Environ. Biosafety Res. 7 (2008) 25–33
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DOI: 10.1051/ebr:2007047

Available online at:
www.ebr-journal.org

Exploration of methods used to describe bacterial communities in silage of maize (*Zea mays*) cultivars

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Different techniques to assess bacterial community structure and diversity were evaluated in silages prepared with four different maize cultivars, three conventional and one transgenic (cv. Tundra, event *Bt*-176). Plants were cultivated in the greenhouse and harvested after 30 days of growth. Silage samples were collected at successive times during fermentation and analyzed for bacterial counts and by various DNA-based fingerprinting techniques. Bacterial counts were similar between cultivars for the total culturable bacteria, sporeforming, and mesophilic and thermophilic lactic acid bacteria (LAB). Further analysis of the species composition of 388 LAB strains by intergenic transcribed spacer (ITS) PCR followed by sequencing of 16S rRNA gene did not reveal differences between cultivars. In contrast, molecular fingerprinting methods targeting whole bacterial communities, such as automated ribosomal intergenic spacers analysis (ARISA) and 16S rRNA gene length heterogeneity-PCR (LH-PCR), indicated that different maize silage batches or cultivars hosted different bacterial communities. Thus, ARISA and LH-PCR fingerprinting techniques offer a fast and sensitive method to compare bacterial communities, and to detect differences in silage bacterial communities.

Keywords: silage fermentation / transgenic plants / maize / methods / bacterial communities

INTRODUCTION

In 2005/2006, about 87.2 millions hectares of genetically modified plants were grown worldwide (Brookes and Barfoot, 2006). Bacterial communities thriving in plant environments are known to be responsive even to small changes in their environmental conditions, including nutrient composition and processing parameters. Changes in plant composition due to phenotypic differences among cultivars may therefore affect the subsequent bacterial community arising in silage, because plant silage is characterized by the bacteria living in close contact with plant material during cultivation. For example, it has been shown that the structure and diversity of the rhizosphere bacterial community vary with plant species and cultivar (Brusetti et al., 2004; Chiarini et al., 1998; Gomes et al., 2001). Hence, transgenic plants also exert cultivar-specific influence on the composition of the rhizosphere bacterial communities (Di Giovanni et al., 1999; Dunfield and Germida, 2001; Gyamfi et al., 2002; Siciliano and Germida 1999).

Silage is made from fine or chopped above-ground plants, which are fermented anaerobically, in a complex interaction between the plant tissue and the epiphytic microflora (Weinberg and Muck, 1996). Among the components of the epiphytic bacterial community naturally present on the plant leaf surface, lactic-acid bacteria (LAB) play a major role in determining the efficiency of silage fermentation process (Cai et al., 1998; 1999; Lin et al., 1991; 1992). After chopping and ensiling, there is a 300-fold increase of LAB cell number within the first 12–24 h of the fermentation (Lin et al., 1992) and, consequently, LAB metabolism leads to the accumulation of lactic acid in the silage, which decreases the pH below 5, stabilizing the product, which can be stored anaerobically for long periods of time.

The objective of this study was to evaluate the suitability of different methods commonly used to characterize bacterial communities, to detect potential changes in the silage fermentative bacterial community. These methods ranged from bacterial counts to fluorescent community fingerprinting techniques, such as automated ribosomal intergenic spacer analysis ((ARISA), Fisher and Triplett, 1999) and length heterogeneity-PCR ((LH-PCR), Ritchie et al., 2000; Suzuki et al., 1998), and to typing isolated LAB by intergenic transcribed spacer (ITS)-PCR followed by partial sequencing of the 16S rRNA gene. We tested the usefulness of these methods in detecting bacterial community differences in maize silages produced with different maize cultivars, including

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one expressing the *Bacillus thuringiensis* Cry1ab toxin (event *Bt*-176; Koziel et al., 1993).

RESULTS

Total bacterial counts

The counts of the total, aerobic sporeforming bacteria and of mesophilic and thermophilic LAB were determined in silages of parental cv. Tundra and cv. Tundra (event *Bt*-176), after 0, 1, 2, 6, 13, 20 and 30 days of fermentation, and in silages of Proxima and Eleonora maize after 30 days of fermentation (Tab. 1). Total cultivable and aerobic sporeforming bacteria and the mesophilic and thermophilic LAB on the fresh plant tissue before ensiling ranged between 0.5 and 3.0 log cfu.g^{-1} fresh weight. In the first 2 days of fermentation, bacteria grew rapidly: total bacteria, aerobic sporeforming and LAB increased up to 7.9, 3.6 and 7.5 log cfu.g^{-1} fresh weight, respectively. The number of total and aerobic sporeforming bacteria and mesophilic and thermophilic LAB showed only sporadic differences among the fermentation batches prepared with the different cultivars (Tab. 1).

LAB diversity

To evaluate if differences could be detected in the LAB species composition within fermentation batches prepared with different cultivars, a total of 388 LAB strains from ensiled cv. Tundra (*Bt*-176) and the parental conventional cv. Tundra were isolated at different times of fermentation and identified by ribosomal DNA analysis. The strains were grouped by ITS-PCR fingerprinting, and one strain for each ITS-PCR profile was identified by partial 16S rRNA gene sequencing. The strains were grouped into 27 different ITS-PCR haplotypes (Tab. 2).

The partial 16S rRNA gene sequences covered between 321 and 556 bp, and Table 2 shows the results of the strain identification. The strains belonged to Lactobacillales, such as *Weissella confusa* (41% of the strains), *Lactobacillus plantarum* (13%), *Pediococcus acidilactici* (10%), *Lb. perolens* (9%), *Lb. brevis* (8%), *P. pentosaceus* (6%), *Enterococcus faecium* (6%), *W. kimchii* (2%), *Lb. paraplantarum* (0.2%), but also rare Bacillales, such as *Bacillus megaterium* (3%). Homofermentative species (*P. acidilactici* and *P. pentosaceus*), facultative heterofermentative species (*Lb. paraplantarum*, *Lb. plantarum* and *Lb. perolens*), and obligate heterofermentative species (*Lb. fermentum*, *Lb. brevis*, *W. kimchii* and *W. confusa*) were found; in particular, homofermentative species were found at the end of fermentation. Changes in species of LAB occurred during the ensiling period. *Bacillus megaterium* disappeared after one week of ensiling, substituted by Lactobacillales such as *Weissella* or *Lactobacillus*. At the late stage of ensiling, *Lactobacillus* and *Pediococcus* were predominant. Homofermentative bacteria such as *P. acidilactici* and *P. pentosaceus* were observable after 20 to 30 days of fermentation. No relevant differences were observed between the fermentation batches in the limited sample size analyzed.

Analysis of the bacterial population structure

The ARISA analysis of silage showed only a low number of peaks (an average of 10 per sample), however, with a very high total fluorescent emission (Tab. 3). The ARISA fragments ranged between 197 and 702 bp. The relatively simple bacterial population structure of the ensiled maize was further confirmed by LH-PCR, which also gave an average of 10 peaks per sample, with a range between 295 and 395 bp (Tab. 3). We previously showed that silage isolates, including *Enterobacteriaceae*, *Bacillaceae*, *Enterococcaceae* and LAB give LH-PCR peak sizes in the range of 344 to 381 bp (Brusetti et al., 2006).

Principal Component Analysis (PCA) of the ARISA peaks is shown as a three dimensional plot (Fig. 1A), explaining 83% of the total variance. The ARISA profiles obtained at different times of sampling from each fermentation batch clustered in compact groups indicating that the bacterial populations were relatively homogeneous, and suggesting that fermentation-specific changes occurred. The scatter plot showed a separation of the silage total microbiota within the different fermentation batches. Analysis of the molecular variance (AMOVA) of the diversity showed that the differences observed in the scatter plot between the fermentation batches were most often significant ($P < 0.05$) both with the data obtained from the ARISA and with LH-PCR techniques (Fig. 1).

DISCUSSION

Plants silage is an environment that facilitates close contact between mechanically disrupted plants and actively growing bacteria, where cultivar-specific induced changes in the bacterial composition can be hypothesized. One relevant question regarding the potential environmental impact of transgenic plants is the effect on the bacterial structure and community diversity in their growth habitat (Nielsen et al., 1998). It should be noted that an observable change in bacterial community composition does not necessarily indicate a negative impact, but rather changes resulting from different cultivar properties. Thus, it is important to improve our knowledge of how plant cultivars interact with their associated microbiological communities. Defining the baseline of microbial

Table 1. Bacterial counts of various fermentation batches containing maize cultivars expressed as average (log cfu.g^{-1} fresh weight, n = 3).

Maize cultivar	Sampling day[1]	Total bacteria[2] S[3]	Sporeforming[4] S[3]	LAB mesophilic[5] S[3]	LAB thermophilic[6] S[3]
Tundra	0	3.00*	1.89	2.19	2.15*
Tundra	1	7.58	3.28	7.57	7.60
Tundra	2	7.66	3.60	7.49	7.49
Tundra	6	7.36*	1.37*	7.80	6.87
Tundra	13	5.66*	1.18*	6.19	6.32
Tundra	20	4.90*	1.37	4.92*	5.00*
Tundra	30	3.92*	1.96	4.39	4.39*
Tundra (*Bt*-176)	0	2.24	1.27	1.33	0.52
Tundra (*Bt*-176)	1	7.18	3.51	7.55	7.60
Tundra (*Bt*-176)	2	7.94	3.29	7.44	7.52
Tundra (*Bt*-176)	6	8.04	2.54	7.30	7.36
Tundra (*Bt*-176)	13	6.65	2.51	7.41	7.51
Tundra (*Bt*-176)	20	6.18	2.00	7.51	6.82
Tundra (*Bt*-176)	30	5.25	2.47	5.96	5.76
Proxima	30	5.64	1.84*	5.54	5.93
Proxima	30	5.14	3.44*	6.09	5.95
Proxima	30	5.15	2.87	6.17	6.12
Proxima	30	5.95	0.66*	5.34	4.48*
Proxima	30	5.11	1.89*	5.10	5.24
Proxima	30	6.10*	2.45	5.94	5.89

[1] Days of maturation of the silage.

[2] Total aerobic bacteria cultivable on Plate Count Agar.

[3] Significant differences between bacterial counts of fermentation batches with conventional varieties *versus* those with cv. Tundra (event *Bt*-176) at the corresponding sampling time; ANOVA and Tukey's tests with $P = 0.05$.

[4] Sporeforming aerobic bacteria cultivable on Plate Count Agar at 28 ˚C.

[5] Mesophilic lactic acid bacteria cultivable at 28 ˚C on MSR medium.

[6] Thermophilic lactic acid bacteria cultivable at 42 ˚C on MSR medium.

* $P < 0.05$.

Table 2. ITS-PCR typing and 16S rRNA gene sequencing of bacterial strains isolated from maize silages.

ITS-PCR haplotypes[1]	N. of strains isolated	Plant cultivar and time of fermentation (days)[2]	Strain identification based on 16S rRNA gene sequence	Closest relative	
				Accession number	% match
1A	1	T1	*Bacillus megaterium*	AY167875	100
1B	7	B0	*B. megaterium*	AY373360	100
1C	2	B6	*B. megaterium*	AY167865	99
2A	1	T20	*Enterococcus faecium*	AY172570	100
2B	8	T0	*E. faecium*	AY172570	99
2C	1	T20	*E. faecium*	AY172570	100
3A	1	T6	*Lactobacillus brevis*	AF515220	100
3B	8	T13	*L. brevis*	AF515220	100
3C	1	T20	*L. brevis*	AF515220	100
3D	6	T30	*L. brevis*	AF515220	100
4A	10	T20	*L. paraplantarum*	AJ306297	99
5A	12	B13	*L. perolens*	Y19167	97
6A	85	T2; B2	*L. plantarum*	AY383631	99
7A	53	T30; B20	*Pediococcus acidilactici*	AJ305322	99
7B	15	B30	*P. acidilactici*	AJ305322	99
8A	15	T30	*P. pentosaceus*	AJ305321	99
8B	9	B20	*P. pentosaceus*	AJ305321	100
8C	17	B0,30	*P. pentosaceus*	AJ305321	100
9A	1	B0	*Weissella* sp.	AF510730	93
9B	6	B6	*W. confusa*	AB023241	100
9C	1	B6	*W. confusa*	AB023241	100
9D	57	T2; B2	*W. confusa*	AB023241	99
9E	63	T1,13; B1,6,13	*W. confusa*	AB023241	100
10A	4	T6	*W. kimchii*	AF312874	100
11A	1	B0	n.d.[3]		
11B	1	B0	n.d.[3]		
11C	2	T6	n.d.[3]		

[1] The number indicates the different species deduced from 16S rRNA gene sequence, and the letter indicates the different haplotypes found for each species by ITS-PCR fingerprinting.

[2] Plant cultivar is indicated by the capital letter (T: Tundra; B: Tundra (*Bt*-176)); the number indicates the day of isolation.

[3] n.d.: Not determined.

Table 3. Characteristics of the ARISA and LH-PCR profiles obtained from the bacterial communities in silage.

		ARISA			LH-PCR		
Cv. of maize	Days[1]	Number of peaks	Range of peak size	Sum of the peak height	Number of peaks	Range of peak size	Sum of the peak height
Tundra	0	11	321–628	8676	6	318–380	5160
Tundra	2	11	297–529	8886	11	303–394	21 412
Tundra	6	8	297–687	11 961	9	318–392	6466
Tundra	13	13	249–702	12 070	11	318–380	8315
Tundra	20	15	249–524	18 239	10	295–380	11 832
Tundra	30	18	197–628	19 514	8	342–395	7363
Tundra (*Bt*-176)	0	7	297–530	18 979	4	345–380	1023
Tundra (*Bt*-176)	2	14	297–565	13 652	9	318–395	8465
Tundra (*Bt*-176)	6	14	297–527	16 987	11	345–380	9796
Tundra (*Bt*-176)	13	17	297–527	17 330	12	295–380	10 449
Tundra (*Bt*-176)	20	11	290–524	14 693	4	342–390	2623
Tundra (*Bt*-176)	30	9	272–524	11 331	9	296–378	12 198

[1] Days of maturation of the silage.

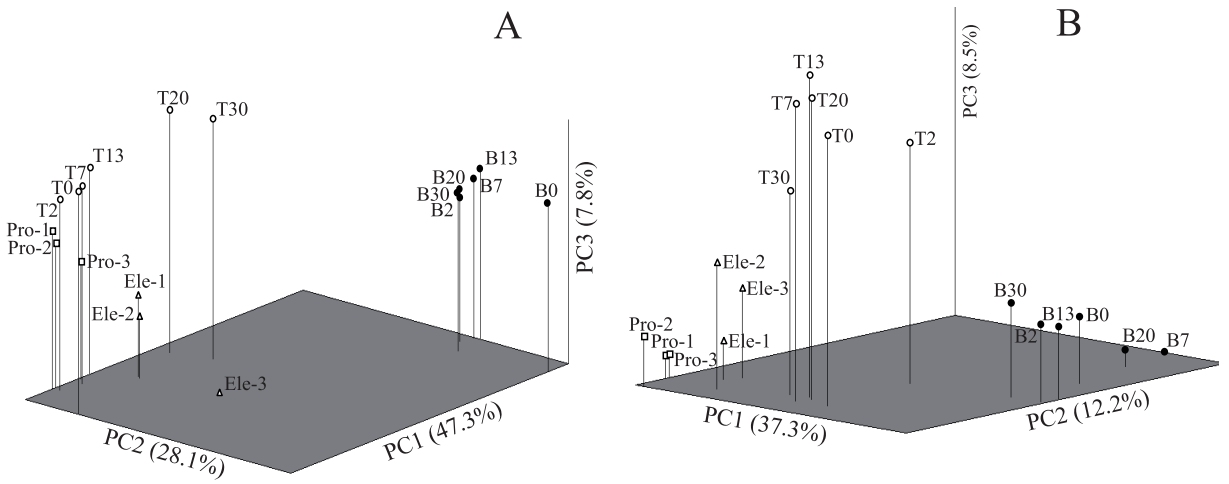

Figure 1. Principal component analysis of ARISA fingerprinting (A) and LH-PCR (B); black circle cv. Tundra (*Bt*-176) silage, white circle cv. Tundra silage, white square cv. Proxima silage, and white triangle cv. Eleonora silage. Community fingerprinting profiles were obtained for cultivars Tundra (*Bt*-176) and Tundra at different days of silage maturation, indicated by the numbers in each point, while for cultivars Proxima and Eleonora after 30 days of fermentation.

community dynamics will help further biosafety assessment of novel plant cultivars, particularly those where a credible and testable hypothesis can be put forward concerning a specific effect on the microbiota.

To assess the composition of bacterial communities associated with plants, researchers have used a wide variety of techniques based on the cultivability of bacteria (bacterial counts on different media, strain genomic fingerprinting, species identification, *etc.*), or based on the analysis of the total bacterial community structure with techniques such as DGGE, SSCP, ARISA or LH-PCR targeting housekeeping genes. In the last years, a polyphasic approach, where several techniques are used in parallel, has been commonly used to describe better the bacterial community structure and diversity. A polyphasic approach was used for instance by Costa et al. (2006), Griffiths et al. (2005), Heuer et al. (2002), Smalla et al. (2001), Smit et al. (2001), who reported differences in bacterial community composition of the rhizosphere associated with different plant cultivars.

In this study, we compared various techniques based on cultivability of bacterial isolates (bacterial counts, strains identification) with cultivation-independent molecular methods (ARISA, LH-PCR) in examining the abundance, diversity and structure of the bacterial population within different fermentation batches during the ensiling of four maize cultivars, one of which was the transgenic cv. Tundra (*Bt*-176). Bacterial counts were done with four different media to assess the density of aerobic copiotrophic bacteria, sporeforming bacteria and mesophilic or thermophilic LAB. The two PCR-based fingerprinting approaches used target either the ITS region of the 16S-23S rRNA gene (ARISA), a region considered to be hypervariable and therefore useful to discriminate bacteria at the subspecies level (Daffonchio et al., 1998), or the 16S rRNA gene (LH-PCR), which is used extensively to distinguish bacterial species. Both ARISA and LH-PCR coupled the characteristics of ITS and 16S rRNA gene analysis with the high sensitivity and precision of capillary electrophoresis. It was previously reported that LH-PCR could display the succession and dynamics of the fermentative microflora during the ensiling process (Brusetti et al., 2006), while ARISA performed with the ITSF/ITSReub PCR primer set performs a powerful evaluation of diversities between different bacterial community structures, reducing the effects due to PCR bias between different environmental samples, maize silage included (Cardinale et al., 2004).

This study demonstrates that both ARISA and LH-PCR show a level of discrimination of the bacterial communities in the different fermentation batches examined, as shown by cultivar-specific clustering (Fig 1). Due to the limited number of batches analyzed and samples taken, we cannot draw any conclusions on whether the differences seen are cultivar-specific or represent random variation among batch cultures. The indications of batch-dependent community structure were not obtained with the traditional methods examining only a proportion of the cultivable bacteria. The overall number of LAB, the most important functional group in silage, did not vary, suggesting that normal fermentation processes occurred within all the silage batches examined. Furthermore, the 16S rRNA gene sequence analysis showed that important

LAB species like *P. acidilactici* and *P. pentosaceous* were frequently found in all fermentation batches.

Although pseudoreplicates from the same silage batch were analyzed, multivariate analysis of ARISA and LH-PCR data showed significant differences between the fermentation batches, indicating that these techniques are sensitive in detecting minor differences in the structure of complex bacterial communities. Biochemical differences in plant tissue compositions could affect the fine structure of microbial communities thriving in silage, with slight effects (especially on the non-dominant species) detectable only with highly sensitive techniques. Some phenotypic features of maize containing *Bt*-toxin, beyond the intended change in the cry protein composition (Saxena et al., 1999), include a higher content of soluble carbohydrates, fructose, nitrogen (Escher et al., 2000) and lignin (Saxena and Stotzky, 2001). These phenotypic changes could potentially influence the less dominant bacterial communities during maize silage fermentation, although the limited sample set analyzed in our experiments was not able to resolve this question.

In conclusion, conventional microbiology techniques based on plating, isolation and sequencing appear to be unable to identify differences between different silage fermentation batches, unless very large sample numbers are used. Moreover, the traditional techniques are very time and labor consuming if adequate samples sizes are to be analyzed. In contrast, automated techniques relying on capillary electrophoresis systems coupled with laser scanning detectors allows representative fractions of community-derived PCR fragments to be separated to provide a broader view of the bacterial community structure developing during ensiling of maize.

MATERIAL AND METHODS

Maize plants, silage preparation and sampling

Three conventional maize lines, cv. Tundra (Tun), Novartis, cv. Proxima (Pro), Novartis, and cv. Eleonora (Ele), Pioneer, and one genetically modified insect-resistant (*Bt*-toxin, Cry1ab protein) maize, cv. Tundra (event *Bt*-176), Novartis were used in the experiments. The maize plants were grown in a greenhouse in pots (Brusetti et al., 2004). Maize plants were harvested after 30 days of growth. The presence of the transgene construct in cv. Tundra (*Bt*-176) was verified by PCR (Rizzi et al., 2001; 2003).

Maize plants without of the root system were chopped (average size 1 cm) with sterile shears, and about 1 kg of this material was pressed into sterile glass bottles in an anaerobic chamber and incubated at 30 ˚C. Silage was sampled (10 g samples) at successive times (0, 2, 7, 13, 20 and 30 days of incubation) by opening the microsilos in an anaerobic chamber.

Bacterial counts

Three two-gram samples were transferred to 18 mL of sterilized salt solution (9 $g.L^{-1}$ NaCl), and mixed for 20 min in a blender. The following bacterial counts were performed in agar-solified media from 10-fold dilutions of each replicate subsample: (i) total aerobic microflora on Plate Count Agar, incubation at 28 ˚C for 24 h; (ii) total sporeforming aerobic bacteria on Plate Count Agar after sample pasteurization, incubation at 28 ˚C for 24 h; (iii) total mesophilic LAB on MRS plus cycloheximide (0.1 $mg.L^{-1}$) (Difco, Italy); incubation at 28 ˚C for 48 h; (iv) total thermophilic LAB on MRS plus cycloheximide (0.1 $mg.L^{-1}$) and incubation at 42 ˚C for 48 h.

DNA sequencing of LAB species

A total of 388 LAB isolates (190 thermophilic and 198 mesophilic) were randomly isolated from MRS agar plates and restreaking two times. Liquid cultures of each strain (2 mL) were used for DNA extraction (Ausubel et al., 1994). Three μL of the DNA solution were used in the subsequent PCR reactions. Bacterial 16S-23S rRNA gene ITS of each strain were amplified with primers ITSF and ITSReub (Cardinale et al., 2004). PCR products were run on an agarose gel, and the different ITS-PCR haplotypes were evaluated. Amplification of the 16S rRNA gene of one strain for each ITS haplotype was performed as described previously (Urzì et al., 2001). ITS-PCR fingerprinting patterns were analyzed with the Diversity Database version 2.1.1 (Biorad, Milan, Italy) to number the different haplotypes amplified. The 16S rRNA gene PCR product was purified with QIAquick PCR Purification kit (Qiagen, Milan, Italy) and about 150 ng of the purified PCR product were used in the sequencing reaction using the universal primer 16S-926F (5'-AAACT(CT)AAA(GT)GAATTGACGG-3'). Each raw sequence was checked manually and compared with known sequences using the BLAST software package (Altschul et al., 1990). The nucleotide sequences were deposited in the EMBL database under the accession numbers AJ784904 to AJ784927.

ARISA and LH-PCR analysis

DNA extraction from silage was done according to Brusetti et al. (2006). The concentration of extracted DNA was determined by agarose gel electrophoresis. Then, 50 ng of environmental DNA were used in ARISA

and LH-PCR amplifications. Amplification of 16S-23S rRNA intergenic spacers of the bacterial community for ARISA (Fisher and Triplett, 1999) was performed as described previously using ITSF and ITSREub universal primer set (Cardinale et al., 2004). For LH-PCR, purified DNA was amplified according to Brusetti et al. (2006) using universal primers 27F and 338R (Ritchie et al., 2000). ARISA and LH-PCR fragments were loaded on an ABI Prism 310 capillary electrophoresis system, and run in denaturing conditions using the POP-4 polymer. The ARISA and LH-PCR data were analyzed with Genescan 3.1.2 software (Applied Biosystems), and a threshold of 50 fluorescent units was used, corresponding to twice the highest peak detected during the negative control run. Sizing was done with the Local Southern Method and light data smoothing. If the baseline varied inconsistently, the sample was rerun.

Statistical analysis

For bacterial counts, means and standard deviations were calculated, and analysis of variance of cfu.g^{-1} fresh weight was made. Individual means in the ANOVA were compared using Tukey's test (Tukey, 1949). The peak matrices corresponding to the ARISA and LH-PCR profiles were subjected to a principal component analysis. Binary 0/1 matrices were created based on the absence or presence of DNA bands. Pairwise distances were calculated with the SimQual option of the NTSYSpc 2.01 computer program (Applied Biostatistics Inc., USA) by employing the Jaccard coefficient for two-state data.

ACKNOWLEDGEMENTS

The work was supported by the Italian Ministry for University and Scientific Research. Partial support comes also from the EU TRANSBAC QLK3-CT-2001-02242 and from Consiglio dei Diritti Genetici in the ambit of the project "Organismi geneticamente modificati ed alimentazione: valutazione degli effetti diretti sull'ospite e sulla microflora intestinale", funded by the Cariplo Foundation. We thank K.M. Nielsen for comments on the manuscript.

Received November 17, 2006; accepted November 12, 2007.

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