A new method for quantitative detection of Lactobacillus casei based on casx gene and its application

Xiaoyang Pang 1,2, Ziyang Jia 2, Jing Lu 2, Shuwen Zhang 2, Cai Zhang 3, Min Zhang 1* and Jiaping Lv 2*

Abstract

Background: The traditional method of bacterial identification based on 16S rRNA is a widely used and very effective detection method, but this method still has some deficiencies, especially in the identification of closely related strains. A high homology with little differences is mostly observed in the 16S sequence of closely related bacteria, which results in difficulty to distinguish them by 16S rRNA-based detection method. In order to develop a rapid and accurate method of bacterial identification, we studied the possibility of identifying bacteria with other characteristic fragments without the use of 16S rRNA as detection targets.

Results: We analyzed the potential of using cas (CRISPR-associated proteins) gene as a target for bacteria detection. We found that certain fragment located in the casx gene was species-specific and could be used as a specific target gene. Based on these fragments, we established a TaqMan MGB Real-time PCR method for detecting bacteria. We found that the method used in this study had the advantages of high sensitivity and good specificity.

Conclusions: The casx gene-based method of bacterial identification could be used as a supplement to the conventional 16s rRNA-based detection method. This method has an advantage over the 16s rRNA-based detection method in distinguishing the genetic relationship between closely-related bacteria, such as subgroup bacteria, and can be used as a supplement to the 16s rRNA-based detection method.

Keywords: TaqMan MGB RT-PCR, Lactobacillus casei, Cas, Rapid detection method, 16s rRNA

Background

In recent years, many studies have confirmed that intestinal flora is associated with a variety of nutritional and metabolic diseases such as obesity [1, 2] and type 2 diabetes [3, 4]. In the field of scientific research, the study of intestinal flora has become of utmost importance. Billions of bacteria in the intestine live in symbiosis with each other for the host’s nutritional and metabolic needs [5, 6]. The intestinal flora of the host have a close relationship with the storage and absorption of nutrition [7, 8], immunity [9], as well as the regulation of sRNA regulation [10]. Through their genes, intermediates and metabolites, these florae affect the host’s nutritional absorption, metabolism, weight, immunity, and several other aspects [11, 12]. Once the balance of intestinal flora is disrupted, a variety of nutritional and metabolic symptoms appears in the host [13, 14]. Although intestinal florae have been shown to be associated with many metabolic diseases, a lot of work still needs to be done in order to establish the differences between related and casual diseases.

Current research on intestinal flora are mostly based on Illumina’s high-throughput sequencing technology; which has the advantages of high throughput, short time, and low cost [15, 16]. However, its low resolution characteristic is a big drawback, and most bacteria can be identified only at genus level. Consequently, at the species level only a few bacteria can be identified using the technology, with an inability to distinguish intestinal flora among sub-species or strains. In fact, the roles of different species of the same genus in a host are remarkably different. For instance, studies have shown that...
different species of the same genus or family exhibit variations to increase or decrease during weight gain in high-fat-fed animals [17, 18]. Obviously, their relationships with the development of obesity cannot be fully elucidated. It is presumed that, while some of the intestinal bacteria are related to obesity, others are not. This suggests that it is necessary to establish a more suitable method with higher resolution to study the relationship between intestinal flora and their hosts. The search for specific gene fragments from the target bacterial genome, and the development of a corresponding detection method, could be the key factor to solve this fundamental problem.

The common strategy for the search of specific fragments of bacteria involves the analysis of bacterial 16s rRNA sequencing, and then find the specific fragments from its variable area [19, 20]; However using this method, it is sometimes difficult to distinguish closely related bacteria such as L. casei and L. rhamnosus, because of the 99% similarity in the 16s rRNA whole sequences (1540/1558). Due to the fact that a specific bacteria fragment from the 16s rRNA sequence is difficult to find, it is necessary to search for new characteristic fragments from other areas of the bacterial genome. In this study, we found that some CRISPR-associated proteins (Cas) are strain-specific and could be used as target gene fragments for the identification of strains. The bacterial identification based on casx gene, could be used as an supplement to the conventional method based on 16s rRNA.

CRISPR is a special-function DNA sequence that widely exists in bacteria and archaea genomes [21, 22]. The sequence covers one leader, multiple short and highly conserved repeats, as well as multiple spacers. CRISPR is considered to be the bacteria’s immune system [23, 24]. After the bacteria are infected by a virus, the surviving bacteria can capture a characteristic DNA fragment from the virus and then integrate it into their genome CRISPR area. At a subsequent viral invasion, the bacteria can quickly identify them according to the CRISPR archive area and then activate the endonuclease to cut the invading virus; equivalently acting as immunity to the virus. Each time a new virus is encountered, the bacteria can capture its characteristic DNA fragments and insert them into their own CRISPR area. The above functions of bacteria are performed by a series of CAS proteins. Although some cas genes (such as the widely known cas9 gene), have great similarities in sequences among different bacteria, several others have low similarities. We selected all the cas genes annotated on the genome of Lactobacillus casei and then aligned them with their corresponding genes of ten Lactobacillus strains. The results showed that a casx gene in the flanking sequence of CRISPR had lower similarity with other Lactobacillus species. Primers and probes for fluorescence quantitative polymerase chain reaction (qPCR) were designed according to the casx gene. Furthermore, the results also showed that L. casei from other intestinal microbes could be accurately distinguished with high sensitivity and reproducibility using this method. In this study, the bacteria from a large microbial flora were accurately identified and their abundance detected using fluorescence qPCR assay based on the casx gene of L. casei. The method is high sensitivity and repeatable. This study established the foundation for the study of the relationships between intestinal microbes and their host via species or subspecies.

Results

The acquisition of Lactobacillus casei specific gene fragments

The CRISPR sequences obtained from this study are shown in Table 1. We compared the CRISPR flanking sequence of L. casei with other strains of Lactobacillus, and found that one casx gene had a conserved region of ~270 bp (Fig. 1). The two L. casei strains in this region had an identical gene sequence (L. casei w56: 2325395–2,325,664; L. casei BL23: 2328749–2,329,018), and was quite different from other Lactobacillus species. Although L. rhamnosus is closely related to L. casei, the casx gene of L. rhamnosus is different from that of L. casei. Therefore, this region could be used as a candidate target gene for the detection of L. casei. In order to verify the specificity of this gene, the 270-bp casx gene fragment was obtained by Blast in the Genbank database. The results showed that the fragment had high similarity with the sequence of the six strains in the genome and Genbank database, and all six strains were L. casei, indicating the species specificity of the sequence.

Fragment-specific validation results

According to the specific fragment in this study, the primers for fluorescence qPCR were designed and named as 06232F and 06232R, while the probe match for the primers was designed and named as 06232P. The probe was linked to a luminophore FAM on the 5’ end and a quencher MGB-NFQ on the 3’ end. The details of the primers and probes are presented in Table 2.

The genetic relationship of 19 Lactobacillus strains was analyzed. The results show that L. casei was closely related to L. brevis, L. plantarum, L. curvatus, L. coryniformis, and L. rhamnosus (Fig. 2). Therefore, six strains of Lactobacillus (L. casei SY13, L. plantarum M15, L. curvatus znj160802, L. coryniformis znj160401, L. rhamnosus YL4, and L. brevis znj160202) were selected and their genomes extracted. The genomes of the six strains were amplified by PCR with 06232F and 06232R primers. As a result, the target fragment of about 90 bp was obtained from L. casei SY13 genome and no target fragment was obtained from the genomes of other bacteria (Fig. 3). This indicated that the specificity of the primers was good.
| Strain                                      | Genbank No. | DR consensus | Position     |
|--------------------------------------------|-------------|--------------|--------------|
| Lactobacillus acidophilus GCF_000934625   | NZ_CP010432.1 | GGATCACCTCCACATACGTGGAGAAAAT | 1,541,318–1,543,298 |
| Lactobacillus acidophilus NCFM            | NC_006814   | GGATCACCTCCACATACGTGGAGAAAAT | 1,541,039–1,543,019 |
| Lactobacillus backii GCF_001663655       | NZ_CP014623  | GATCATTTTACGTGAAACTAACTGACGTTAACACG | 844,462–846,673 |
| Lactobacillus brevis KB290                | NC_020819   | GTATCTCCCACTATGGGAGGGGATGATCC | 1,071,990–1,072,505 |
| Lactobacillus casei W56                   | NC_018641   | GCTCTGGAACAGTTGATCGATCTACATCGGACAG | 2,323,692–2,325,115 |
| Lactobacillus casei BL23                  | NC_010999   | GCTCTGGAACAGTTGATCGATCTACATCGGACAG | 2,327,048–2,328,469 |
| Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842 | NC_008054 | GTATCTCCCACTATGGGAGGGGATGATCC | 764,071–766,562 |
| Lactobacillus fermentum F-6               | NC_021235   | GGATCACCCCATATACATGGGAGGACAC | 1,348,092–1,352,667 |
| Lactobacillus helveticus GCF_0001006025  | NZ_CP011386  | GATCATTTTACGTGAAACTAACTGACGTTAACACG | 1,344,308–1,346,001 |
| Lactobacillus plantarum ZJ316             | NC_020229   | GCTCTGGAACAGTTGATCGATCTACATCGGACAG | 359,930–360,361 |
| Lactobacillus rhamnosus GG                | NC_013198   | GCTCTGGAACAGTTGATCGATCTACATCGGACAG | 2,265,855–2,267,474 |
| Lactobacillus salivarius CECT 5713        | NC_017481   | GGATCACCCCATATACATGGGAGGACAC | 121,320–123,253 |

**Fig. 1** The alignment result of the Lactobacillus CRISPR flanking sequence.
Establishment of the fluorescent qPCR detection method

A 93 bp DNA fragment was amplified from the genome of \textit{L. casei} SY13 using the 06232F and 06232R primers. The fragment was ligated with pMD19T plasmid and transformed into \textit{E. coli} DH5α. Positive clones were screened on lysogeny broth plates which contained 50.0 \(\mu\)g/mL ampicillin, extracted and correctly sequenced. The target DNA of standard substance pMD19T-CS was assayed to determine its concentration using Spark 20 M Multiscan Spectrum. The concentration of pMD19T-CS was 30.05 ng/\(\mu\)L and the unit was converted to copies/\(\mu\)L according to formula:

\[
\text{plasmid concentration (copies/\(\mu\)L)} = \frac{\text{plasmid concentration (ng/\(\mu\)L)} \times 6.02 \times 10^{23}}{\text{pMD19T-CS length (bp)} \times 660 \text{g/mol}}
\]

The DNA standard was diluted from \(10^3\) to \(10^8\) copies/\(\mu\)L and used to generate the standard curve (Fig. 4). The regression equation was:

\[
Y = -3.53\lg C + 45.28
\]

Where \(R^2 = 0.998\), \(Y\) represents \(C_T\), while \(C\) represents the concentration of standard DNA. The efficiency of amplification was 92.011\% and the detection limit was \(10^2\) copies/\(\mu\)L.

Analysis of mice experiment

Balb/c mice were fed with \textit{L. casei} SY13 for 7d, and then sacrificed at the end of the feeding trial. The content of the different parts of their intestines were analyzed to quantify \textit{L. casei} SY13. The results showed that the target bacteria were not found in the intestinal tract of the negative control group, which implied that there were no endogenous \textit{L. casei} in the intestines of the mice. However, the target bacteria were detected in the experimental group, and the highest numbers were found in the cecum. Interestingly, the target bacteria were not detected in the ileum (Table 3). The quantities of \textit{L. casei} subsp. \textit{casei} SY13 in the jejunum, cecum, and colon were \(1.6 \times 10^5\) copies/g, \(2.1 \times 10^6\) copies/g, and \(1.7 \times 10^6\) copies/g respectively. The results indicated that the fluorescence qPCR method based on the \textit{casx} gene could specifically detect \textit{L. casei} from the intestinal microbial flora of mice.

Discussion

The effort to search for the specific gene fragments of bacteria had plagued the researchers of environmental microbiology for a long time. In the past, the conventional strategy was to search the 16 s rRNA sequence and then select the conserved region sequence as the

| Table 2 Amplification primers and Taqman–MGB probes used for specific detection |
|-----------------|------------------|--------|
| Primers or probes | Nucleotide sequence (5′ → 3′) | Amplicon size |
| Primers | 06232F TCAACCGTGACTGGCAAGT | 91 |
| 06232R AGCGGCTTGTCGAACTGA | |
| M13–47 CGCCAGGGTTTTCCAGTCACGAC | 247 |
| M13–48 AGCGGATAACAATTTCCACACAGGA | |
| Probes | 06232P FAM-CTCAAAAAATGGATCTTGG-MGB-NFQ | |

Fig. 2 Phylogenetic analysis on 16s rRNA of 19 \textit{Lactobacillus} strains

Lactobacillus buchneri JCM1115
Lactobacillus hilgardii JCM 1155
Lactobacillus fructivorans NBRC 13954
Lactobacillus brevis ATCC 14869
Lactobacillus plantarum C
Lactobacillus rhamnosus Bacterium XV
Lactobacillus curvatus LC-20
Lactobacillus casei DSM 20011
Lactobacillus rhamnosus strain JCM 1136
Lactobacillus salivarius i24
Lactobacillus fermentum B39
Lactobacillus viridescens
Lactobacillus acidophilus N02-6
Lactobacillus helveticus IMAU05040
Lactobacillus jensenii ATCC25258
Lactobacillus delbrueckii subsp lactis strain BH1525
Lactobacillus delbrueckii subsp bulgaricus LG1
Lactobacillus delbrueckii strain UVF H2b20
Lactobacillus leichmannii LMG 13136

0.08 0.09 0.1 0.11 0.12 0.13 0.14 0.15 0.16 0.17 0.18 0.19 0.2 0.21 0.22 0.23 0.24 0.25 0.26 0.27 0.28 0.29 0.3 0.31 0.32 0.33 0.34 0.35 0.36 0.37 0.38 0.39 0.4 0.41 0.42 0.43 0.44 0.45 0.46 0.47 0.48 0.49 0.5 0.51 0.52 0.53 0.54 0.55 0.56 0.57 0.58 0.59 0.6 0.61 0.62 0.63 0.64 0.65 0.66 0.67 0.68 0.69 0.7 0.71 0.72 0.73 0.74 0.75 0.76 0.77 0.78 0.79 0.8 0.81 0.82 0.83 0.84 0.85 0.86 0.87 0.88 0.89 0.9 0.91 0.92 0.93 0.94 0.95 0.96 0.97 0.98 0.99 1.0
target fragment for detection [25–27]. However, this method is insufficient to distinguish closely related bacteria. The current microbiome technique involves the use of high-throughput sequencing technology based on the V3–V4 region of the 16 s rRNA of the bacteria, to distinguish the different bacteria in the sample [28]. Although this method identified most of the microorganisms in the sample, however it cannot distinguish bacteria that are closely related in the same genus [29].

In order to identify bacteria more quickly and accurately, many researchers had explored several other options. For instance, multiplex PCR was used to detect L. casei [30] in such a way that two sets of primers were designed to ensure the specificity of L. casei ATCC 393 in the multiplex PCR system. However, this method was not able to quantify L. casei ATCC 393; thus it is usually combined with conventional microbiological cultivation. It is usually a difficult and labor-intensive procedure. FISH probe and hybridization were also used to detect Lactobacillus. Slides were made from intestinal-tract samples and examined using an Olympus BH2 epifluorescence microscope [31]. Without precision, the researchers visually recognized only the number of Lactobacillus that adhered to the intestinal tract. Thus, the development of a simple, highly efficient, and highly specific method was urgently required. We used the casx gene of L. casei and developed a method for the rapid and accurate detection of L. casei by fluorescence qPCR. The core of this method is to find the appropriate casx gene fragment on the flank of CRISPR. In order to verify the general applicability of this method, we tested it on Legionella pneumophila. L. pneumophila includes two main subspecies, one is L. pneumophila subsp. fraseri, the other is L. pneumophila subsp. pneumophila. Traditional methods based on 16S rRNA are difficult to distinguish these two kinds of bacteria. First of all, we searched for CRISPR region in the whole genome of L. pneumophila subsp. fraseri GCF_001886795 and L.pneumophila subsp. pneumophila GCF_001592705 respectively. The results are shown in Additional file 1: Table S1. Then, 3000 bp was taken from CRISPR’s flank as candidate sequence, the extracted sequences are shown in Additional file 2: Table S2. Using Clustal X 2.0 to align the extracted flank sequence. Based on the sequence alignment results, a 256 bp specific sequence was found on L.pneumophila subsp. pneumophila GCF_001592705, which only existed in L.pneumophila subsp. pneumophila GCF_001592705 genome, but not in L. pneumophila subsp. fraseri GCF_001886795, the sequence information is shown in Additional file 3: Table S3. In order to verify the specificity of the sequence, we used blast tool to align the 256 bp
sequence in GenBank, the results are shown in Additional file 4: Figure S1. It can be seen from the results that the 256 bp fragment has good specificity and can distinguish *L. pneumophila* subsp. *pneumophila* from *L. pneumophila* subsp. *fraseri*. The above case in *L. pneumophila* can prove that the method provided in this study is not only applicable to *Lactobacillus casei*, but also applicable to other bacteria.

**Conclusions**

In this study, we used the *casx* gene of *L. casei* and developed a method for the rapid and accurate detection of *L. casei* by fluorescence qPCR. *L. casei* and *L. rhamnosus* were easily distinguished with the use of this method. There is an extremely high similarity between the two bacteria in 16 s rRNA sequences, therefore, it is difficult to distinguish them from each other based on the 16 s rRNA method. The *casx* gene-based method of identification developed in this study was able to rapidly and accurately distinguish the two bacteria. Finally, we validated the accuracy and sensitivity of the method using mouse experiments. This method has an advantage over the 16 s rRNA-based detection method in distinguishing the genetic relationship between closely-related bacteria, such as subgroup bacteria, and can be used as a supplement to the 16 s rRNA-based detection method.

### Methods

**Bacteria strains, plasmids, and mice**

The bacteria and plasmids used in this study are shown in Table 4. *Lactobacillus* strains were statically cultured in MRS broth (Cat. No. CM187, Beijing Land Bridge Technology Co., Ltd., China) at 37 °C. *Escherichia coli* DH5α was grown at 37 °C in LB broth (1.0% peptone, 0.5% yeast extract powder, 1.0% NaCl; pH 7.4), SPF BALB/c mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China).

**Acquisition and alignment of cas sequences of Lactobacillus**

The CRISPR sequence of *Lactobacillus* was derived from the CRISPR database (http://crispr.i2bc.paris-saclay.fr). Due to the fact that some *cas* genes are not annotated in the genome of *Lactobacillus*, the CRISPR flank 3000 bp was selected as the analysis sequence to prevent the loss of some key *cas* genes. ClustalX 2.0 was used for the alignment of sequence.

**Design of primers and TaqMan-MGB probes**

According to the alignment results, we searched for the characteristic fragment of *L. casei*. The primers and TaqMan-MGB probe to detect *L. casei* were designed by Primer Express 3.0 based on the characteristic fragment. The syntheses of primers and probes were entrusted to the Beijing Genomics Institute (BGI). In this study, the specificity of the characteristic fragment was verified from two procedures. Firstly, the characteristic fragment was subjected to Blast in Genbank to examine whether the sequence matched the bacteria other than *L. casei*. Secondly, 19 *Lactobacillus* strains were used to reconstruct a phylogenetic tree based on their 16S rRNA sequences.
nucleotide sequences using MEGA 6.0. The five closely related strains to L. casei were selected to verify the specificity of the primers.

Establishment of the fluorescent qPCR detection method
Bacterial genome DNA was extracted and purified using a nucleic acid extraction kit (Cat. No. DP302, TIANGEN Biotech, Beijing, China) according to the manufacturer’s instructions. Genome DNA was extracted from the intestinal contents and purified with the TIANamp Stool DNA Kit (Cat. No. DP328, TIANGEN Biotech, Beijing, China) according to the manufacturer’s instructions.

PCR was performed to obtain target DNA fragments using primers 06232F and 06232R. Target DNA fragments were recovered from agarose gels using the TIANgel Gel Purification Kit (Cat. No. DP209, TIANGEN Biotech, Beijing, China). The target fragment was then inserted into the pMD19T vector (Cat. No. 6013, Takara Biomedical Technology (Beijing) Co., Ltd, Beijing, China). The ligation products were transformed into DH5α competent cells using the heat shock method. Positive clones were chosen and inoculated into the LB broth containing 100 μg/mL ampicillin and then cultured overnight in an incubator at 37 °C and 200 rpm. The TIANprep Plasmid Kit (Cat. No. DP103, TIANGEN Biotech, Beijing, China) was used to extract plasmids from the cultured bacteria suspension. The extracted plasmids were sequenced to verify the inserted fragments. Sequencing was entrusted to BGI. Sequencing validates the correct transformants as the DNA standard. The concentration of the standard DNA was detected on Spark 20 M Multiscan Spectrum (Tecan Group Ltd., Switzerland).

The DNA standard was used to generate a standard curve and analyze assay sensitivity. The DNA diluted from 10² to 10⁶ copies/μL was used as a template to perform RT-PCR. A TaqMan-MGB probe was used to detect the Ct value. A standard curve between the Ct value, dilution gradient and linear regression equation was generated automatically by ABI 7500 Real-Time PCR System. The coefficient of determination (R²) value was also demonstrated. The DNA standard diluted from 10⁶ to 10⁴ copies/μL was tested to observe the detection limit.

Mice experiment
After the Balb/c mice were treated with L. casei for 7.0 d, we collected the cecum and colon contents and extracted the genomic DNA, to further test the specificity of the primers and probes in the intestinal flora. The number of L. casei in different parts of the intestinal tract was measured by fluorescence qPCR using the extracted genomic DNA as templates. The mice fed without L. casei were used as negative control. Six male mice were used in this experiment. They were randomly divided into two groups; three in each cage. They were left for 7.0 d to adapt to their environment, and water and basal diets were freely given. At the onset of the treatment, the experimental group was gavage-induced with 10⁹ cfu L. casei SY13, while the negative group was administered sterile water. Mice were sacrificed 7 days after treatment. Carbon dioxide method was used to euthanize mice. The methods were referenced to previously published literature [32]. The sacrificed mice were dissected; the jejunum, ileum, cecum, and colon were extracted and preserved in liquid nitrogen. The genome DNA of the intestinal contents was also extracted. The quantity of the target bacteria was measured by RT-PCR. Data analysis was conducted using SPSS 20.0 (IBM Corporation, Armonk, NY, USA).

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12896-019-0587-6.

Additional file 1: Table S1. CRISPR region of Legionella pneumophila.
Additional file 2: Table S2. Flanking sequence of CRISPR region.
Additional file 3: Table S3. Specific sequence selected from sequence alignment results.
Additional file 4: Figure S1. The result of sequence BLAST.

Abbreviations
Cas proteins: CRISPR-associated proteins; QPCR: Quantitative Polymerase Chain Reaction; rRNA: Ribosomal RNA; RT-PCR: Realtime Polymerase Chain Reaction

Acknowledgments
We thank China Agricultural University Laboratory Animal Service Center for its help with the animal experiments. We also showed our thanks to the anonymous reviewers for their helpful comments on the manuscript.

Authors’ contributions
XP, MZ, JLv designed all the experiments; JLu, SZ, CZ performed the experiments. SZ, CZ analyzed the data. XP and ZJ wrote the paper. All authors read and approved the final manuscript.

Funding
This study was supported by National Key R&D Program of China (2017YFC1600903) and National Natural Science Foundation of China (31871833). These funds were used for collection of materials, analysis data, the interpretation and the writing/publication of the manuscript.

Availability of data and materials
The datasets used and analyzed for the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate
Mice experiments were conducted in the China Agricultural University Laboratory Animal Service Center and were approved by the animal ethics committee of the university (No. CAU20161020-3, 20/10/2016). All the experiment processes were done under the supervision of the China Agricultural University Laboratory Animal Ethics Committee.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.
Author details
1Beijing Advanced Innovation Center for Food Nutrition and Human Health, Beijing Technology & Business University (BTBU), Beijing 100048, China.
2Institute of Food Science and Technology, Chinese Academy of Agricultural Science, Beijing 100193, China.
3Laboratory of Environment and Livestock Products, Henan University of Science and Technology, Luoyang 471023, China.

Received: 10 July 2019 Accepted: 15 November 2019

Published online: 10 December 2019

References
1. Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, Bindels LB, Guiot Y, Derrien M, Mucilli GC, Delbenne NW, et al. Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. P Natl Acad Sci USA. 2013;110(22):9066–71.
2. Bleau C, Karels AD, St-Pierre DH, Lamontagne L. Crosstalk between intestinal microbiota, adipose tissue and skeletal muscle as an early event in systemic low-grade inflammation and the development of obesity and diabetes. Diabetes Metab Res Rev. 2017;33(6):545–61.
3. Sabatino A, Regolisti G, Cosola C, Gesualdo L, Fiaccadori E. Intestinal microbiota in type 2 diabetes and chronic kidney disease. Curr Diab Rep. 2017;17(3):16.
4. Wu T, Trahar LG, Little TJ, Bound MJ, Zhang X, Wu H, Sun Z, Horowitz M, Rayner CK, Jones KL. Effects of Vildagliptin and metformin on blood pressure and heart rate responses to small intestinal glucose in type 2 diabetes. Diabetes Care. 2017;40(5):702–5.
5. Gaiy YP, Chuang TW, Liao CW, Lee VL, Akivale OP, Orok A, Ajibaye O, Babasola AJ, Cheng PC, Chou CM, et al. Intestinal parasitic infections: current status and associated risk factors among school aged children in an archetypal African urban slum in Nigeria. J Microbiol Immunol Infect. 2019;52(1):106–113.
6. Marcial-Coba MS, Marshall JP, Schreiber L, Nielson DS. High-Quality Draft Genome Sequence of Lactobacillus casei strain 211, Isolated from a Human Adult Intestinal Bypass Sample. Genome Announc. 2017;5(8):e00634–17.
7. Volynets V, Kuper MA, Strahl S, Maier IB, Spruss A, Wagnerberger S, Konigsrainer A, Bischoff SC, Bergheim I. Nutrition, intestinal permeability, and blood ethanol levels are altered in patients with nonalcoholic fatty liver disease (NASH). Dig Dis Sci. 2012;57(7):1932–41.
8. Kelly P. Nutrition, intestinal defence and the microbiome. Proc Nutr Soc. 2010;69(2):261–8.
9. Che C, Pang X, Hua X, Zhang B, Shen J, Zhu J, Wei H, Sun L, Chen P, Cui L, et al. Effects of human fecal flora on intestinal morphology and mucosal immunity in human flora-associated piglet. Scand J Immunol. 2009;69(3):223–33.
10. Sesto N, Koutero M, Cossart P. Bacterial and cellular RNAs at work during Listeria infection. Future Microbiol. 2014;9(9):1025–37.
11. Fernandes PC, Dolinger EJ, Abdallah VO, Resende DS, Gontijo Filho PP, Brito MT. Late onset sepsis and intestinal bacterial colonization in very low birth weight infants receiving long-term parenteral nutrition. Rev Soc Bras Med Trop. 2011;44(4):447–50.
12. Levine A. Exclusive enteral nutrition: clues to the pathogenesis of Crohn’s disease. Nestle Nutr Inst Workshop Ser. 2014;79:131–40.
13. Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Ja W, Perterson S. Host-gut microbiota metabolic interactions. Science. 2012;336(6086):1262–7.
14. Rastall RA, Gibson GR. Recent developments in prebiotics to selectively impact beneficial microbes and promote intestinal health. Curr Opin Biotechnol. 2015;32:42–6.
15. Li Y, Poryok Y, Yan Z, Pan L, Feng Y, Zhao P, Xie Z, Hong L. Characterization of intestinal microorganisms of Hirschsprung’s disease patients with or without Enterocolitis using Illumina-MiSeq high-throughput sequencing. PLoS One. 2016;11(19):e0162079.
16. Moreau MM, Eades SC, Reinemeyer CR, Fugaro MN, Onishi JC. Illumina sequencing of the V4 hypervariable region 16S rRNA gene reveals extensive changes in bacterial communities in the cecum following carbohydrate oral infusion and development of early-stage acute laminitis in the horse. Vet Microbiol. 2014;168(2–4):436–41.
17. Barbosa A, Reiss A, Jackson B, Warren K, Paparini A, Gillespie G, Stokeld D, Irvin P, Ryan U. Prevalence, genetic diversity and potential clinical impact of blood-borne and enteric protozoan parasites in native mammals from northern Australia. Vet Parasitol. 2017;238:94–105.
18. Barrios C, Beaumont M, Pallister T, Villar J, Goodrich JK, Clark A, Pascual J, Ley RE, Spector TD, Bell JT, et al. Gut-microbiota-metabolite Axis in early renal function decline. PLoS One. 2015;10(8):e0134311.
19. Momose Y, Maruyama A, Iwasaki T, Miyamoto Y, Itoh K. 16S rRNA gene sequence-based analysis of costudia related to conversion of gemiflur to the normal state. J Appl Microbiol. 2009;107(6):2086–97.
20. De Hertogh G, Aerssens J, de Hoogt R, Peeters P, Verhaesel P, Van Eyken P, Ectors N, Vermeire S, Rutgeerts P, Coulie B, et al. Validation of 16S rDNA sequencing in microdissected bowel biopsies from Crohn’s disease patients to assess bacterial flora diversity. J Pathol. 2006;204(4):532–9.
21. Beneke T, Madden R, Makin L, Vail J, Sunter J, Gluecz E A. CRISPR Cas9 high-throughput genome editing toolkit for kinetoplastids. R Soc Open Sci. 2017;4(5):170095.
22. Comfort N. Genome editing: That’s the way the CRISPR crumbles. Nature. 2017;546(7656):30–1.
23. Hryhorowicz M, Lipinski D, Zeyland J, Slomsk M. CRISPR/Cas9 immune system as a tool for genome engineering. Arch Immunol Ther Exp. 2017;65(3):232–40.
24. Rath D, Amlinger L, Rath A, Lundgren M. The CRISPR-Cas immune system: biology, mechanisms and applications. Biochimie. 2015;117:119–28.
25. Overmann J, Coolen MJ, Tuschak C. Specific detection of different phylogenetic groups of chemocline bacteria based on PCR and denaturing gradient gel electrophoresis of 16S rRNA gene fragments. Arch Microbiol. 1999;172(2):89–94.
26. Zhan XY, Hu CH, Zhu QY. Targeting single-nucleotide polymorphisms in the 16S rRNA gene to detect and differentiate legionella pneumophila and non-legionella pneumophila species. Arch Microbiol. 2016;198(6):591–4.
27. Ravasi D, Peduzzi S, Guidi V, Peduzzi R, Wirth SB, Gilli A, Tonolla M. Development of a real-time PCR method for the detection of fells 16S rDNA fragments of phototrophic sulfur bacteria in the sediments of Lake Cadagno. Geobiology. 2012;10(3):196–204.
28. Parikh HI, Koparde VN, Bradley SP, Buck GA, Sheh NU. MeFiT: merging and filtering tool for illumina paired-end reads for 16S rRNA amplicon sequencing. BMC Bioinformatics. 2016;17(1):491.
29. Hua G, Cheng Y, Kong J, Li M, Zhao Z. High-throughput sequencing analysis of bacterial community spatiotemporal distribution in response to clogging in vertical flow constructed wetlands. Bioresour Technol. 2018;248:104–112.
30. Karapetas A, Vavoulidis E, Galanis A, Sandaltzopolous R, Koukoutsou Y. Rapid detection and identification of probiotic Lactobacillus casei as ATCC 393 by multiplex PCR. J Mol Microbiol Biotechnol. 2010;18(3):156–61.
31. Wang B, Li JS, Li QR, Zhang HY, Li N. Isolation of adhesive strains and evaluation of the colonization and immune response by Lactobacillus casei ATCC 393 by published maps and institutional affiliations.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.