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Identification of species belonging to the *Bifidobacterium* genus by PCR-RFLP analysis of a *hsp60* gene fragment

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Abstract

Background: *Bifidobacterium* represents one of the largest genus within the *Actinobacteria*, and includes at present 32 species. These species share a high sequence homology of 16S rDNA and several molecular techniques already applied to discriminate among them give ambiguous results.
The slightly higher variability of the *hsp60* gene sequences with respect to the 16S rRNA sequences offers better opportunities to design or develop molecular assays, allowing identification and differentiation of closely related species. *hsp60* can be considered an excellent additional marker for inferring the taxonomy of the members of *Bifidobacterium* genus.

Results: This work illustrates a simple and cheap molecular tool for the identification of *Bifidobacterium* species. The *hsp60* universal primers were used in a simple PCR procedure for the direct amplification of 590 bp of the *hsp60* sequence. The *in silico* restriction analysis of bifidobacterial *hsp60* partial sequences allowed the identification of a single endonuclease (HaeIII) able to provide different PCR-restriction fragment length polymorphism (RFLP) patterns in the *Bifidobacterium* spp. type strains evaluated. The electrophoretic analyses allowed to confirm the different RFLP patterns.

Conclusions: The developed PCR-RFLP technique resulted in efficient discrimination of the tested species and subspecies and allowed the construction of a dichotomous key in order to differentiate the most widely distributed *Bifidobacterium* species as well as the subspecies belonging to *B. pseudolongum* and *B. animalis*.

Keywords: *Bifidobacterium* spp, *hsp60*, PCR-RFLP, Taxonomy

Background

Members of the genus *Bifidobacterium* are Gram-positive, obligate anaerobic, non-motile, non-spore forming bacteria [1], and are the most important constituents of human and animal intestinal microbiota [2,3]. Recently, news species of bifidobacteria have been described [4-6] and now more than 30 species have been included in this genus.

Bifidobacterium spp. can be detected in various ecological environments, such as intestines of different vertebrates and invertebrates, dairy products, dental caries and sewage. Considering the increasing application of *Bifidobacterium* spp. as protective and probiotic cultures [7-9], and the fast enlargement of the genus, easy identification tools to discriminate new isolates are essential. Moreover, their correct taxonomic identification is of outmost importance for their use as probiotics [2]. Conventional identification and classification of *Bifidobacterium* species have been based on phenotypic and biochemical features, such as cell morphology, carbohydrate fermentation profiles, and polyacrylamide gel electrophoresis analysis of soluble cellular proteins [10]. In the last years several molecular techniques have been proposed in order to identify bifidobacteria. Most available bifidobacterial identification tools are based on 16S rRNA gene sequence analysis, such as ARDRA [11,12], DGGE [13] and PCR

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Table 1 Type-strains investigated

Species	International culture collection
Bifidobacterium adolescentis	ATCC 15703
Bifidobacterium angulatum	ATCC 27535
Bifidobacterium animalis subsp. *animalis*	ATCC 25527
Bifidobacterium animalis subsp. *lactis*	DSM 10140
Bifidobacterium asteroides	ATCC 25910
Bifidobacterium bifidum	ATCC 29521
Bifidobacterium boum	ATCC 27917
Bifidobacterium breve	ATCC 15700
Bifidobacterium catenulatum	ATCC 27539
Bifidobacterium choerinum	ATCC 27686
Bifidobacterium coryneforme	ATCC 25911
Bifidobacterium cuniculi	ATCC 27916
Bifidobacterium dentium	ATCC 27534
Bifidobacterium gallicum	ATCC 49850
Bifidobacterium gallinarum	ATCC 33777
Bifidobacterium indicum	ATCC 25912
Bifidobacterium longum subsp. *longum*	ATCC 15707
Bifidobacterium longum subsp. *infantis*	ATCC 15697
Bifidobacterium longum subsp. *suis*	ATCC 27533
Bifidobacterium minimum	ATCC 27539
Bifidobacterium merycicum	ATCC 49391
Bifidobacterium pseudolongum subsp *pseudolongum*	ATCC 25526
Bifidobacterium pseudolongum subsp. *globosum*	ATCC 25865
Bifidobacterium pseudocatenulatum	ATCC 27919
Bifidobacterium pullorum	ATCC 27685
Bifidobacterium ruminantium	ATCC 49390
Bifidobacterium subtile	ATCC 27537
Bifidobacterium thermacidophilum subsp. *porcinum*	LMG 21689
Bifidobacterium thermacidophilum subsp. *thermacidophilum*	LMG 21395
Bifidobacterium thermophilum	ATCC 25525

with the use of species-specific primers [14-16]. However, 16S rDNA of *Bifidobacterium* spp. has a high similarity, ranging from 87.7 to 99.5% and bifidobacterial closely related species (e.g. *B. catenulatum* and *B. pseudocatenulatum*) or subspecies (e.g. *B. longum* and *B. animalis* subspecies) even possess identical 16S rRNA gene sequences [17,18]. For this reason different molecular approaches have been tested based on repetitive genome sequences amplification, such as ERIC-PCR [19,20], BOX-PCR [21,22] or RAPD fingerprinting analysis [23]. These fingerprinting methods have the disadvantage of a low reproducibility, and they need

Table 2 List of strains investigated to confirm the conservation of RFLP profiles (strains belonging to BUSCoB collection)

Species*	Strain	Source
Bifidobacterium animalis subsp. *animalis*	T169	Rat
Bifidobacterium animalis subsp. *animalis*	T6/1	Rat
Bifidobacterium animalis subsp. *lactis*	P23	Chicken
Bifidobacterium animalis subsp. *lactis*	F439	Sewage
Bifidobacterium animalis subsp. *lactis*	Ra20	Rabbit
Bifidobacterium animalis subsp. *lactis*	Ra18	Rabbit
Bifidobacterium animalis subsp. *lactis*	P32	Chicken
Bifidobacterium bifidum	B1764	Infant
Bifidobacterium bifidum	B2091	Infant
Bifidobacterium bifidum	B7613	Preterm infant
Bifidobacterium bifidum	B2009	Infant
Bifidobacterium bifidum	B2531	Infant
Bifidobacterium breve	B2274	Infant
Bifidobacterium breve	B2150	Infant
Bifidobacterium breve	B8279	Preterm infant
Bifidobacterium breve	B8179	Preterm infant
Bifidobacterium breve	Re1	Infant
Bifidobacterium catenulatum	B1955	Infant
Bifidobacterium catenulatum	B684	Adult
Bifidobacterium catenulatum	B2120	Infant
Bifidobacterium pseudocatenulatum	B1286	Infant
Bifidobacterium pseudocatenulatum	B7003	
Bifidobacterium pseudocatenulatum	B8452	
Bifidobacterium dentium	Chz7	Chimpanzee
Bifidobacterium dentium	Chz15	Chimpanzee
Bifidobacterium longum subsp.*longum*	PCB133	Adult
Bifidobacterium longum subsp. *infantis*	B7740	Preterm infant
Bifidobacterium longum subsp. *infantis*	B7710	Preterm infant
Bifidobacterium longum subsp. *suis*	Su864	Piglet
Bifidobacterium longum subsp. *suis*	Su932	Piglet
Bifidobacterium longum subsp. *suis*	Su905	Piglet
Bifidobacterium longum subsp. *suis*	Su908	Piglet
Bifidobacterium pseudolongum subsp. *pseudolongum*	MB9	Chicken
Bifidobacterium pseudolongum subsp. *pseudolongum*	MB10	Mouse
Bifidobacterium pseudolongum subsp. *pseudolongum*	MB8	Chicken
Bifidobacterium pseudolongum subsp. *globosum*	Ra27	Rabbit
Bifidobacterium pseudolongum subsp. *globosum*	VT366	Calf

Table 2 List of strains investigated to confirm the conservation of RFLP profiles (strains belonging to BUSCoB collection) *(Continued)*

Bifidobacterium pseudolongum subsp. *globosum*	T19	Rat
Bifidobacterium pseudolongum subsp. *globosum*	P113	Chicken

* previously assigned taxonomic identification.

strict standardization of PCR conditions. The use of different polymerases, DNA/primer ratios or different annealing temperatures may lead to a discrepancy in the results obtained in different laboratories [24].

In recent years alternative molecular markers have been proposed for bifidobacteria identification (e.g. *hsp60*, *recA*, *tuf*, *atpD*, *dnaK*) and Ventura et al. [18] developed a multilocus approach, based on sequencing results, for the analysis of bifidobacteria evolution. The *hsp60* gene, coding for a highly conserved 60 kDa heat-shock-protein (a chaperonin), has been evaluated for phylogenetic analysis in bifidobacteria by Jian et al. [25]. The sequence comparison of this gene has been already used for species identification and phylogenetic analysis of other genera (e.g. *Staphylococcus*, *Lactobacillus*) and enteric pathogens [26-28]. A chaperonin database (cpnDB) is available on line, collecting bacterial and eukaryotic sequences (http://www.cpndb.ca/cpnDB/home.php) [29].

Table 3 Expected fragment sizes obtained with *in silico* digestion of the *hsp60* gene sequences

***Bifidobacterium* species**	**GenBank entry**	**Predicted fragment sizes**	**Profile**
B. adolescentis	AF210319	31-36-81-103-339	
B. angulatum	AF240568	42-54-59-139-296	
B. animalis subsp. *animalis*	AY004273	17-53-86-97-114-223	
B. animalis subsp. *lactis*	AY004282	71-86-96-114-223	
B. asteroides	AF240570	30-38-75-97-109-242	
B. bifidum	AY004280	22-31-59-181-297	
B. boum	AY004285	22-117-200-251	
B. breve	AF240566	106-139-139-200	
B. catenulatum	AY004272	53-198-338	
B. choerinum	AY013247	36-42-51-52-54-59-97-200	
B. coryneforme	AY004275	16-32-54-158-338	
B. cuniculi	AY004283	16-42-53-70-128-281	
B. dentium	AF240572	22-31-42-68-130-139-158	
B. gallicum	AF240575	42-253-297	
B. gallinarum	AY004279	16-31-42-81-139-281	
B. indicum	AF240574	16-32-36-42-45-123-296	
B. longum subsp. *longum*	AF240578	42-113-138-139-158	*
B. longum subsp. *infantis*	AF240577	42-113-138-139-158	*
B. longum subsp. *suis*	AY013248	42-113-138-139-158	*
B. merycicum	AY004277	22-31-42-59-139-297	
B. minimum	AY004284	16-51-60-66-70-327	
B. pseudocatenulatum	AY004274	42-53-198-297	
B. pseudolongum subsp *pseudolongum*	AY004282	17-22-30-32-42-42-109-297	
B. pseudolongum subsp. *globosum*	AF286736	16-17-22-30-32-42-109-323	
B. pullorum	AY004278	16-31-36-42-81-87-297	
B. ruminantium	AF240571	31-106-114-339	
B. subtile	Not available	Not avaiable	+
B. thermacidophilum subsp *porcinum*	AY004276	20-42-53-59-97-139-180	*†
B. thermacidophilum subsp *thermacidophilum*	AY004276	20-42-53-59-97-139-180	*†
B. thermophilum	AF240567	54-59-117-139-222	

+ hsp60 sequence of *B. subtile* type strain was not available in the press-time.
† the available sequences at GeneBank and cpnDB belonged to *B. thermacidophilum* (with no distinction in subspecies).
*subspecies not discernable.

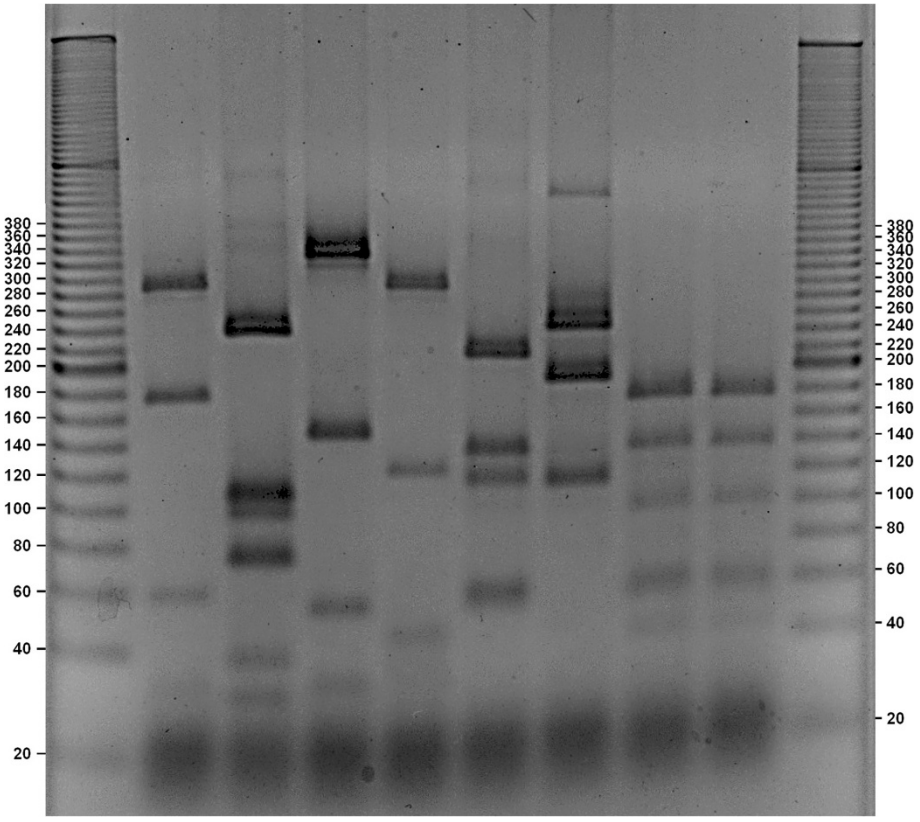

Figure 1 Agarose gel electrophoresis of digested *hsp60* DNA fragments with HaeIII (negative image). Lane1, ladder 20 bp (Sigma-Aldrich); Lane 2, *B. bifidum* ATCC 29521; Lane 3, *B. asteroides* ATCC 25910, Lane 4, *B. coryneforme* ATCC 25911; Lane 5, *B. indicum* ATCC 25912; Lane 6, *B. thermophilum* ATCC 25525; Lane 7, *B. boum* ATCC 27917; Lane 8, *B. thermacidophilum* subsp. *porcinum* LMG 21689; Lane 9, *B. thermacidophilum* subsp. *thermacidophilum* LMG 21395; Lane 10, ladder 20 bp (Sigma-Aldrich).

The purpose of this study is the development of a rapid, reproducible and easy-to-handle molecular tool for the identification of *Bifidobacterium* species isolated from various environments. The protocol is based on the restriction endonuclease analysis of the PCR-amplified *hsp60* partial gene sequence (*hsp60* PCR-RFLP) with the use of a single restriction enzyme and has been tested on the 30 most widely distributed *Bifidobacterium* species and subspecies. A diagnostic dichotomous key to speed up profile interpretation has also been proposed.

Methods

Bacterial strains and culture conditions

The type strains used to develop the technique are listed in Table 1, whereas the strains used to validate the method are reported in Table 2. The strains, belonging to BUSCoB (Bologna University Scardovi Collection of Bifidobacteria) collection, were isolated from faeces of human and animals and from sewage. Bacteria were maintained as frozen stocks at −80°C in the presence of skim milk as cryoprotective agent. Working cultures were prepared in TPY medium [1], grown anaerobically at 37°C and harvested at logarithmic phase.

In silico analysis

An *in silico* analysis was performed for the evaluation of a suitable restriction enzyme. Available *hsp60* sequences had been retrieved from cpnDB database and GeneBank, thanks to the work of Jian et al. [25]. *In silico* digestion analysis was carried out on fragments amplified by universal primers H60F-H60R [30] using two on-line free software: webcutter 2.0 (http://rna.lundberg.gu.se/cutter2) and http://insilico.ehu.es/restriction softwares [31]. Blunt end, frequent cutter enzymes that recognize not degenerated sequences have been considered in order to find a suitable enzyme for all the species (e.g. RsaI, HaeIII, AluI, AccII). However *in silico* analysis had been performed also on sticky end enzymes (e.g. AatII, Sau3AI, PvuI).

DNA extraction from pure cultures

10 ml of culture were harvested and washed twice with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.6), resuspended in 1 ml TE containing 15 mg lysozyme and incubated at 37°C overnight. Cells were lysed with 3 ml of lysis buffer (100 mM Tris–HCl, 400 mM NaCl, 2 mM EDTA, pH 8.2), 220 μl SDS (10% w/v) and 150 μl

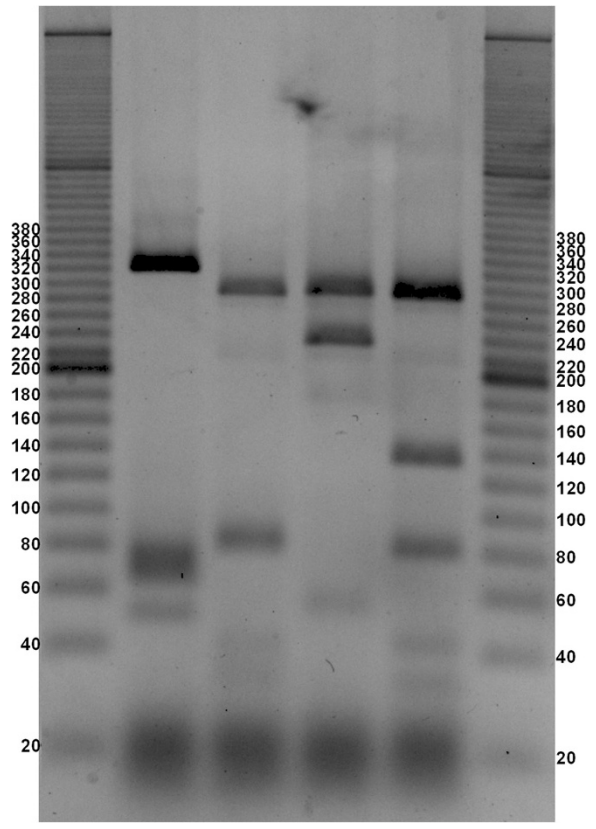

Figure 2 Agarose gel electrophoresis of digested *hsp60* DNA fragments with HaeIII (negative image). Lane1, ladder 20 bp (Sigma-Aldrich); Lane 2, *B. minimum* ATCC 27539; Lane 3, *B. pullorum* ATCC 27685, Lane 4, *B. subtile* ATCC 27537; Lane 5, *B. gallinarum* ATCC 33777; Lane 6, ladder 20 bp (Sigma-Aldrich).

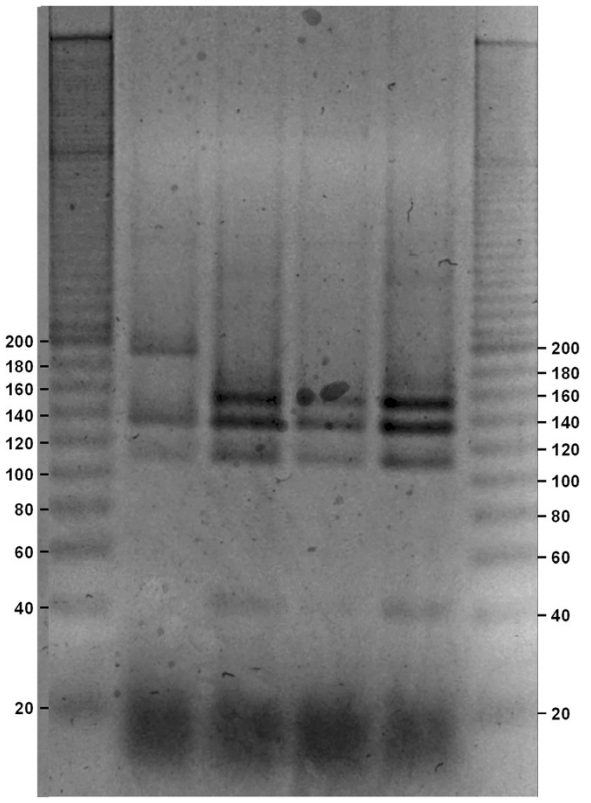

Figure 3 Agarose gel electrophoresis of digested *hsp60* DNA fragments with HaeIII (negative image). Lane1, ladder 20 bp (Sigma-Aldrich); Lane 2, *B. breve* ATCC 15700; Lane 3, *B. longum* subsp. *infantis* ATCC 15697; Lane 4, *B. longum* subsp. *longum* ATCC 15707; Lane 5, *B. longum* subsp. *suis* ATCC 27533; Lane 6, ladder 20 bp (Sigma-Aldrich).

proteinase K (>600 mAU/ml, solution) and incubated for 2 hours in water bath at 60°C. One ml of saturated NaCl solution was added and the suspension was gently inverted twice. Pellets were harvested through centrifugation (5000 × g) at room temperature for 15 minutes. After the transfer of clean supernatants in new tubes, DNA was precipitated with 2.5 volumes of cold ethanol (95%) and resuspended in 300 μl of TE buffer [32].

Amplification of gene *hsp60* and restriction with HaeIII

Universal primers were used to amplify approximately 600 bp of the *hsp60* gene in the *Bifidobacterium* spp. investigated. These primers H60F (5'-GG(ATGC)GA(CT)GG (ATGC)AC(ATGC)AC(ATGC)AC(ATGC)GC(ATGC)A C(ATGC)GT-3') and H60R (5'-TC(ATGC)CC(AG)AA (ATGC)CC(ATGC)GG(ATGC)GC(CT)TT(ATGC)AC (ATGC)GC-3') were designed by Rusanganwa et al. [30] on the basis of the conserved protein sequences GDGTTATV and AVKAPGFGD in HSP60. Amplifications were performed in 20 μl volumes with 1.5 μM of each primer (Eurofins MWG Operon, Ebersberg, Germany), 10 μl 2X HotStarTaq Plus Master Mix (Qiagen, Italy) (1,5 mM $MgCl_2$, 1 U Taq, 0.2 mM dNTP, final concentration) and 150 ng/μl DNA. The PCR cycle consisted of an initial denaturation of 5 min at 95°C followed by 35 cycles of denaturation (30s at 94°C), annealing (30s at 61°C) and extension (45 s at 72°C). The PCR was completed with a final elongation of 10 min at 72°C. The PCR amplification was performed with a PCR Verity 96-well thermal cycler (Applied Biosystems, Milan, Italy). After amplification, the product was visualized via agarose gel (1.3% w/v) in 1X TBE buffer and visualized with ethidium bromide under UV light. A 100 bp DNA ladder (Sigma-Aldrich) was used as a DNA molecular weight marker. Bands were excised from agarose gel (Additional file 1: Figure S1) and DNA was eluted with NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel GmbH & Co. KG, Germany) in order to avoid possible non-specific amplifications. 3 μl of the eluted DNA was re-amplified in a 30 μl PCR reaction (see above). BSA was added to the reaction (5% v/v, Fermentas). The PCR products (2 μl) were checked for non-specific amplification on agarose gel. 20 μl (~6 μg) of PCR amplicons were digested with HaeIII enzyme. Restriction digestion was carried out for 2 h at 37°C in 30 μl

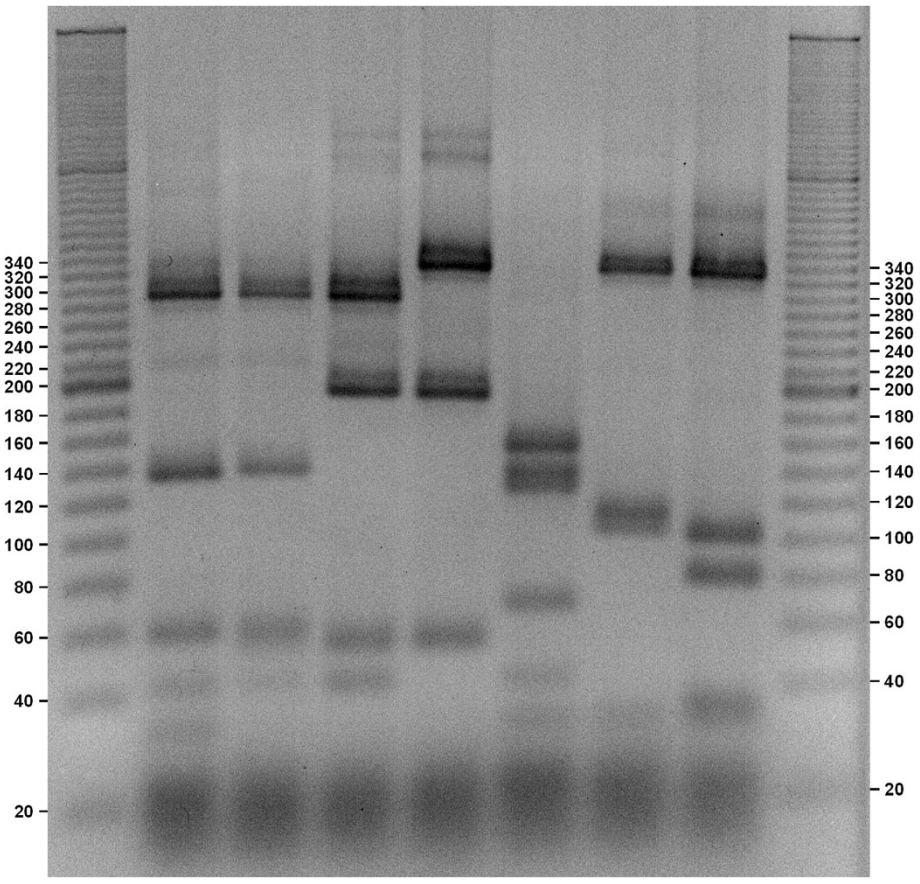

Figure 4 Agarose gel electrophoresis of digested *hsp60* DNA fragments with HaeIII (negative image). Lane1, ladder 20 bp (Sigma-Aldrich); Lane 2, *B. merycicum* ATCC 49391; Lane 3, *B. angulatum* ATCC 27535, Lane 4, *B. pseudocatenulatum* ATCC 27919; Lane 5, *B. catenulatum* ATCC 27539; Lane 6, *B. dentium* ATCC 27534; Lane 7, *B. ruminantium* ATCC 49390; Lane 8, *B. adolescentis* ATCC 15703; Lane 9, ladder 20 bp (Sigma-Aldrich).

reaction mixture with 1X SM Restriction Buffer (Sigma-Aldrich), 1.5 μl HaeIII (10 U/μl, Sigma-Aldrich) and water. Digestion products were stained with ethidium bromide and visualized under UV-light (GelDoc™, BioRad), after agarose gel electrophoresis (3.0% agarose (w/v), TBE 1X) at 210 V (3 h). A 20 bp DNA ladder (Sigma-Aldrich) was used. The obtained pictures were elaborated with a free software GNU Image Manipulation Program (Gimp 2.8) only to invert colors and increase contrast.

Precast gradient polyacrylamide gels (4-20%) (Lonza Group Ltd, Switzerland) were also used to obtain RFLP profiles, in order to have a comparison with agarose gels. The vertical electrophoresis apparatus used was P8DS™ Emperor Penguin (Owl, Thermo Scientific) with an adaptor for Lonza precast gels. The run was performed at 100 V in TBE 1X.

Diagnostic key

A dichotomous key was developed comparing *in silico* digestion results and the evaluation of visible bands with the use of ImageLab™ 2.0 software (Bio-Rad Laboratories, Inc.).

Results and discussion

In silico analysis

The analysis and comparison of restriction profiles obtained with *in silico* digestion of bifidobacterial *hsp60* sequences allowed the identification of a set of appropriate frequent-cutter endonucleases that recognize non degenerated sequences. The restriction enzyme HaeIII was found to give the clearest and most discriminatory profiles in theoretical PCR-RFLP patterns, discriminating the majority of *Bifidobacterium* type-strains tested (Table 3). Furthermore, the profiles of other strains, belonging to the investigated species, have been analyzed to confirm the conservation of RFLP profiles within species.

Amplification and restriction analysis of *Bifidobacterium* spp.

Theoretical restriction profiles have been confirmed *in vitro* on agarose gel. The obtained fragments ranged from 16 bp to 339 bp (Table 3). Fragments lower than 25 bp were not considered as they did not help in species discrimination and in addition they co-migrate with

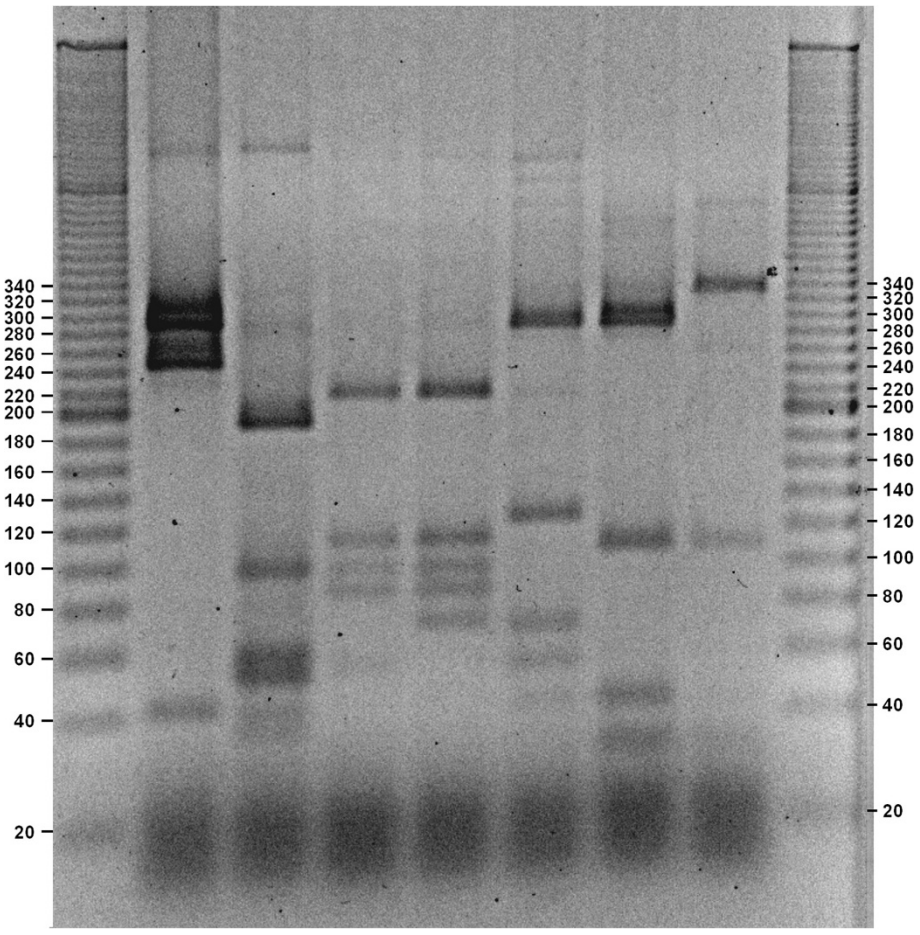

Figure 5 Agarose gel electrophoresis of digested *hsp60* DNA fragments with HaeIII (negative image). Lane1, ladder 20 bp (Sigma-Aldrich); Lane 2, *B. gallicum* ATCC 49850; Lane 3, *B. choerinum* ATCC 27686, Lane 4, *B. animalis* subsp. *lactis* DSM 10140; Lane 5, *B. animalis* subsp. *animalis* ATCC 25527; Lane 6, *B. cuniculi* ATCC 27916; Lane 7, *B. pseudolongum* subsp. *pseudolongum* ATCC 25526; Lane 8, *B. pseudolongum* subsp. *globosum* ATCC 25865; Lane 9, ladder 20 bp (Sigma-Aldrich).

primers. Time course analysis of restricted samples showed the formation of a band of ~200 bp in several species due to an over-digestion (data not shown) and this invalidated the RFLP profiles. For this reason the protocol has been optimized at 2 hours restriction time. Fragments greater than 360 bp were also not considered due to a possible incomplete digestion of such long fragments.

The obtained gels (Figures 1, 2, 3, 4 and 5) show species-specific profiles for all type-strains other than *B. longum* and *B. thermacidophilum* subspecies. This technique does not allow the identification of the subspecies belonging to these species, which displayed identical RFLP profiles. Matsuki et al. [14,17] proposed specific primers to differentiate the subspecies of the species *B. longum*, while *B. thermacidophilum* subsp. *porcinum* and *B. thermacidophilum* subsp. *thermacidophilum* can be differentiated according to Zhu et al. [33]. The proposed restriction analysis is efficient in discriminating very closely related species and subspecies as *B. catenulatum*/*B. pseudocatenulatum*, *B. pseudolongum* subsp. *pseudolongum*/*B. pseudolongum* subsp. *globosum* and *B. animalis* subsp. *animalis*/*B. animalis*. subsp. *lactis*.

The same method has been applied with the use of precast gradient polyacrylamide gels. The resolution was greater than that obtained on agarose gels, loading only 4 μl of the restriction reaction instead of the 30 μl used in horizontal electrophoresis. This may allow to reduce the volume of amplification reactions with a consequent reduction of costs.

The comparison between *in silico* digestion and the obtained gel profiles allowed to develop a dichotomous key (Figure 6) for a faster interpretation of the restriction profiles.

Validation of PCR-RFLP analysis on bifidobacterial isolates

39 strains belonging to 12 different species/subspecies (Table 2) have been investigated to validate the PCR-

1. Distinct fragment between 320 and 360 bp……2
- No fragment between 320 and 360 bp……7
2. One fragment ≥ 340 bp……3
- No fragment ≥ 340 bp……*B. minimum*
3. Fragments between 280 and 140 bp……4
- No fragments between 280 and 140 bp……5
4. One fragment at approximately 200 bp……*B. catenulatum*
- No fragment at 200 bp……*B. coryneforme*
5. Fragment at approximately 80 bp……*B. adolescentis*
- No fragment at 80 bp……6
6. Two fragments near 100 bp……*B. ruminantium*
- One fragment near 100 bp……*B. pseudolongum* subsp. *globosum*
7. Fragment at approximately 280- 300bp……8
- No fragment at 280-300 bp ……18
8. One fragment between 150-260 bp……9
- No fragment between 150-260 bp……12
9. Fragment at 250 bp and 40 bp ……*B. gallicum*
- No fragment at 250 bp and 40 bp……10
10. Fragment at approximately 240 bp……*B. subtile*
- No fragment at approximately 240bp……11
11. Fragment at approximately 200bp……*B. pseudocatenulatum*
- No fragment at approximately 200 bp……*B. bifidum*
12. Fragment at 140 bp……13
- No fragment at 140 bp……15
13. One fragment at 80 bp……*B. gallinarum*
- No fragment at 80 bp……14
14. One fragment at 30 bp……*B. merycicum*
- No fragment at 30 bp……*B. angulatum*
15. Fragment between 100-200 bp……16
- No Fragment between 100-200 bp……*B. pullorum*
16. Fragment ≥ 120bp……17
- No fragment ≥ 120 bp……*B. pseudolongum* subsp. *pseudolongum*
17. Fragment at approximately 70 bp……*B. cuniculi*
- No fragment at 70 bp……*B. indicum*
18. Fragment ≥ 220 bp……19
- No fragment ≥ 220 bp……23
19. At least one fragment ≥ 240 bp……20
- No fragment ≥ 240 bp……21
20. Fragment at approximately 190 bp……*B. boum*
- No fragment at 190 bp……*B. asteroides*
21. Fragment at approximately 140-135 bp……*B. thermophilum*
- No fragment at 140-135 bp……22
22. Fragment at approximately 70bp……*B. animalis* subsp. *lactis*
- No fragment at 70 bp……*B. animalis* subsp. *animalis*
23. Fragment at 140 bp……24
- No fragment 140 bp……*B. choerinum*
24. Fragment at approximately 160bp……25
- No fragment at 160 bp……26
25. Fragment at 70 bp……*B. dentium*
- No fragment at 70 bp……*B. longum* spp.
26. Fragment at 60 e 40 ……*B. thermacidophilum* spp.
- No fragment at 60 e 40 bp……*B. breve*

Figure 6 Dichotomous key to identify species of *Bifidobacterium* based upon HaeIII restriction digestion of ~590 bp of the *hsp60* gene.

RFLP technique. Most of the strains tested were previously identified using biochemical tests and in some cases also molecular techniques (species-specific PCR, 16S rDNA sequencing). The obtained data confirmed a conservation of the profiles concerning the species and subspecies tested. Two figures are available as Additional files (Additional file 2: Figure S2: strains belonging to *B. animalis* subsp. *lactis* and *B. animalis* subsp. *animalis*. Additional file 3: Figure S3: strains belonging to *B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, *B. longum* subsp. *suis*). About 95% of the strains confirmed the taxonomic identification previously assigned. Two strains, B1955 and Su864, previously classified as *B. catenulatum* and *B. longum* subsp. *suis* respectively, gave different profiles from those expected. The RFLP profiles of B1955 turned out to be the same of *B. adolescentis* ATCC 15703 (T), the dichotomous key confirmed the assignment to the *B. adolescentis* species. In addition, Su864 was identified as a *B. breve* strain. These results were also verified through a species-specific PCR [14].

Conclusions

In this work a PCR-RFLP based method to identify *Bifidobacterium* spp. was developed and tested on strains belonging to different species. The technique could efficiently differentiate all the 25 species of *Bifidobacterium* genus and the subspecies belonging to *B. pseudolongum* and *B. animalis*, with the support of an easy-to-handle dichotomous key. The technique turned out to be fast and easy, and presented a potential value for a rapid preliminary identification of bifidobacterial isolates.

Additional files

Additional file 1: Figure S1. Example of agarose gel electrophoresis of hsp60 amplicons from different bifidobacterial strains.

Additional file 2: Figure S2. Agarose gel electrophoresis of digested *hsp60* DNA fragments with HaeIII (negative image). Lane1, ladder 20 bp (Sigma-Aldrich); Lane 2–6, *B. animalis* subsp.*lactis* strains Ra20, Ra18, F439, P23, P32; Lane 7–8, *B. animalis* subsp. *animalis* strains T169, T6/1; Lane 9, ladder 20 bp (Sigma-Aldrich).

Additional file 3: Figure S3. Agarose gel electrophoresis of digested *hsp60* DNA fragments with HaeIII (negative image). Lane1, ladder 20 bp (Sigma-Aldrich); Lane 2–4, *B. longum* subsp. *suis* strains Su864, Su908, Su932; Lane 5–6, *B. longum* subsp. *longum* strains PCB133, ATCC 15707 (T); Lane 7–9, *B. longum* subsp. *infantis* strains ATCC 15697 (T), B7740, B7710; Lane 9, ladder 20 bp (Sigma-Aldrich).

Abbreviations

PCR: Polymerase chain reaction; RFLP-PCR: Restriction fragment length polymorphism; HSP60: Heat-shock protein 60; rDNA: Ribosomal DNA; ARDRA: Amplified ribosomal DNA restriction analysis; DGGE: Denaturing gradient gel electrophoresis; ERIC-PCR: Enterobacterial repetitive intergenic consensus-PCR; RAPD: Random amplified polymorphic DNA; cpnDB: Chaperonin database; TPY medium: Tryptone phytone, yeast medium; BUSCoB: (Bologna University Scardovi Collection of Bifidobacteria).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LB conceived the study. LB, VS and ES carried out all the bioinformatics, RFLP analyses, DNA extractions and culture handling. VS conceived the dichotomous key. MM and PM provided some of the strains tested together with the extracted DNA. DDG and FG supervised the work. LB, VS, DDG and FG contributed to paper writing. All authors read and approved the final manuscript. BB supported the research.

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