Assessment of *Bifidobacterium* Species Using *groEL* Gene on the Basis of Illumina MiSeq High-Throughput Sequencing

Lujun Hu 1,2,†, Wenwei Lu 1,2,†, Linlin Wang 1,2, Mingluo Pan 1,2, Hao Zhang 1,2,3, Jianxin Zhao 1,2,3,* and Wei Chen 1,2,3,4

1 State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China; 7130112038@vip.jiangnan.edu.cn (L.H.); luwenwei@jiangnan.edu.cn (W.L.); wanglllynn09@163.com (L.W.); mingluo@163.com (M.P.); zhanghao@jiangnan.edu.cn (H.Z.); chenwei66@jiangnan.edu.cn (W.C.)

2 School of Food Science and Technology, Jiangnan University, Wuxi 214122, China

3 National Engineering Research Center for Functional Food, Jiangnan University, Wuxi 214122, China

4 Beijing Innovation Centre of Food Nutrition and Human Health, Beijing Technology and Business University, Beijing 100048, China

* Correspondence: jxzhao@jiangnan.edu.cn; Tel.: +86-510-8591-2155

† These authors contributed equally to this work.

Received: 31 October 2017; Accepted: 15 November 2017; Published: 21 November 2017

**Abstract:** The next-generation high-throughput sequencing techniques have introduced a new way to assess the gut’s microbial diversity on the basis of 16S rRNA gene-based microbiota analysis. However, the precise appraisal of the biodiversity of *Bifidobacterium* species within the gut remains a challenging task because of the limited resolving power of the 16S rRNA gene in different species. The *groEL* gene, a protein-coding gene, evolves quickly and thus is useful for differentiating bifidobacteria. Here, we designed a *Bifidobacterium*-specific primer pair which targets a hypervariable sequence region within the *groEL* gene that is suitable for precise taxonomic identification and detection of all recognized species of the genus *Bifidobacterium* so far. The results showed that the novel designed primer set can specifically differentiate *Bifidobacterium* species from non-bifidobacteria, and as low as 10^4 cells of *Bifidobacterium* species can be detected using the novel designed primer set on the basis of Illumina Miseq high-throughput sequencing. We also developed a novel protocol to assess the diversity of *Bifidobacterium* species in both human and rat feces through high-throughput sequencing technologies using *groEL* gene as a discriminative marker.

**Keywords:** *Bifidobacterium*; *groEL*; species level; biodiversity; MiSeq high-throughput sequencing

1. Introduction

Members of *Bifidobacterium* species, characterized by high G + C Gram-positive, non-motile, non-gas-producing, non-sporulating, anaerobic bacteria, constitute a group of the commensal bacterium of human and animal intestinal microbiota. The contribution of bifidobacteria in maintaining or improving human and animal health has been accepted and some members of the *Bifidobacterium* species have been added as probiotics to various foods [1,2]. Therefore, it is vital to detect and identify bifidobacterial strains precisely and to assess their diversity and population size in the gastrointestinal tract. Within this context, assessment of the *Bifidobacterium* species in complex samples such as feces from humans and animals has attracted great interest from researchers.

With the advent of massive parallel sequencing technologies, the cultivation-independent methods based on the 16S rRNA gene with the help of next-generation sequencing technologies have been extensively recognized as a useful tool for the assessment of *Bifidobacterium* species [3–7]. Among the next-generation sequencing technologies, the MiSeq Illumina sequencing platform had the lowest error rates
and highest throughput per run as compared to the 454 GS Junior and Ion Torrent PGM [8]. However, the resolvability of the 16S rRNA gene sequences among closely related bacterial strains is limited. In general, bacterial strains showing more than 97% 16S rRNA gene sequence similarity, markers with higher discriminating power are necessary. The internal transcribed spacer (ITS) region provides a high resolution and can be used to assess population biodiversity in bacterial communities [9]. However, the ITS marker for studying the environmental samples is problematic in that the presence of operon copy number heterogeneity within a genome and the possibility of intragenomic variation in ITS sequence and length may lead to skewed estimates of bacterial communities [10,11].

Alternative target genes, such as atpD [12], recA [13], tuf [14], dnaK [15], tal [16], xfy [17], rpoC [18], and groEL [19–22] have been used for the differentiation and identification of Bifidobacterium species. These gene markers have been demonstrated to have similar or even higher resolution ability for Bifidobacterium species than the 16S rRNA gene. Compared to other molecular markers, the groEL gene has more advantages. The groEL gene is a ubiquitous housekeeping gene in the genus Bifidobacterium, that encodes the heat shock proteins (Hsp60, also known as Copn60 or GroEL), which play an important role in response to cellular stress. Additional sequences of bifidobacterial strains are available in the Chaperonin Sequence Database [23]. Many experiments have proved that Bifidobacterium species have just one copy of the groEL gene [24–26], which facilitates quantitative analyses of Bifidobacterium species. Furthermore, the groEL gene sequence identities between Bifidobacterium species were much lower than those of 16S rDNA, thus possessing higher resolution power for Bifidobacterium species than the 16S rRNA gene.

In this study, a novel protocol was described to assess Bifidobacterium species through high-throughput sequencing technologies using groEL as a discriminative marker. To test the robustness of the novel designed primer set, we analyzed Bifidobacterium species in human and rat fecal samples using the designed primer set on the basis of the MiSeq Illumina sequencing platform.

2. Materials and Methods

2.1. Bacterial Strains, Culture Media and DNA Extraction

The bifidobacterial strains used in the study were as follows: B. adolescentis CCFM626, B. animalis subsp. animalis CCFM624, B. animalis subsp. lactis BB12, B. bifidum CCFM641, B. breve CCFM623, B. dentium FJSNT63M4, B. longum subsp. infantis CCFM666, B. longum subsp. longum CCFM642, B. pseudacatenulatum CCFM749, and B. pseudolongum FJSWX2M9. All the bifidobacterial strains were grown in de Man, Rogosa and Sharp (MRS) broth with addition of 0.05% of L-cysteine hydrochloride monohydrate at 37 °C. Seven non-bifidobacterial strains were also used in this study: Actinomyces odontolyticus HNSQ3B4, Bacteroides uniformis CCFM792, Escherichia coli CCFM21, Enterococcus faecalis CCFM596, Lactobacillus acidophilus CCFM137, L. plantarum ST-III (CGMCC no. 0847) and Rothia dentocariosa JSWX1B7. All the bifidobacterial strains were grown in de Man, Rogosa and Sharp (MRS) broth with addition of 0.05% of L-cysteine hydrochloride monohydrate at 37 °C. Seven non-bifidobacterial strains were also used in this study: Actinomyces odontolyticus HNSQ3B4, Bacteroides uniformis CCFM792, Escherichia coli CCFM21, Enterococcus faecalis CCFM596, Lactobacillus acidophilus CCFM137, L. plantarum ST-III (CGMCC no. 0847) and Rothia dentocariosa JSWX1B7. All the bifidobacterial strains were cultured in MRS broth at 37 °C. E. coli was cultured at 37 °C in Luria-Bertani medium. A. odontolyticus, B. uniformis, E. faecalis and R. dentocariosa were grown in Brain Heart Infusion Broth at 37 °C. All the bacteria used in this study came from the Culture Collection of Food Microorganisms of Jiangnan University (Wuxi, China).

Genomic DNA of these bacteria was extracted using the method described previously and subjected to further phenol/chloroform purification using an established protocol [27,28].

2.2. Fecal Sample Collection and Genomic DNA Extraction

Feces from humans and from rats were collected in this study. All stool samples from adult humans were collected in sterile containers within 20 min after defecation, transported to the laboratory on ice, and stored at −80 °C until genomic DNA was extracted. Fresh feces from rats were collected in individual sterile EP tubes on ice, which were taken to the laboratory within 2 h and stored at −80 °C for further observation. Fecal samples were homogenized and then subjected to bacterial genome
DNA extraction using FastDNA SPIN Kit for Feces (MP Biomedicals; Carlsbad, CA, USA) as per the manufacturer’s protocols. The protocols of the study were approved by the Ethical Committee of Jiangnan University (Wuxi, China).

2.3. Phylogenetic Analysis

The phylogenetic relationships among the genus Bifidobacterium were constructed using groEL gene. The best fitted substitution model for each partition was estimated using Akaike information criterion (AIC) implemented in jModeltest [29]. The model of TIM1 + I + G was chosen for Maximum likelihood (ML) analyses, which were performed with RAxML BlackBox web servers [30].

2.4. Bifidobacterium groEL-Specific Primer Design

All available bifidobacterial and some actinobacterial groEL gene sequences were retrieved from the GenBank and European Molecular Biology Laboratory (EMBL) nucleotide sequence databases and aligned using the ClustalW software program [31]. To identify Bifidobacterium species, a region of 487 or 496 base pairs (bp) located at positions 1066 to 1552 (B. animalis) or 1561 (B. breve) of the complete groEL gene of ca. 1600 bp was chosen as discriminative target sites. Degenerate primers Bif-groEL-F (5-TCC GAT TAC GA Y CGY GAG AAG CT-3)/Bif-groEL-R (5-CSG CYT CGG TSG TCA GGA ACA G-3) for the genus Bifidobacterium were manually designed according to multiple sequence alignment. Specificity testing was carried out using PRIMER-BLAST which allows in silico PCR amplification using the National Center for Biotechnology Information (NCBI) nonredundant database as a template [32]. An additional specificity test was conducted by PCR amplification using genomic DNA extracted from known bacterial species: A. odontolyticus, B. uniformis, E. coli, E. faecalis, L. acidophilus, L. plantarum and R. dentocariosa. The primers were synthesized by the Shanghai Sangon Biological Science & Technology Company (Shanghai, China) and used for the PCR amplification.

2.5. PCR Amplification, Quantification, and Sequencing

The selected partial groEL gene sequences from microbial genome DNA were PCR amplified using the barcoded fusion primers Bif-groEL-F/Bif-groEL-R designed during this study. PCR amplifications were performed using a 50 µL total volume consisting of 1 µL of the target DNA, 25 µL Premix Taq (TaKaRa, Dalian, China), 1 µL of each primer (20 µM) and 22 µL of double distilled water (ddH2O). The PCR amplification procedures were pre-denaturation at 95 °C for 4 min, followed by 30 cycles consisting of denaturation at 95 °C for 30 s, annealing for 30 s at 60 °C, extension for 50 s at 72 °C and the final step at 72 °C for 10 min. In addition, The V3–V4 hypervariable sequence region of the 16S rRNA gene was amplified with the forward primer (341F: 5-CCT A YG GGR BGC ASC AG-3) and reverse primer (806R: 5-GGA CTA CNN GGG TAT CTA AT-3) according to the previous protocols [33].

All the PCR amplification products obtained following amplification of the groEL and 16S rRNA gene sequences were excised from the agarose gel, purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and quantified by Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer’s recommendations. DNA amplicon libraries were prepared using TruSeq DNA LT Sample Preparation Kit (Illumina, San Diego, CA, USA) and sequenced on the MiSeq Illumina platform using the MiSeq v3 Reagent Kit (600 cycles) following instructions provided by the manufacturer.

2.6. Evaluation of the Sensitivity of the Novel Designed Primer Set

The detection sensitivity and accuracy of the primer set Bif-groEL-F/Bif-groEL-R were evaluated employing known DNA amounts, ranging from 0.01 to 40 ng, of the artificial sample from 10 different bifidobacterial taxa. The groEL gene copy numbers were estimated using “Calculator for Determining the Number of Copies of a Template” (URI Genomics & Sequencing Center) [34]. Thus, colony-forming units (CFU) could be calculated on the basis of the groEL gene copy numbers predicted above.
2.7. Bioinformatic Sequence Analysis

Sequence reads were processed with the QIIME package version 1.9.1 (Quantitative Insights Into Microbial Ecology, Flagstaff, AZ, USA) [35]. The raw sequences with a lower quality score and short-read length compared to target sequences were first removed. Sequences were also removed if they contained ambiguous bases or mismatches in primers. Only pair-end reads overlapping longer than 10 bp and without any mismatch were assembled following their overlap sequences, and the unassembled reads were discarded. Barcode and sequencing primers from the above assembled sequences were trimmed.

Each sample’s high-quality reads were clustered into operational taxonomic units (OTUs) for further taxonomic analyses. Taxonomic identification of OTUs for the \textit{groEL} sequences was performed through comparison to the Chaperonin Sequence Database [23]. The OTUs of V3–V4 region sequences were assigned to a taxonomy with the naive Bayes classifier of the Ribosomal Database Project (RDP) [36], and all OTUs with representative sequences from each cluster were combined and aligned against the Greengenes core set in QIIME with the PyNAST aligner [35,37]. Similarities among the microbial communities were estimated using cluster heatmap analysis with the R software for statistical computing [38].

2.8. Real-Time qPCR

The total and main bifidobacterial numbers of human feces were determined by SYBR Green-based qPCR using a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) as previously described with some modifications [22,39]. The total bifidobacteria and the main bifidobacteria of \textit{B. pseudocatenulatum} and \textit{B. longum} subsp. \textit{longum} from human feces were quantified through qPCR amplifications using the primers previously described in 20 µL volume using 96-well plates in triplicate [22]. For quantification of the genus \textit{Bifidobacterium}, \textit{B. longum} subsp. \textit{longum} CCFM642 was used as the standard strain. \textit{B. pseudocatenulatum} CCFM749 and \textit{B. longum} subsp. \textit{longum} CCFM642 were also used as qPCR controls for species-specific quantification. The target bifidobacterial population was expressed as Log\textsubscript{10} bifidobacteria per gram of wet stool.

2.9. Statistical Analysis

All data were expressed as means ± standard deviation (SD). The statistical analyses of differences between two groups were analyzed using Student’s \textit{t}-test. The analyses were carried out with SPSS version 16.0 (SPSS Inc., Chicago, IL, USA), and statistical significance was accepted at least at the 5% level.

3. Results

3.1. Comparative Analysis of the \textit{groEL} and 16\textit{S} rRNA Gene

The \textit{groEL} and 16\textit{S} rRNA gene nucleotide sequences of \textit{Bifidobacterium} species (download from NCBI and EMBL databases) were compared by BLAST [40] (Table 1). When comparing the two sets of sequences, we found that the lowest value of the pairwise similarities of the \textit{groEL} gene is 79.1% (\textit{B. magnum} and \textit{B. tsurumiense}) and the average pairwise similarities value is 86.3% whereas the lowest value of the pairwise similarities of the 16\textit{S} rRNA gene is 90.9% (\textit{B. magnum} and \textit{B. crudilactis}) and the average pairwise similarities value is 93.8%. Thus, the \textit{groEL} gene provides higher resolution power than that provided by the 16\textit{S} rRNA gene. We also plotted the average pairwise similarities values targeted for the \textit{groEL} gene against the average pairwise similarities values corresponding to 16\textit{S} rRNA gene sequences for classification of microorganisms at the same taxonomic rank. Using the regression shown in Figure 1, we found that the variation trend of the average pairwise similarities values were similar between the \textit{groEL} and 16\textit{S} rRNA genes.
### Table 1. List of *Bifidobacterium* strains used for comparative analysis.

| Number | Bifidobacterium Species | Strain a | GenBank Accession no. of groEL Gene Sequences | GenBank Accession no. of 16S rRNA Gene Sequences |
|--------|-------------------------|----------|---------------------------------------------|-----------------------------------------------|
| 1      | *B. adolescentis*       | ATCC 15703 | AF009526                                    | NR_074802                                    |
| 2      | *B. angulatum*          | JCM 796   | AF012322                                    | LC071846                                     |
| 3      | *B. animalis* subsp. *animalis* | ATCC 25527 = LMG 10508 | CP002567                                    | JGZM01000004                                  |
| 4      | *B. animalis* subsp. *lactis* | BB12     | CP001853                                    | GU116483                                     |
| 5      | *B. bifidum*            | DSM 3969  | NZ_KQ03859                                  | EF187231                                     |
| 6      | *B. bifidum*            | PRL2010  | CP001840                                    | CP001840                                     |
| 7      | *B. bohemicum*          | R3250    | FMAM01000002                                | FMAM01000014                                 |
| 8      | *B. brevis*             | JCM 1192  | AP012324                                    | LC071799                                     |
| 9      | *B. catenulatum*        | DSM 23973 | AP012325                                    | JGYS01000004                                 |
| 10     | *B. catteslachus*       | DSM 16992 | AP012325                                    | NR_041875                                    |
| 11     | *B. cecorum*            | ATCC 27666 = LMG 10510 | JGZU01000001 | D861386                                     |
| 12     | *B. coryneforme*        | Bna6     | KXQ03865                                    | EF187237                                     |
| 13     | *B. coryneforme*        | DSM 26699 | NZ_HAL10000002                              | NZ_HAL10000001                               |
| 14     | *B. coryneforme*        | DSM 11973 | JGYS10000008                                | JGYS10000001                                 |
| 15     | *B. coryneforme*        | JCM 1195  | AP012326                                    | LC071795                                     |
| 16     | *B. gallicum*           | DSM 20093 = LMG 11596 | NZ_ABX01000002 | ABX01000004                        |
| 17     | *B. gallicum*           | DSM 20670 = JCM 6291 | NZ_JGDN10000004                              | D86191                                       |
| 18     | *B. indolicum*          | LMG 11597 = DSM 2214 = JCM1302 | CP006018                                    | D861388                                     |
| 19     | *B. magnus*             | DSM 20222 = JCM 1228 | NZ_ATV01000001                              | D86193                                       |
| 20     | *B. magnus*             | DSM 6492 = JCM 8219 | NZ_JDIT10000006                              | D86192                                       |
| 21     | *B. minimum*            | DSM 20102 = ATCC 27538 | NZ_ATXM01000001 | M59741                                   |
| 22     | *B. minimum*            | DSM 20102 = ATCC 27538 | NZ_ATXM01000001 | M59741                                   |
| 23     | *B. minimum*            | DSM 20102 = ATCC 27538 | NZ_ATXM01000001 | M59741                                   |
| 24     | *B. lautus*             | BM2-1    | AB579933                                    | AB491757                                     |
| 25     | *B. longum* subsp. *infantis* | ATCC 15697 | CP001095                                    | NR_043437                                    |
| 26     | *B. longum* subsp. *longum* | BMM168   | CP002286                                    | GQ380695.1                                   |
| 27     | *B. mongoliense*        | DSM 21395 | JGZM01000001                                | AB433896                                     |
| 28     | *B. pseudocatenulatum*  | JCM 1210  | AP012330                                    | LC071796                                     |
| 29     | *B. pseudolongum*       | CP007457 | CP007457                                    | CP007457                                     |
| 30     | *B. pullorum*           | DSM 20433 = JCM 1214 | NZ_JDUN01000001 | D86198                       |
| 31     | *B. reuteri*            | DSM 23975 | NZ_JDUN01000002                              | NZ_JDUN01000019                              |
| 32     | *B. reuteri*            | DSM 23975 | NZ_JDUN01000002                              | NZ_JDUN01000019                              |
| 33     | *B. ruminantium*        | DSM 6489 = JCM 8222 | NZ_JHWQ10000003                              | D86197                                       |
| 34     | *B. saccharolyticum*    | DSM 6531  | JGZM01000001                                | D89932                                       |
| 35     | *B. scaredii*           | JCM 12489 | AP012331                                    | AP012331                                     |
| 36     | *B. schoenleinii*       | DSM 3968  | JGZP01000003                                | JGZP01000012                                 |
| 37     | *B. suis*               | JCM 15918 | JGZQ10000008                                | NZ_JDUX01000017                              |
| 38     | *B. thermacidophilum* subsp. *porcinum* | LMG 21689 | JGZS01000003                                | NZ_JGZS01000003                              |
| 39     | *B. thermophilum*       | BRL67    | CP004346                                    | DQ340557                                     |
| 40     | *B. turanense*          | DSM 17777 = OMB115 | NZ_AUCL01000007 | AR241106                       |

a ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; JCM, Japanese Collection of Microorganisms; LMG, Laboratorium voor Microbiologie, University of Ghent.
3.2. Phylogenetic Analysis of the Partial groEL Gene

The study determined the selected partial sequences of the groEL gene and V3–V4 region of the 16S rRNA gene of 120 bifidobacterial strains from 54 species (subspecies) (Table S1). The results by BLAST revealed that the lowest value of the pairwise similarities of the partial groEL gene is 74.9% (B. commune R-52791 and B. animalis subsp. lactis BB12) and the average pairwise similarities value is 84.9%. We used the MEGA software (Version 5.1) to align the selected partial groEL gene and V3–V4 region sequences determined in this study [41]. As shown in Figure 2, a ML analysis of groEL gene sequences from the bifidobacterial strains conducted in the phylogenetic dendrogram revealed that the Bifidobacterium species were grouped into six clusters. Moreover, the closely related Bifidobacterium species (i.e., subspecies) fell into the same clusters, whereas different Bifidobacterium species were categorized into different clusters. Specifically, the closely related taxa such as members of B. longum subsp. longum, B. longum subsp. infantis and B. longum subsp. suis, as well as B. animalis subsp. animalis and B. animalis subsp. lactis can be distinguished on the basis of the selected partial groEL gene, confirming that the partial groEL gene possessed the high taxonomic and phylogenetic resolution for identification and differentiation of the Bifidobacterium species. However, as shown in Figure S1, the neighbor-joining tree on the basis of the V3–V4 region sequences of the 16S rRNA gene showed that some of the same Bifidobacterium species were grouped into different clusters, demonstrating that the V3–V4 region sequences of the 16S rRNA gene lacked sufficient resolution for distinguishing different Bifidobacterium species.
3.3. Specificity, Accuracy and Sensitivity of the Novel Designed Primer Set

*In silico* PCR through PRIMER-BLAST generates only an amplicon for bifidobacterial genomes, suggesting the bifidobacterial specificity of the primer set. We also performed PCR amplification using genomic DNA extracted from known bacterial species including ten bifidobacterial strains and seven non-bifidobacterial strains. As shown in Figure 3, the results revealed that a PCR amplification product was obtained only when template DNA was extracted from *Bifidobacterium* species, whereas no PCR amplification product was observed when DNA genome from any of the other investigated non-bifidobacterial strains was used as a template. Therefore, the novel designed primer set can specifically differentiate *Bifidobacterium* species from other bacterial species tested in this study.
which corresponds to primer set, and the amplicons were sequenced on the MiSeq Illumina sequencing platform. Figure 4A shows strong correlation of the relative abundances of taxa through comparison between known samples consisting of known DNA amounts of different bifidobacterial species. The genomic DNA was 104 CFU/mL (Figure 4B).

To evaluate the accuracy and sensitivity of the novel designed primer set, we developed artificial samples consisting of known DNA amounts of different bifidobacterial species. The genomic DNA from these Bifidobacterium species served as a template for PCR amplification with the novel designed primer set, and the amplicons were sequenced on the MiSeq Illumina sequencing platform. Figure 4A shows strong correlation of the relative abundances of taxa through comparison between known bifidobacterial composition of the artificial samples and retrieved results through groEL-profiling analysis. Specifically, the minimum DNA amount of detectable bifidobacterial species was 0.05 ng, which corresponds to Bifidobacterium species of 10^4 CFU at concentration. Therefore, the limit of detection (LOD) of the novel designed primer set based on the MiSeq Illumina sequencing platform was 10^4 CFU/mL (Figure 4B).

![Figure 3. Specificity of PCR amplification of the selected partial groEL gene using the novel designed primer set. M, marker; 1, A. odontolyticus, 2, B. uniformis, 3, E. coli, 4, E. faecalis, 5, L. acidophilus, 6, L. plantarum; 7, R. dentocariosa, 8, B. adolescentis; 9, B. animalis subsp. animalis; 10, B. animalis subsp. lactis; 11, B. bifidum; 12, B. breve; 13, B. dentium; 14, B. longum subsp. infantis; 15, B. longum subsp. longum; 16, B. pseudocatenulatum; 17, B. pseudolongum; C, control.

Figure 4. Accuracy and the limit of detection of the novel designed primer set. (A) Relationship between normalized relative abundance predicted of Bifidobacterium species and relative abundance observed through groEL-profiling analysis. (B) The limit of detection (LOD) of the novel designed primer set based on the selected partial groEL gene. CFU: Colony-forming units.

3.4. Comparison of Resolving Power between the Partial groEL Gene and V3–V4 Region of 16S rRNA

To evaluate the efficacy of the groEL-based primer set designed in the study, we sequenced the partial groEL gene and V3–V4 region of 16S rRNA amplicons obtained by PCR amplification using the same genomic DNA from human and rat fecal samples. As shown in Table 2, MiSeq sequencing analysis of feces samples from eight humans generated 136,488 and 181,257 high-quality and classifiable sequences corresponding to the groEL gene and 16S rRNA gene, respectively, and average sequence reads of
the two genes were 17,061 and 22,657 per sample. For eight rat fecal samples, 150,875 and 152,033 high-quality and classifiable sequences were obtained for the groEL gene and 16S rRNA gene, respectively, and average sequence reads of the two genes were 18,859 and 19,004 per sample.

To evaluate the robustness of the designed primer pair in determining the bifidobacterial community composition in complex ecosystems, the bifidobacterial profiles identified were compared using the new designed primer pair Bif-groEL-F/Bif-groEL-R and the bifidobacterial profiles obtained with the primer set 341F/806R as described previously in each case using the same genomic DNA. As shown in Figure 5A,B, when assessing the diversity of bifidobacteria, using the universal primer primer set of the V3–V4 region of 16S rDNA at the genus level.

As shown in Figure 5A,B, when assessing the diversity of bifidobacteria, using the universal primer primer set of the V3–V4 region of 16S rDNA at the genus level. When using the primer pairs Bif-groEL-F/Bif-groEL-R, we amplified a region of the groEL gene from Bifidobacterium species, and the results revealed that almost all the sequences could be assigned to the genus Bifidobacterium. When using the primer pairs Bif-groEL-F/Bif-groEL-R, we amplified a region of the groEL gene from Bifidobacterium species, and the results revealed that almost all the sequences could be assigned to the genus Bifidobacterium (Figure 5C,D). Furthermore, the primer pairs targeted to the partial groEL gene could identify bifidobacteria at the species level, in contrast to the universal primer set of the V3–V4 region of 16S rDNA at the genus level.

| Sample ID | Sequence Number a (16S) | OTU Number b (16S) | Sequence Number (groEL) | OTU Number (groEL) |
|-----------|-------------------------|---------------------|--------------------------|---------------------|
| H1        | 8774                    | 3187                | 8583                     | 2044                |
| H2        | 29297                   | 1566                | 20519                    | 3050                |
| H3        | 26457                   | 1511                | 23778                    | 2755                |
| H4        | 28524                   | 2632                | 18206                    | 3176                |
| H5        | 18197                   | 970                 | 15605                    | 2536                |
| H6        | 19625                   | 2045                | 15297                    | 3943                |
| H7        | 13352                   | 1137                | 14950                    | 3457                |
| H8        | 37031                   | 3429                | 19550                    | 4349                |

Table 2. Overview of sequencing results for each sample.
Table 2. Cont.

| Sample ID | Sequence Number a (16S) | OTU Number b (16S) | Sequence Number (groEL) | OTU Number (groEL) |
|-----------|-------------------------|--------------------|-------------------------|--------------------|
| R1        | 13986                   | 1351               | 9341                    | 736                |
| R2        | 9721                    | 1279               | 9385                    | 815                |
| R3        | 13187                   | 1462               | 34297                   | 1665               |
| R4        | 16230                   | 1404               | 27903                   | 1545               |
| R5        | 43429                   | 936                | 17599                   | 967                |
| R6        | 19969                   | 596                | 19369                   | 1060               |
| R7        | 18249                   | 723                | 19835                   | 1111               |
| R8        | 17262                   | 930                | 13146                   | 870                |

a The sequence number refers to the count of assembled sequences after quality filtering. b The OTU (Operational Taxonomic Units) number is presented for all sequences without rarefaction.

3.5. Comparison of Bifidobacterium Species between Humans and Rats

As shown in Figure 5C,D, 12 Bifidobacterium species are contained in human fecal samples, and nine are contained in rat fecal samples. Notably, the predominant Bifidobacterium species for human gut bacteria were B. pseudocatenulatum and B. longum subsp. longum. The dominate gut bifidobacteria of rats were B. animalis subsp. animalis. Furthermore, when cluster heatmap analysis (Figure 6) was performed to visualize the differences in the composition of Bifidobacterium species from human and rat fecal samples, these Bifidobacterium species from humans and rats formed two distinct blocks on the heatmap. Notably, the relative abundances of B. pseudocatenulatum and B. longum subsp. longum (humans) and B. animalis subsp. animalis (rats) are significantly different among gut bifidobacteria from humans and rats (p < 0.05) (Figure 7).

Figure 6. Relative abundance of Bifidobacterium species was visualized using a heatmap determined using the sequence data obtained from human and rat fecal samples, with a high percentage of species belonging to the genus Bifidobacterium indicated in red and low percentages in blue. Each row on the y-axis represents a Bifidobacterium species, and each column on the x-axis represents a sample.
was similar to the one based on the 16S rRNA gene sequences [3,42]. A significant correlation existed with those from the new method of using the designed primer set on the basis of the MiSeq Illumina sequencing platform. According to the multiple sequence alignment method used in the study, we selected a fragment of about 490 bp for PCR amplification with the designed degenerate primers, and the selected partial groEL nucleotide sequence identities among different Bifidobacterium species ranged from 74.3 to 96.7% (mean 85.0%). In addition, the phylogenetic tree depicted using the selected region of the groEL gene used during the study displayed 98.2%, respectively. In contrast, the selected region of the groEL gene used during the study displayed 94.1% and 98.2%, respectively. In contrast, the selected region of the groEL gene used during the study displayed 98.5% nucleotide sequence similarity between the two organisms in the selected region of the study showed that Bifidobacterium species. * p < 0.05; ** p < 0.01 according to one-way analysis of variance and Duncan’s multiple comparisons test.

3.6. qPCR-Based Determination of Main Bifidobacterial Numbers

To further quantify the main bifidobacterial numbers in human feces, qPCR analysis was carried out in a CFX96 real-time PCR detection system. As shown in Table S2, the results of the main bifidobacterial community composition in human feces from qPCR were in accord with those from the new method of using the designed primer set on the basis of the MiSeq Illumina sequencing platform.

4. Discussion

To identify Bifidobacterium species on the basis of the MiSeq Illumina sequencing platform, the following criteria should be met: (1) The target gene must be ubiquitous in the genus Bifidobacterium; (2) the target gene must have high resolution power; (3) the target gene used for primer binding must include a sequence containing a hypervariable region flanked by two constant regions; (4) the PCR amplification region in the target gene must comprise no more than 500-bp nucleotide sequences; and (5) many sequences of the target gene must be available.

The groEL gene, a single-copy housekeeping gene, is ubiquitous in the genus Bifidobacterium. According to the multiple sequence alignment method used in the study, we selected a fragment of about 490 bp for PCR amplification with the designed degenerate primers, and the selected partial groEL nucleotide sequence identities among different Bifidobacterium species ranged from 74.3 to 96.7% (mean 85.0%). In addition, the phylogenetic tree depicted using the selected region of the groEL gene or even complete groEL gene nucleotide sequences (about 1600 bp) of Bifidobacterium species [18,20,21] was similar to the one based on the 16S rRNA gene sequences [3,42]. A significant correlation existed between the genetic distances of the groEL gene nucleotide sequences and those of the 16S rDNA nucleotide sequences [21]. Furthermore, the 16S rDNA nucleotide sequence identities among all the Bifidobacterium species ranged from 90.6 to 99.9% [42]. Notably, the resolving ability of the 16S rRNA gene is limited among some closely related Bifidobacterium species. For example, B. catenulatum and B. pseudocatenulatum cannot be distinguished because they share 98.5% nucleotide sequence similarity in their 16S rRNA genes sequences [18]. However, the phylogenetic dendrogram delineated in this study showed that B. catenulatum and B. pseudocatenulatum could be easily distinguished because of the 93.9% nucleotide sequence similarity between the two organisms in the selected region of the groEL gene. Moreover, at the subspecies level, B. animalis subsp. animalis and B. animalis subsp. lactis, as well as B. longum subsp. infantis, B. longum subsp. longum and B. longum subsp. suis, share 16S rRNA nucleotide sequence similarities are all above 99% [43]. The selected region of the groEL gene nucleotide
sequence identities between \textit{B. animalis} subsp. \textit{animalis} and \textit{B. animalis} subsp. \textit{lactis}, as well as \textit{B. longum} subsp. \textit{infantis} and \textit{B. longum} subsp. \textit{longum} were 94.1\% and 98.2\%, respectively. In contrast, the selected region of the \textit{groEL} gene used during the study displayed a considerably higher resolving ability between these closely related species in \textit{Bifidobacterium} species than did the 16S rRNA gene. What is more, the reference databases (NCBI, EMBL and Chaperonin Sequence Database) provide ample nucleotide sequences of the \textit{groEL} gene for further sequence alignment. Overall, the selected region of the \textit{groEL} gene fulfils all of the prerequisites to serve as a reliable alternative target marker gene for distinguishing different \textit{Bifidobacterium} species based on the MiSeq Illumina sequencing platform.

To characterize the specificity and LOD of the developed method using the novel designed primer set on the high-throughput sequencing platform, we prepared samples spiked with the known concentrations of \textit{Bifidobacterium} species and other bacteria for sequencing based on the MiSeq Illumina platform. The results demonstrated that the developed method could discriminate different \textit{Bifidobacterium} species, and concentrations of \textit{Bifidobacterium} species as low as $10^4$ CFU/mL could be detected.

To prove the robustness of the novel designed primer set, two kinds of different fecal samples derived from humans and rats were selected for further analysis. Based on the MiSeq Illumina sequencing platform, we identified the \textit{Bifidobacterium} species of both sets of fecal samples. The results showed that the diversity of bifidobacterial composition in human samples is greater than that in rat samples. One possible reason may be that diets varied among different people, whereas diets in different rats did not vary.

It is known that there may be drawbacks to each method. Maybe there is no one gene that can differentiate all \textit{Bifidobacterium} species. One possible limitation of \textit{groEL} gene is that a high similarity of \textit{groEL} gene similarity values between different bifidobacterial species is present. For example, there are high similarity values between \textit{B. thermophilum} and \textit{B. thermacidophilum} subsp. \textit{thermacidophilum}, \textit{B. longum} subsp. \textit{infantis} and \textit{B. longum} subsp. \textit{longum}. Consequently, we should be careful when differentiating these different \textit{Bifidobacterium} species using \textit{groEL} gene. Maybe \textit{groEL} gene combined with more genes like 16S rRNA, \textit{rpoB} and \textit{clpC} are good in differentiating these different bifidobacterial species with a high similarity of \textit{groEL} similarity values [44].

5. Conclusions

In conclusion, a \textit{Bifidobacterium}-specific primer pair that targets a hypervariable region of about 490 bp within the \textit{groEL} gene can specifically differentiate \textit{Bifidobacterium} species from bacterial species, and the LOD of the novel designed primer set based on the MiSeq Illumina sequencing platform was $10^4$ CFU/mL. In addition, the novel designed primer pair can identify bifidobacteria at the species level from human and rat fecal samples on the basis of the MiSeq Illumina sequencing platform. The results demonstrated that the predominant \textit{Bifidobacterium} species for human gut bacteria were \textit{B. pseudocatenulatum} and \textit{B. longum} subsp. \textit{longum}, and the predominant gut bifidobacteria of rats were \textit{B. animalis} subsp. \textit{animalis}.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4425/8/11/336/s1. Figure S1: Phylogenetic tree based on the V3–V4 region sequences of the 16S rRNA gene. The tree was constructed by the neighbor-joining distance method with bootstrap values calculated from 1000 trees, Table S1: List of \textit{Bifidobacterium} strains used for phylogenetic analysis of the selected partial \textit{groEL} gene and the V3–V4 region of the 16S rRNA gene.

Acknowledgments: The work was supported by the Key Program of the National Natural Science Foundation of China (Grant No. 31530056).

Author Contributions: Lujun Hu, Wenwei Lu and Jianxin Zhao conceived and designed the experiments; Lujun Hu and Wenwei Lu performed the experiments; Lujun Hu and Jianxin Zhao analyzed the data; Linlin Wang, Mingluo Pan, Hao Zhang and Wei Chen contributed reagents/materials/analysis tools; and Lujun Hu and Jianxin Zhao wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.
References

1. Arvanitoyannis, I.S.; Van Houwelingen-Koukaliaroglou, M. Functional foods: A survey of health claims, pros and cons, and current legislation. Crit. Rev. Food Sci. Nutr. 2005, 45, 385–404. [CrossRef] [PubMed]

2. Backhed, F.; Ley, R.E.; Sonnenburg, J.L.; Peterson, D.A.; Gordon, J.I. Host-bacterial mutualism in the human intestine. Science 2005, 307, 1915–1920. [CrossRef] [PubMed]

3. Turroni, F.; Marchesi, J.R.; Foroni, E.; Guenimonde, M.; Shanahan, F.; Margolles, A.; van Sinderen, D.; Ventura, M. Microbiomic analysis of the bifidobacterial population in the human distal gut. ISME J. 2009, 3, 745–751. [CrossRef] [PubMed]

4. Fouhy, F.; Guinane, C.M.; Hussey, S.; Wall, R.; Ryan, C.A.; Murphy, B.; Ross, R.P.; Fitzgerald, G.F.; Stanton, C.; et al. High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. Antimicrob. Agents Chemother. 2012, 56, 5811–5820. [CrossRef] [PubMed]

5. Claesson, M.J.; Jeffery, I.B.; Conde, S.; Power, S.E.; O’Connor, E.M.; Cusack, S.; Harris, H.M.B.; Coakley, M.; Lakshminarayanan, B.; O’Sullivan, O.; et al. Gut microbiota composition correlates with diet and health in the elderly. Nature 2012, 488, 178–184. [CrossRef] [PubMed]

6. Milani, C.; Hevia, A.; Foroni, E.; Duranti, S.; Turroni, F.; Lugli, G.A.; Sanchez, B.; Martin, R.; Guenimonde, M.; van Sinderen, D.; et al. Assessing the fecal microbiota: An optimized ion torrent 16S rRNA gene-based analysis protocol. PLoS ONE 2013, 8, e68739. [CrossRef] [PubMed]

7. Mao, B.; Li, D.; Ai, C.; Zhao, J.; Zhang, H.; Chen, W. Lactulose differently modulates the composition of luminal and mucosal microbiota in C57BL/6j mice. J. Agric. Food Chem. 2016, 64, 6240–6247. [CrossRef] [PubMed]

8. Loman, N.J.; Misra, R.V.; Dallman, T.J.; Constantinidou, C.; Gharbia, S.E.; Wain, J.; Palen, M.J. Performance comparison of benchtop high-throughput sequencing platforms. Nat. Biotechnol. 2012, 30, 434–439. [CrossRef] [PubMed]

9. Milani, C.; Lugli, G.A.; Turroni, F.; Mancabelli, L.; Duranti, S.; Viappiani, A.; Mangifesta, M.; Segata, N.; van Sinderen, D.; Ventura, M. Evaluation of bifidobacterial community composition in the human gut by means of a targeted amplicon sequencing (ITS) protocol. FEMS Microbiol. Ecol. 2014, 90, 493–503. [CrossRef] [PubMed]

10. Stewart, F.J.; Cavanaugh, C.M. Intragenomic variation and evolution of the internal transcribed spacer of the rRNA operon in bacteria. J. Mol. Evol. 2007, 65, 44–67. [CrossRef] [PubMed]

11. Crosby, L.D.; Criddle, C.S. Understanding bias in microbial community analysis techniques due to RRN operon copy number heterogeneity. Biotechniques 2003, 34, 790–802. [PubMed]

12. Ventura, M.; Canchaya, C.; van Sinderen, D.; Fitzgerald, G.F.; Zink, R. Bifidobacterium lactis DSM 10140: Identification of the atp (atpBEFHAGDC) operon and analysis of its genetic structure, characteristics, and phylogeny. Appl. Environ. Microbiol. 2004, 70, 3101–3121. [CrossRef] [PubMed]

13. Ventura, M.; Zink, R. Comparative sequence analysis of the tuf and recA genes and restriction fragment length polymorphism of the internal transcribed spacer sequences supply additional tools for discriminating Bifidobacterium lactis from Bifidobacterium animalis. Appl. Environ. Microbiol. 2003, 69, 7517–7522. [CrossRef] [PubMed]

14. Ventura, M.; Canchaya, C.; Meylan, V.; Klaenhammer, T.R.; Zink, R. Analysis, characterization, and loci of the tuf genes in Lactobacillus and Bifidobacterium species and their direct application for species identification. Appl. Environ. Microbiol. 2003, 69, 6908–6922. [CrossRef] [PubMed]

15. Ventura, M.; Zink, R.; Fitzgerald, G.F.; van Sinderen, D. Gene structure and transcriptional organization of the dnaK operon of Bifidobacterium breve UCC 2003 and application of the operon in bifidobacterial tracing. Appl. Environ. Microbiol. 2005, 71, 487–500. [CrossRef] [PubMed]

16. Requena, T.; Burton, J.; Matsuki, T.; Munro, K.; Simon, M.A.; Tanaka, R.; Watanabe, K.; Tannock, G.W. Identification, detection, and enumeration of human Bifidobacterium species by PCR targeting the transaldolase gene. Appl. Environ. Microbiol. 2002, 68, 2420–2427. [CrossRef] [PubMed]

17. Yin, X.; Chambers, J.R.; Barlow, K.; Park, A.S.; Wheatcroft, R. The gene encoding xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (xpf) is conserved among Bifidobacterium species within a more variable region of the genome and both are useful for strain identification. FEMS Microbiol. Lett. 2005, 246, 251–257. [CrossRef] [PubMed]
18. Ventura, M.; Canchaya, C.; Del Casale, A.; Dellaglio, F.; Neviani, E.; Fitzgerald, G.F.; van Sinderen, D. Analysis of bifidobacterial evolution using a multilocus approach. Int. J. Syst. Evol. Microbiol. 2006, 30, 734–759. [CrossRef] [PubMed]

19. Zhu, L.; Li, W.; Dong, X. Species identification of genus Bifidobacterium based on partial HSP60 gene sequences and proposal of Bifidobacterium thermacidophilum subsp. porcinum subsp. nov. Int. J. Syst. Evol. Microbiol. 2003, 53, 1619–1623. [CrossRef] [PubMed]

20. Masco, L.; Ventura, M.; Zink, R.; Huys, G.; Swings, J. Polyphasic taxonomic analysis of Bifidobacterium animalis and Bifidobacterium lactis reveals relatedness at the subspecies level: Reclassification of Bifidobacterium animalis as Bifidobacterium animalis subsp. animalis subsp. nov. and Bifidobacterium lactis as Bifidobacterium animalis subsp. lactis subsp. nov. Int. J. Syst. Evol. Microbiol. 2004, 54, 1137–1143. [PubMed]

21. Ventura, M.; Canchaya, C.; Zink, R.; Fitzgerald, G.F.; van Sinderen, D. Characterization of the groEL and groES loci in Bifidobacterium breve UCC 2003: Genetic, transcriptional, and phylogenetic analyses. Appl. Environ. Microbiol. 2004, 70, 6197–6209. [CrossRef] [PubMed]

22. Junick, J.; Blaut, M. Quantification of human fecal Bifidobacterium species by use of quantitative real-time PCR analysis targeting the groEL gene. Appl. Environ. Microbiol. 2012, 78, 2613–2621. [CrossRef] [PubMed]

23. Hill, J.E.; Penny, S.L.; Crowell, K.G.; Goh, S.H.; Hemmingsen, S.M. cpnDB: A chaperonin sequence database. Genome Res. 2004, 14, 1669–1675. [CrossRef] [PubMed]

24. Schell, M.A.; Karmirantzou, M.; Snel, B.; Berger, B.; Pessi, G.; Zwahlen, M.C.; Desiere, F.; Bork, P.; Delley, M.; et al. The genome sequence of Bifidobacterium longum reflects its adaptation to the human gastrointestinal tract. Proc. Natl. Acad. Sci. USA 2002, 99, 14422–14427. [CrossRef] [PubMed]

25. Garrigues, C.; Johansen, E.; Pedersen, M.B. Complete genome sequence of Bifidobacterium animalis subsp. lactis BB-12, a widely consumed probiotic strain. J. Bacteriol. 2010, 192, 2467–2468. [CrossRef] [PubMed]

26. Zhurina, D.; Zomer, A.; Gleinser, M.; Brancaccio, V.F.; Auchter, M.; Waidmann, M.S.; Westermann, C.; Canchaya, C.; Del Casale, A.; Dellaglio, F.; Neviani, E.; Fitzgerald, G.F.; van Sinderen, D.; van Sinderen, D. Complete genome sequence of Bifidobacterium breve UCC 2003: Genetic, transcriptional, and phylogenetic analyses. Appl. Environ. Microbiol. 2004, 70, 6197–6209. [CrossRef] [PubMed]

27. Ventura, M.; Reniero, R.; Zink, R. Specific identification and targeted characterization of Bifidobacterium lactis from different environmental isolates by a combined multiplex-PCR approach. Appl. Environ. Microbiol. 2001, 67, 2760–2765. [CrossRef] [PubMed]

28. Sambrook, J.; Russell, D.W. Molecular Cloning: A Laboratory Manual, 3rd ed.; Cold Spring Harbor Laboratory Press: New York, NY, USA, 2001.

29. Posada, D. jModelTest: Phylogenetic model averaging. Mol. Biol. Evol. 2008, 25, 1253–1256. [CrossRef] [PubMed]

30. Stamatakis, A.; Hoover, P.; Rougemont, J. A rapid bootstrap algorithm for the RAxML web servers. Syst. Biol. 2008, 57, 758–771. [CrossRef] [PubMed]

31. Chenna, R.; Sugawara, H.; Koike, T.; Lopez, R.; Gibson, T.J.; Higgins, D.G.; Thompson, J.D. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. 2003, 31, 3947–3950. [CrossRef] [PubMed]

32. Ye, J.; Coulouris, G.; Zaretskaya, I.; Cutcutache, I.; Rozen, S.; Madden, T.L. Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics 2012, 13, 134. [CrossRef] [PubMed]

33. Jia, H.R.; Geng, L.L.; Li, Y.H.; Wang, Q.; Diao, Q.Y.; Zhou, T.; Dai, P.L. The effects of Bt CryIe toxin on bacterial diversity in the midgut of Apis mellifera ligustica (Hymenoptera: Apidae). Sci. Rep. 2016, 6, 24664. [CrossRef] [PubMed]

34. Staroslic, A. Calculator for Determining the Number of Copies of a Template. Available online: http://cels.uri.edu/gsc/cndna.html (accessed on 8 February 2017).

35. Caporaso, J.G.; Bittinger, K.; Bushman, F.D.; DeSantis, T.Z.; Andersen, G.L.; Knight, R. PyNAST: A flexible tool for aligning sequences to a template alignment. Bioinformatics 2010, 26, 266–267. [CrossRef] [PubMed]

36. R Development Core Team. R: A Language and Environment for Statistical Computing; R Foundation for Statistical Computing: Vienna, Austria, 2011.
39. Lewisa, Z.T.; Bokulicha, N.A.; Kalanetraa, K.M.; RuizMoyanoa, S.; Underwoodb, M.A.; Mills, D.A. Use of bifidobacterial specific terminal restriction fragment length polymorphisms to complement next generation sequence profiling of infant gut communities. *Anaerobe* 2013, 19, 62–69. [CrossRef] [PubMed]

40. Altschul, S.F.; Madden, T.L.; Schäffer, A.A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 1997, 25, 3389–3402. [CrossRef] [PubMed]

41. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 2011, 28, 2731–2739. [CrossRef] [PubMed]

42. Miyake, T.; Watanabe, K.; Watanabe, T.; Oyaizu, H. Phylogenetic analysis of the genus *Bifidobacterium* and related genera based on 16S rDNA sequences. *Microbiol. Immunol.* 1998, 42, 661–667. [CrossRef] [PubMed]

43. Jian, W.; Zhu, L.; Dong, X. New approach to phylogenetic analysis of the genus *Bifidobacterium* based on partial HSP60 gene sequences. *Int. J. Syst. Evol. Microbiol.* 2001, 51, 1633–1638. [CrossRef] [PubMed]

44. Duranti, S.; Mangifesta, M.; Lugli, G.A.; Turrioni, F.; Anzalone, R.; Milani, C.; Mancabelli, L.; Ossiprandi, M.C.; Ventura, M. *Bifidobacterium vansinderenii* sp. nov., isolated from faeces of emperor tamarin (*Saguinus imperator*). *Int. J. Syst. Evol. Microbiol.* 2017, 67, 3987–3995. [CrossRef] [PubMed]

© 2017 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).