 out of 15 indicators (Table 3). The principal (most sensitive) indicators generally used in our study, *E. avium* EA5 strain as well as

L. innocua LMG 13568, were resistant to the strains tested. The growth of *S. enterica* PT4 was not inhibited by the substances produced by the tested enterococci from Japanese quails. The growth of *S. aureus* CB40 as well as *C. renale* CCM 5740 was inhibited (before dry-freezing).

Discussion

It seems (also from our previous studies) that the phenotyping of *E. faecium* is in harmony with its genotyping (unpublished data). However, there were differences found in phenotyping and genotyping of the species *E. casseliflavus* or *E. avium* (Kirschner et al. 2001). *E. faecium* is a frequently determined species from the genus Enterococcus among poultry faecal samples (Strompfová et al. 2003, 2007; Tejedor-Junco et al. 2005; Lauková et al. 2008). There are suggestions that especially plasmid-encoded properties such as antibiotic resistance or bacteriocin production could be missing or changed by subsequent passaging of the strains when they are re-vitalized after storage (Lauková and Kuncová 1991). Under our conditions, unchanged LA amounts were detected in enterococci after their long-term storage. This stability could be probably supported by the chromosomal encoding of LA as has been found e.g. for rifampicin resistance in enterococcci (Lauková and Kuncová 1991; Rice et al. 1991). Detection of the structural enterocin genes by PCR is a method that was not available years ago. Therefore it was interesting to determine it when the strains tested were found to produce an antimicrobial substance. There are differences in the ent genes occurrence among *E. faecium* species isolated from different ecosystems. Strompfová and Lauková (2007) reported the presence of only ent A and P structural genes among 5 bacteriocin producing *E. faecium* strains isolated from the caecum, ileum and crop of chickens. Moreover, contrary to the results presented here, ent P gene was the most frequently occurring gene among 234 enterococcal strains from different sources (Strompfová et al. 2008). Among rabbit enterococci, ent A, P, L50B genes were determined but not ent B (Simonová and Lauková 2007). On the other hand, ent B gene was found in enterococci isolated from horses (Lauková et al. 2008). Taking into account bacteriocin production and structural genes detection, EEP4 strain was the most active; although it could be probably discussed that it produces one among enterocins tested and/or a new substance; it will be researched in more detail in our further studies. Interestingly, *S. aurues* CB40 as well as *C. renale* CCM 5740 were mostly inhibited before dry-freezing. That is, bacteriocin production as well as antibiotic resistance are plasmid-encoded properties which as formerly mentioned could be lost by subsequent manipulation (Lauková and Kuncová 1991). In the case of our strains this phenomenon could be hypothetically supposed, although in preliminary studies no plasmids were determined (unpublished data).

Changes in antibiotic sensitivity or resistance in the strains after strain re-vitalization could be explained by different ways; e.g. by the molecular way - the structural gene transport (Lauková et al. 1990), or pragmatically by better quality of modern disks. It is also interesting that polyresistant EEP4 strain showed the best antimicrobial activity; it could be due to bacteriocin but also due to LA production (the highest value among 6 strains tested). The knowledge on bacteriocin production among the isolates presented give us opportunity to search for the probable new substance, to spread information in association with space research as well as to select the strain for other e.g. probiotic testing. It can be concluded that the tested enterococci maintained stability of the properties determined before as well as after their long-term storage by dry-freezing; moreover, additional properties were determined.

Enterokoky z japonských prepelíc vystavených podmienkam mikrogravitácie a stabilita ich vlastností

Enterokoky izolované z hrvoľa a céka japonských prepelíc (vystavených po dobu 7 dní podmienkam mikrogravitácie) boli lyofilizované pre dlhodobé uskladnenie. Kmene boli

izolované z japonských prepelíc po ich návrate z letu na palube orbitálnej stanice Mir počas experimentu v auguste roku 1991v rámci výskumného vesmírneho programu Interkozmos-Inkubátor 2. Pretože taxonómia ako aj poznatky o bakteriocínoch, najmä o tých, ktoré sú produkované enterokokmi sa rokmi vyvíjali, cieľom predkladanej práce bolo genotypizáciou overiť taxonomickú príslušnosť uskladnených kmeňov, prehodnotiť ich stabilitu a vlastnosti po dlhodobom uskladnení ako aj zistiť u nich prítomnosť génov pre produkciu niektorých doposiaľ známych enterocínov. Vyselektovaných 6 kmeňov bolo genotypizáciou priradených k druhu *Enterococcus faecium*. Intenzita produkcie kyseliny mliečnej ostala nezmenená po dlhodobom skladovaní testovaných kmeňov. Enterokoky boli citlivé na vankomycín a rezistentné na kanamycín. Variabilita v citlivosti či rezistencii k testovaným antibiotikám bola zistená pred i po ich skladovaní lyofilizáciou. Každý testovaný kmeň obsahoval aspoň jeden štrukturálny gén pre produkciu testovaného enterocínu. Štrukturálne gény pre produkciu enterocínov A, P, B, L50B boli detegované u kmeňa *E. faecium* EP7. Kmene EP2, EEP4 obsahovali gény pre enterocíny A, B, L50B. Gén pre produkciu enterocínu P bol zistený len u kmeňa EP7. Najčastejšie bol detegovaný ent A gén, potom gény pre produkciu enterocínov B a L50B. Všetky kmene inhibovali rast najmenej 4 z 15 indikátorových mikroorganizmov. Enterokoky si zachovali svoje vlastnosti i po dlhodobom skladovaní v lyofilizovanej forme. Naviac, boli u nich detegované i ďalšie vlastnosti.

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