Lait 87 (2007) 59–69
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DOI: 10.1051/lait:2007002

Original article

Demonstration of the cellular viability and safety of *Enterococcus faecium* CRL 183 in long-term experiments

Veridiana P.S. CANO[a], Sandro R. VALENTINI[a], Katia SIVIERI[b]*, Elizeu A. ROSSI[b]**

[a] UNESP São Paulo State University, Department of Biology, Araraquara, S.P, Brazil
[b] UNESP São Paulo State University, Department of Food Nutrition, Araraquara, S.P, Brazil

Received 17 July 2006 – Accepted 21 December 2006

Abstract – *Enterococcus faecium* CRL 183, a strain isolated from NSLAB cheese starter, has been the focus of much research on its potential probiotic capacity, although its survival through the gastrointestinal tract has not been demonstrated so far. In order to determine the capacity of *E. faecium* CRL 183 to survive such conditions, this strain was administered daily to rats for 30 weeks. The experimental animals were divided into Group I: those that did not receive *E. faecium*, Group II: those that received a pure culture of *E. faecium* CRL 183 and Group III: animals that received *E. faecium* CRL 183 in the form of a fermented soy-based product. Faecal samples were collected at the beginning and at the 50%, 75% and 100% stages of the experimental period. Isolation and counts of *Enterococcus* were carried out on KF selective media. To distinguish the various *Enterococcus* species in the faeces, biochemical (API Strep 20) and molecular (PCR) tests were performed. Initially, *E. faecium* was absent from the intestinal flora of the rats; however, after 15 weeks of administration, *E. faecium* could be recovered from the faeces of Groups II and III, demonstrating that *E. faecium* CRL 183 was able to survive gastrointestinal transit under the study conditions. This is further evidence of the probiotic qualities of this strain. The safety of the strain was also investigated with regard to body weight and serum biochemical analysis.

probiotic / *Enterococcus faecium* / rat / survival

摘要 – 粪肠球菌 CRL 183 细胞存活能力和安全性的研究。以从干酪的非发酵剂乳酸菌(NSLAB)中分离出来的一株粪肠球菌CRL 183 (*Enterococcus faecium*)为目标，研究了该菌株潜在的益生特性。到目前为止尚未证明粪肠球菌 CRL 183 通过胃肠道后的存活情况。为了证明粪肠球 CRL 183 在胃肠道中存活能力，将这株菌进行了大鼠 30 周的喂养试验，每天喂养一次。将实验动物分成 3 组，第一组为对照组，第二组为喂养纯粪肠球菌 CRL 183 细胞，第三组为喂养粪肠球菌 CRL 183 在大豆培养基中的培养物。分别在试验的第一天、 50%、75% 和 100% 实验周期的时间收集大鼠的粪便。在 KF 选择性培养基上分离和计数肠球菌，并采用生物化学和 PCR 方法对肠球菌菌株进行鉴定。在实验的 1–15 周内，三个试验组大鼠粪便中粪肠球菌数量相对较小，然而喂养 15 周后，第二和第三试验组动物粪便中的肠球菌全部是粪肠球菌 CRL 183。由此证明在本研究的试验条件下，粪肠球菌 CRL 183 能够通过胃肠道并存活下来。本研究也进一步证明了粪肠球 CRL 183 的益生性，以及通过对大鼠的体重和血清的生化分析证明了该菌株的安全性。

益生菌 / 粪肠球菌 / 大鼠 / 存活

* Corresponding author (通讯作者): katiasiv@hotmail.com
** Current address: Rodovia Araraquara/Jaú, Km 1, Araraquara, São Paulo, Brazil, cep: 14801-902

Résumé – Démonstration de la viabilité cellulaire et de l'innocuité de *Enterococcus faecium* CRL 183 dans des expériences sur le long terme. *Enterococcus faecium* CRL 183, souche isolée à partir de bactéries lactiques non levain en transformation fromagère, a été l'objet de nombreuses recherches sur sa capacité à être un probiotique potentiel, bien que sa survie dans le tractus digestif ne soit pas encore bien démontrée à ce jour. Afin de déterminer la capacité de *E. faecium* CRL 183 à survivre dans de telles conditions, cette souche a été administrée quotidiennement à des rats pendant 30 semaines. Les animaux ont été divisés en 3 groupes expérimentaux : le groupe I ne recevant pas *E. faecium*, le groupe II recevant une culture pure de *E. faecium* CRL 183, et le groupe III recevant *E. faecium* CRL 183 sous la forme d'un produit fermenté à base de soja. Les échantillons de fécès ont été collectés au début et à 50 %, 75 % et 100 % de la période d'expérimentation. L'isolement et la numération des *Enterococcus* ont été réalisés sur milieu sélectif KF. Des tests biochimiques (API Strep 20) et moléculaires (PCR) ont été réalisés pour distinguer les espèces isolées des fécès. Initialement, *E. faecium* était absent de la flore intestinale des rats ; cependant, après 15 semaines d'administration, *E. faecium* a pu être retrouvé dans les fécès des groupes II et III, démontrant que *E. faecium* CRL 183 était capable de survivre dans les conditions du transit gastrointestinal. L'innocuité de la souche a également été étudiée au regard du poids corporel et des analyses biochimiques plasmatiques.

probiotique / *Enterococcus faecium* / rat / survie

1. INTRODUCTION

The gastrointestinal tract of animals is colonised by a complex bacterial ecosystem, which has not been completely determined. Most of the microorganisms are anaerobic and many of them (60%–80%) cannot be cultured readily in traditional media [25]. On the other hand, there is much agreement between researchers about the components of the human microflora that may play an important role in the development of certain diseases.

Lactic acid bacteria (LAB) are supposed to offer several health benefits, such as prevention and treatment of diarrhoea, decrease in lactose intolerance, reduction of seric cholesterol, stimulation of the immune system, anti-colon cancer effects, reduction of inflammatory reactions and regulation of gut motility [23, 39]. However, some strains of this group are considered important pathogens. This dual nature means that the beneficial effects cannot be derived from all LAB strains. Hence, selection and characterisation of strains are essential to guarantee their safe use as probiotics [28, 39]. *Enterococcus* comprises various species that produce L(+)–lactic acid and are, therefore, considered part of the LAB group. *E. faecalis* has been described as the major nosocomial pathogen causing bacteraemia, endocarditis and other infections, for example, in the urinary tract [37]. On the other hand, enterococci have been safely used in food processing and there is biotechnological interest in their production of bacteriocins and their probiotic characteristics.

The beneficial properties of enterococci used in development of flavour in cheeses is well known. Enterococci have been proposed as part of the defined starter cultures for several European cheeses, such as Mozzarella [9], Cebreiro [6] and Feta cheeses [21]. Sarantinopoulos et al. [37] showed that the inclusion of *E. faecium* strains positively affected the flavour, texture, aroma and colour of mature Feta cheese. *E. faecium* CRL 183 has been investigated by our research group for 12 years with the objective of defining its functional properties. Some properties have been demonstrated, such as breast cancer [40] and osteoporosis prevention [2]. Moreover, Rossi et al. [32] proved that in vitro *E. faecium* CRL 183 can reduce cholesterol levels by 53%. Encouraged by these results, these researchers developed a soy product fermented with *E. faecium* CRL 183, associated with *Lactobacillus jugurti*, with organoleptic and technological properties similar to traditional milk fermented products. The hypocholesterolemic effect of this product was tested in male rabbits. The results showed that a daily dose of 10 mL for 30 d was able to reduce the

cholesterol level by 18.4% and increase the HDL fraction by 17.8% [33, 34]. Besides, Rossi et al. [35] evaluated the intake of a 200-mL daily dose of soy fermented juice containing *E. faecium* CRL 183 and *Lactobacillus jugurti* by adult men with normal cholesterol levels, for a period of 6 weeks. This study not only showed the maintenance of normal cholesterol levels and LDL fractions but also showed an increase of 10% in the HDL fraction.

Several studies are being done in order to prove the probiotic characteristics of *E. faecium* CRL 183. The survival of this microorganism during gastrointestinal transit is one of the conditions for a microorganism to be considered probiotic [22], since the viability of bacteria can be reduced by exposure to gastric acid and bile salts [5].

The aim of the present study is to investigate the capacity of *E. faecium* CRL 183 to survive gastrointestinal passage by monitoring rat faecal samples by microbiological, biochemical and molecular analysis, as well as its safety in long-term experiments.

2. MATERIALS AND METHODS

2.1. Processing of fermented products

Processing was carried out at the Soy Products Development and Production Unit (Unisoja) of the Department of Food and Nutrition, School of Pharmaceutical Sciences, UNESP (São Paulo, Brazil) [31]. The soy juice was fermented with 1.5% (v/v) *E. faecium* CRL 183 culture and 1.5% (v/v) *Lactobacillus helveticus* ssp. *jugurti* 416 (technological support) culture, at 37 °C, until the product reached pH 4.5.

2.2. Preparation of *Enterococcus faecium* CRL 183 (pure culture)

E. faecium CRL 183 was reinoculated weekly into Tryptic Soy Broth (TSB, Acumedia, Baltimore, USA). The cells were centrifuged (3000 rpm) and the supernatant discarded. The cells were rinsed and kept in peptone water until administered.

2.3. Measurement of cell viability of the fermented product and the pure culture suspension

The fermented product and the pure culture suspension were prepared weekly and their cell viability estimated as follows. One gram of fermented product or centrifuged culture was suspended in 9 mL of phosphate buffer (pH 7.2) and serial decimal dilutions were carried out. Appropriate dilutions were spread on M17 Agar (Difco, Le-Pont-de-Claix, France) plates which were incubated aerobically for 24 h at 37 °C.

2.4. Animals

Thirty male Wistar rats, weighing approximately 90 g (± 2 g), were randomly divided into three groups. The animals were kept in polypropylene cages (5 animals/cage) on a ventilated shelf with air filtration and internal control of temperature (23 °C ± 1 °C). The room was maintained at 23 °C ± 1 °C with a 12:12 light/dark photocycle.

Before the experiment began, the animals were acclimatised. First, they were given a sweet solution, followed by an acid solution and, finally, the taste of the fermented soy product, using a placebo (the same product without the lactic bacteria). Both the pure culture solution and the fermented product were given to the animals by gavage for 30 weeks.

The animals were randomly divided into 3 groups ($n = 10$) that were treated as follows:

(a) Group I (Control) did not receive *Enterococcus faecium* CRL 183.

(b) Group II received the pure culture of *E. faecium* CRL 183 (3 mL·kg^{-1} body weight·day^{-1}).

(c) Group III received *E. faecium* CRL 183 in the fermented soy product (3 mL·kg^{-1} body weight·day^{-1}).

The animals had free access to daily water and food, both sterilised. The food was Purina commercial rat chow, with 23% protein, 49% carbohydrate, 4% fat, 5% fibre, 7% ashes and 6% vitamin C. At the end of the experimental period, the animals

were killed by CO_2 inhalation. The University Ethical Committee for Animal Research approved the methods used in this study (protocol no. 46/2004).

2.5. Biochemical tests

Immediately before killing, serum blood was collected from the animals for biochemistry tests. Tests were carried out by biochemists of hepatic function (aminotransferase-GOT, serum alanine amino-transferases-ALT and alkaline phosphatase-ALP), according to the method described by [26]. For renal function the urea test was carried out by the method of urease and creatinin as described by [4]. Also, the seric calcium concentration was determined in a Bayer Technicon autoanalyser Ra-100, according to [29].

2.6. Collection of faecal samples

The rat faecal samples were collected in sterile flasks at the beginning and at the 50%, 75% and 100% stages of the experimental period and prepared according to [22].

2.7. Counts of *Enterococcus* and identification of the species

Nine grams of animal faeces were mixed and suspended in 90 mL of phosphate buffer (pH 7.2) and serial decimal dilutions were carried out. The analyses were realised in triplicate. The isolation was done as described by [9] at the beginning and at the 50%, 75% and 100% stages of the experimental period.

KF *Streptococcus* Agar medium (Acumedia, Baltimore, USA) was used to count the enterococci. The plates were incubated at 37 °C for 48 h. Four colonies with distinct morphologies were picked out and transferred to Bile Esculin Azide Agar medium (Acumedia, Lasing, USA) for confirmation of the *Enterococcus* genus. API Strep 20 (Biomerieux, Basingstoke, UK) was used to identify the species of enterococci. Colonies identified as *E. faecium* were cultured on TSB medium (Acumedia, Baltimore, Maryland) for genomic DNA extraction (Fig. 1).

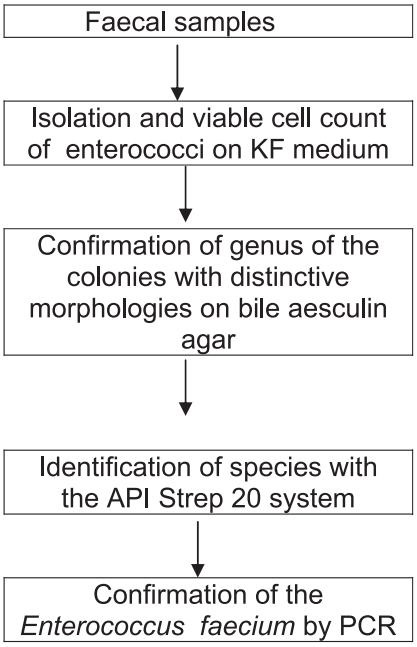

Figure 1. Methodology used for the isolation, counting and identification of the species of enterococci.

2.8. 16S rDNA amplification

Species confirmation of *E. faecium* was performed by PCR amplification of the genomic DNA that was extracted with the Dneasy Kit (Qiagen, Valencia, USA) from colonies isolated on KF medium, from faecal samples collected at the 0, 50, 75 and 100% stages of the experimental period.

DNA fragments encoding the 16S-23S rRNA spacer region were amplified, using the primer combination Enf 1 (5'-ATTACGGAGACTACACACTTTG-3') and Ent 2 (5'-TAGCCATAGAAGTTACATCAAG-3'), as detailed in [17].

PCR reactions were performed in a mixture of 1.5 mmol·L^{-1} $MgCl_2$, deoxynucleotides at a concentration of 100 µmol, 1 µmol of each primer, 100 ng of genomic DNA and 2 U of the enzyme Taq DNA polymerase (GE Healthcare) in a final reaction volume of 50 µL. The following

Table I. Food consumption and body-weight gain during the experimental period.

Group	Food consumption ($g \cdot rat^{-1} \cdot day^{-1}$)				Body-weight gain (g)		
	Start	50%	75%	100%	50%	75%	100%
I	13.0	24.0	25.0	27.7	354.6	386.2	424.2
II	12.8	22.0	23.0	24.0	243.0	273.4	322.6
III	13.0	23.5	24.3	24.7	310.6	361.8	410.4

Group I: animals that did not receive the *E. faecium*; Group II: animals that received cultured *E. faecium* CRL 183; Group III: animals that received *E. faecium* CRL 183 in a fermented soy-based product.

Table II. Biochemical analysis of blood serum at the end of the experimental period.

Groups	Calcium ($mg \cdot dL^{-1}$)	Urease ($mg \cdot dL^{-1}$)	Creatinin ($mg \cdot dL^{-1}$)	GOT ($U \cdot L^{-1}$)	ALT ($U \cdot L^{-1}$)	ALP ($U \cdot L^{-1}$)
I	$9.71^a \pm 0.88$	$32.30^a \pm 7.00$	$0.7^a \pm 0.01$	$113.60^a \pm 18.12$	$31.70^a \pm 6.46$	$148.00^a \pm 19.26$
II	$11.01^a \pm 0.45$	$46.10^a \pm 8.66$	$0.8^a \pm 0.04$	$167.80^a \pm 45.47$	$40.70^a \pm 10.59$	$170.00^a \pm 27.10$
II	$9.64^a \pm 0.30$	$32.00^a \pm 6.02$	$0.7^a \pm 0.03$	$82.28^a \pm 7.86$	$29.00^a \pm 9.64$	$145.00^a \pm 61.61$

Group I: animals that did not receive the *E. faecium*; Group II: animals that received cultured *E. faecium* CRL 183; Group III: animals that received *E. faecium* CRL 183 in a fermented soy-based product. Means with the same letters in the same row are not significantly different ($P \leq 0.05$).

amplification programme was used: 30 cycles of 1 min at 94 °C, 30 s at 56 °C and 1 min at 72 °C. The programme also included pre-incubation at 94 °C for 1 min, 1 min at 72 °C and a final extension at 72 °C for 5 min.

PCR products were separated by 1.5% agarose gel electrophoresis in TAE buffer (40 $mmol \cdot L^{-1}$ Tris, 11% glacial acetic acid and 1 $mmol \cdot L^{-1}$ EDTA) and stained with ethidium bromide. DNA was visualised by UV fluorescence and photographed on a UV transillumination.

2.9. Statistical analysis

The mean values of all the evaluated parameters were submitted to analysis of variance (ANOVA) and Tukey tests, significance being declared at $P \leq 0.05$, using the statistical program Sigma Stat 5.0.

3. RESULTS

3.1. General observation

All rats survived until the end of the experiment. The food consumption was similar for all groups during the experimental period (Tab. I). The body-weight gain was 424.2, 322.6 and 410.4 for Groups I, II and III, respectively. To analyse a possible adverse effect of *E. faecium* treatment some biochemical parameters were evaluated. Calcium, urease, creatinin, GOT, ALT and ALP were not altered by *E. faecium* CRL 183 treatment (Tab. II).

3.2. Cell viability

All 30 weekly batches of the soy product fermented with *E. faecium*, as well as the pure suspension culture of *E. faecium,* showed viable counts of 10^7 $CFU \cdot mL^{-1}$. The products were stable over two weeks.

3.3. Isolation and counts of enterococci

The faecal viable count for total enterococci increased more than tenfold by the 75% stage of the period of ingestion of the fermented product or the pure culture of *E. faecium* CRL 183, relative to the initial

Table III. Viable cell count (log CFU·g^{-1}) of enterococci in faeces of rats during the experimental period (start, 50, 75 and 100%).

Group	Start	50%	75%	100%
I	$6.86^{aB} \pm 0.04$	$8.89^{aA} \pm 0.01$	$8.44^{cA} \pm 0.01$	$8.21^{aA} \pm 0.06$
II	$7.05^{aA} \pm 0.07$	$8.58^{aB} \pm 0.07$	$8.78^{cB} \pm 0.05$	$7.77^{bA} \pm 0.06$
III	$7.18^{aA} \pm 0.16$	$8.03^{bB} \pm 0.11$	$8.45^{cB} \pm 0.11$	$7.84^{bA} \pm 0.14$

Group I: animals that did not receive the *E. faecium*; Group II: animals that received cultured *E. faecium* CRL 183; Group III: animals that received *E. faecium* CRL 183 in a fermented soy-based product.
Means with the same capital letters in the same row are not significantly different ($P \leq 0.05$).
Means with the same lower-case letters in the same column are not significantly different ($P \leq 0.05$).

Table IV. Identification of species, with their respective percentages, of the colonies with distinctive morphologies on KF selective media, at the start and at the 50, 75 and 100% stages of the experimental period.

Time:	Start	50%	75%	100%
Group				
I	*E. faecalis* (57%) *E. durans* (43%)	*E. faecalis* (75%) *E. durans* (24%)	*E. faecalis* (50%) *E. durans* (50%)	*E. faecalis* (58%) *E. cassiflavus* (42%)
II	*E. faecalis* (60%) *E. avium* (40%)	*E. faecium* (100%)	*E. faecium* (100%)	*E. cassiflavus* (100%)
III	*E. faecalis* (100%)	*E. faecium* (60%) *E. faecalis* (40%)	*E. faecium* (100%)	*E. cassiflavus* (100%)

Group I: animals that did not receive *E. faecium*; Group II: animals that received cultured *E. faecium* CRL 183; Group III: animals that received *E. faecium* CRL 183 in a fermented soy-based product.

count (Tab. III). In Groups II and III the counts of enterococci were statistically the same at the beginning and end of the experimental period. In the control group, however, there was a hundredfold increase by the 50% stage of the experimental period, and this count remained the same up to the end.

The animals in the different groups did not differ statistically in their enterococcal counts at the beginning or at the 75% stage of the experimental period, while at the 50% stage of the period, the count for Group III was significantly lower than those of Groups I and II. At the end of the experimental period, the count for the control group (Group I) was significantly higher than those for the groups that had received *E. faecium* (Groups II and III).

The colonies selected for their distinct morphologies on KF medium during the experimental period were biochemically identified by the API Strep 20 system (Tab. IV). At the beginning of the experiment, the predominant flora of enterococci belonged to the *faecalis* species. At the intermediate sampling times, Groups II and III showed the presence of *E. faecium*. At the end of the period, enterococci were identified in the faeces of all groups belonging to *faecalis* and *cassiflavus.* Table III shows the absence of *E. faecium* in the faeces at the end of the experimental period in Groups II and III. In Group II, 41.79, 51.96 and 100% of the ingested *E. faecium* was retained at the 50, 75 and 100% stages of the experimental period, respectively, while in Group III, 61.17, 63.82 and 100% of the bacteria were retained at these times.

3.4. PCR confirmation

Genomic DNA obtained from colonies identified as *E. faecium* by the API Strep

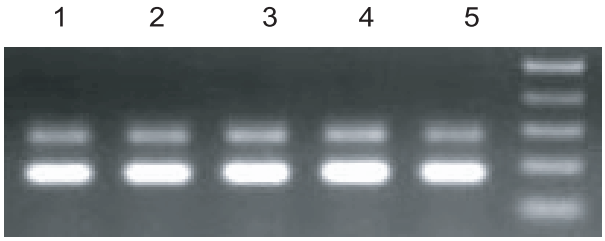

Figure 2. Agarose gel electrophoresis of PCR products obtained from colonies isolated from faecal samples. Lane 1, *E. faecium* from GII 50%; Lane 2, *E. faecium* from GII 75%; Lane 3, *E. faecium* from GIII 50%; Lane 4, *E. faecium* from GIII 75%; Lane 5, *E. faecium* CRL 183.

system was used to amplify the 16S-23S rRNA gene spacer region. Figure 2 shows the PCR products obtained from colonies isolated from faecal samples of both Groups II and III at the 50 and 75% stages of the experimental period.

As can be seen, the Enf 1-Ent 2 primer combination generated two amplification products: one of 200 bp and a 300 bp fragment of lower yield. However, a previous analysis of PCR reactions with Enf 1 and Ent 2 primers demonstrated fragments of different lengths [16]. Since the strains used in that study differed from *E. faecium* CRL 183, a second strain (CRL 39) was introduced into the present study, to observe what size pattern would be generated with the same primer combination.

Figure 3 demonstrates the detection of the DNA fragments amplified from the genomic DNA of *E. faecium* CRL 39. The observation of two bands at 300 bp and 400 bp is in perfect agreement with results published by Jin et al. [16] and clearly shows differences between strain CRL 39 and the strain isolated from the faecal samples.

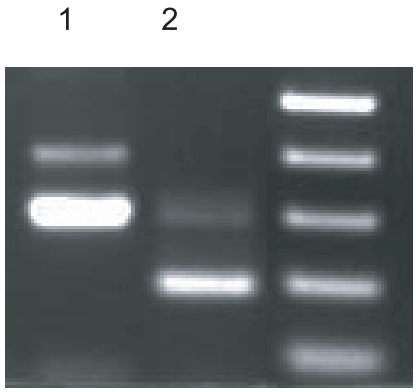

Figure 3. Agarose gel electrophoresis of PCR products obtained from *E. faecium strains* by using Enf 1 and Ent 2 primers. Lane 1, *E. faecium* CRL 39; Lane 2, *E. faecium* CRL 183.

4. DISCUSSION

Many studies have demonstrated the beneficial effects of the ingestion of lactic acid bacteria (LAB) by humans [10, 15, 18, 20, 24, 29, 38], as long as the bacteria survive gastrointestinal transit [8]. Nevertheless, the viability of LAB can be depleted upon contact with gastric acid or bile salts [22]. In order to confirm the probiotic effects of a microorganism, rats are widely used as a model to evaluate its survival through the gastrointestinal tract [18, 25, 30].

The strain *E. faecium* CRL 183 used in this experiment has been the object of research to confirm its probiotic effect. Some results have already been achieved and are reported as breast cancer inhibition [40], cholesterol control [35], osteoporosis prevention [2] and immune system stimulation [40]. For a microorganism to be considered as probiotic, it has to be able to withstand gastrointestinal conditions and colonise the intestine [12]. In this study we investigated the ability of *E. faecium* CRL 183 to do this.

All processed lots of the fermented product and culture suspension containing viable

E. faecium CRL 183 had an enterococcal count of 10^7 CFU·mL^{-1}, an adequate number to promote a probiotic effect [11]. In this study *Enterococcus faecium* CRL 183 treatment did not induce any toxicity in rats after a 30-week exposure period, as evidenced by unaltered body and serum biochemical analyses, demonstrating the safety of this microorganism in long-term studies.

All the animals had, on average, 90 g of initial weight; however, the animals from Group II gained little weight when compared with the other groups. An antilipogenic effect was observed on the fat pad, in animals that had consumed fermented soy product supplemented with isoflavones [23]; therefore, in hypothesis the consumption of pure culture of *E. faecium* CRL 183 can increase this effect.

The microbiological results showed that during the 30-week experimental period all the treatment groups showed similar profiles for counts of enterococci in faecal samples. Initially, all groups showed similar counts, and the counts of enterococci increased in all groups during the experimental period. The daily intake of *Enterococcus faecium* CRL 183 did not alter the general flora of these animals quantitatively, confirming the report published by [22], who also observed that a daily intake of *faecium* by humans for 10 days resulted in dominance of the total flora by enterococci, proving that it was capable of altering the intestinal microflora qualitatively.

At the end of the experimental period, the control group maintained the count of total enterococci at the same level, but the groups that received daily doses of CRL 183 enterococci showed decreases of about one log cycle. The control group results are similar to those reported by many authors [1, 13, 36], both in human and young and adult animal flora. Viable counts of the predominant bacteria in the faeces of adults and children highlighted differences in microbiota composition [14]. In this context, with regard to the animals' age, they are considered young at the beginning of the experimental period, adults at the 50% and 75% stages of the period and old at the 100% stage, according to criteria from the literature.

The biochemical results showed that the initial flora of the rats under study did not include the *faecium* species. This was confirmed by molecular biology (PCR). The initial flora of enterococci had the *faecalis, durans* and *avium* species, the same composition as reported by Bejuk et al. [3], who analysed 153 strains of enterococci isolated from human faeces and observed that 90% were *faecalis.* Linaje et al. [19] characterised the enterococcal species in rabbit faeces and verified that the isolated species were 60% *faecalis*, 34% *faecium* and 4% *durans.* In our study, during the experimental period, there was a change in the enterococcal flora quality in the groups that received *E. faecium* CRL 183 daily (Groups II and III): the initial flora of *E. faecalis*, *E. durans* and E. *avium* was substituted by *E. faecium.* These results corroborate those of Lund et al. [22] in humans.

E. faecium was not found in the faeces of the animals of Groups II and III at the end of the experimental period. Considering that the experimental period was very extended (30 weeks), it is probable that the conditions of the intestinal mucosa were altered, creating a significant change in the habitat. Hopkins and Macfarland [13] reported changes in the intestinal flora of animals related to age, but the metabolism of such changes has not been thoroughly studied. Hopkins et al. [14] suggested that some bacterial strains could take advantage of new ecological niches, thereby inducing a shift in the composition of the gut microflora.

The level of survival of *E. faecium* was not statistically different between the groups that were given a daily dose of the microorganism. This was expected, since all the animals took the same amount of viable microorganism (3 mL·kg^{-1} weight) throughout the experimental period.

Biochemical similarities among many enterococci have made species identification a challenge. Clinical isolates have been identified by conventional biochemical tests [7]. In this context, the present study addressed the problem of species identification for *E. faecium*, using PCR amplification in combination with the results obtained by the API Strep system. After isolation and

biochemical characterisation of single colonies obtained from faecal samples at the 50 and 75% stages of the experimental period, PCR amplification of the 16S-23S rRNA gene spacer region with specific primers for *E. faecium* yielded two amplification products: one of 200 bp and the other of 300 bp. However, these results differ from those of Langa et al. [17], who used the same primer combination, but different strains. Those authors found two bands, an expected one of 300 bp and an unexpected fragment of 400 bp, and that result was reproduced in this study by PCR amplification of *E. faecium* CRL 39. The production of more than one band may be explained by differences in the region of insertion of the gene in the chromosome [17].

Considering the contrasting sizes and intensities obtained, it was not clear whether the fragments found in our study corresponded to amplified rDNA, since the yield of the 200 bp fragment was significantly smaller than the ones amplified by [17]. Thus, only the 200 bp product obtained from amplification of CRL 183 and the 300 bp product from CRL 39 were partially sequenced. Sequence analysis was carried out with the Blastin program (www.ncbi.nlm.nih.gov/BLAST). The nucleotide sequences obtained for both PCR fragments revealed the 16S-23S rRNA gene spacer of *E. faecium*. Moreover, these results suggest that the strain isolated from faecal samples is the same as that administered during the experimental period, and therefore *E. faecium* CRL 183.

In a study that used PCR and direct sequence analysis of the amplification product, 300 bp fragments were obtained for *E. faecium* strains and ATCC 19434, which corresponds to the 16S-23S larger spacer region without the $tRNA^{ala}$ gene. In contrast to ATCC 19434, LMA631 contains no spacer region 1 with the $tRNA^{ala}$ gene and, therefore, no 400 bp can be observed by PCR amplification of this strain. Interestingly, *E. hirae* contains rrn operons that vary in length, and are represented by rrn operons without a 107 bp fragment without the $tRNA^{ala}$ gene, and with the 107 bp insert; or without the 107 bp but with a $tRNA^{ala}$ [27]. Since *E. hirae* and *E. faecium* share a high level of similarity (96%) between their 16S-23S gene spacer regions, our findings may represent such a situation, since heterogeneities are observed among members of the same species [27]. From this viewpoint, the CRL 183 strain must contain the rrn operon without a 100 bp insertion.

Our results demonstrate that *E. faecium* CRL 183 may survive the conditions while passing through the gastrointestinal tract. This is probably an indication of the colonising capacity of a probiotic *E. faecium* strain, indicating an additional characteristic of its probiotic potential. On the other hand, the results also demonstrated that the consumption of *E. faecium* CRL 183 in the form of fermented product and pure culture for 30 weeks did not provoke deleterious effects in the animals in the study, demonstrating the safety of this microorganism in long-term studies.

Acknowledgements: This study was supported by the Fundação de Amparo a Pesquisa do Estado de São Paulo. The authors wish to thank Ms. Roseli Ap. Pinto for technical assistance.

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