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Investigation Of 16S rRNA Gene And Gene Segments For The Determination Of Probiotics

Amar Ćosić
Altijana Hromic Jahjefendic

Faculty of Engineering and Natural Sciences,
International University of Sarajevo,
Hrasnicka Cesta 15, Ilidža 71210 Sarajevo,
Bosnia and Herzegovina
amar_cosic@outlook.com
ahromic@ius.edu.ba

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Abstract

The 16s-rRNA consists of hypervariable regions (V1 – V9) that demonstrate considerable sequence diversity among different bacteria. Species-specific sequences within a given hypervariable region constitute useful targets for diagnostic assays and other scientific investigations. Usually the size of the gene region is 1500 bp, which is large enough to be analyzed using bioinformatic tools and applied for detection. The need to advance the knowledge of the 16s-rRNA gene segments in bacterial strains would allow better understanding and better diagnostic possibilities when dealing with them. This could also be the basis for investigation of pathogenic microorganisms.

1. INTRODUCTION

Today, DNA-based analysis remains a central method in microbiology, used not only to explore microbial diversity but also as a day-to-day method for bacterial identification. Identification methods are conceptually easier to interpret than molecular phylogenetic analyses and are often preferred when the groups are well understood [1]. Reason for this is the vast amount of phenotypic differences abacteria can show on its surface, which leads to hard identification even with the best laboratories and expertise. This shows highimportance and need for better and more precise knowledge about the human microbiome.

Bacteria used in this investigation are classified as probiotics which are live bacteria that have positive impact on the digestive tract and the human gut microbiome[4]. Probiotics can be classified into two main genera, *Lactobacillus* and *Bifidobacterium.* Probiotics as good bacteria have a vast amount of beneficial impact on the human health, such as increase in the food nutrient value and helping with diseases such as irritable bowel syndrome or lactose intolerance [2]–[5].

In this research we investigated 15 different probiotics soys represented in Table 1. These are:*Bacillussubtilis, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus delbruecki ssp.bulgaricus, Lactobacillus casei,Lactobacillus plantarum, Lactobacillus rhamnosus, Lactobacillus helveticus, Lactobacillus salivarius, Lactococcus lactis ssp. Lactis, Streptococcus thermophilus, Lactobacillus sporogenes.*

Table 1: List of bacteria and their nomenclature

Name of the bacteria	International Code of Nomenclature ofBacteria
Bacillus subtilis	PXN 21
Bifidobacterium bifidum	PXN 23
Bifidobacterium breve	PXN 25
Bifidobacterium infantis	PXN 27
Bifidobacterium longum	PXN 30
Lactobacillus acidophilus	PXN 35
Lactobacillus delbruecki ssp.bulgaricus	PXN 39
Lactobacillus casei	PXN 37
Lactobacillus plantarum	PXN 47/CECT 7315/CECT 7316
Lactobacillus rhamnosus	PXN 54
Lactobacillus helveticus	PXN 45
Lactobacillus salivarius	PXN 57
Lactococcus lactis ssp. lactis	PXN 63
Streptococcus thermophilus	PXN 66

2. MATERIALS AND METHODS

Bacteria were provided from multiple suplement pills:Biokult-Oktal Pharm (Croatia), Prolife Capsule-Zeta Farmaceutici Group (Italy), AB-IMMUNO -AB Biotics (Spain), Feminabiane-PiLeje (France). The growth media MRS both broth and agar were provided by Sigma Aldrich (Germany), while Homofermentative-Heterofermentative differential (HHD) agar was made according to L.C.Mcdonald et. al [6]. Every ingredient for this medium was provided by Sigma Aldrich (Germany). DNA isolation kit (EXTRACTME GENOMIC DNA KIT) and Gel-out kit(EXTRACTMEDNA GEL-OUT KIT) were both acquired from Blirt (Poland). Polymerase Chain Rection primers were acquired from Sigma Aldrich (Germany).

Bacterial Growth

Probiotics were grown in the incubator (Innova 42 Incubator Shaker Series, Eppendorf North America, USA), at 37°C for 24h. Handling of the bacteria during the inoculation and enumeration was done in sterile environment under the hood (NÜVE | MN 120). These bacteria were grown in two different mediums. DeMan, Rogosa and Sharpe Agar (MRS) as the main medium for *Lactobacillus.*

HHD agar was mainly used in separation manner. This medium works on the principle of Bromocresol green and the change in the pH that happens when bacteria is making products. Homofermentative will produce only one product, which is usually lactic acid, and it will show a green color in the medium. Heterofermentative will produce more than one product which does not change the color of the medium and it remains blue. [6]

DNA Isolation

Bacterial DNA was isolated using the previously mentioned ExtractMe Genomic DNA, which is a fastbp for the primers which were targeting the whole gene, and the 500 bp and 350 bp for the hypervariable regionand reliable way to get high concentrated DNA from bacteria. Expected purity ratio is between 1.7 and 1.9 for the absorbance on 260/270 nm of wavelength. The overall time for the whole protocol is 40-60 minutes with incubation time included.

Polymerase Chain Reaction

The primers were designed based on previous studies [7][8][9]. Table 2 shows the primer sequence and their reference studies. Amplification target were split into two categories: whole gene where the point was to get as close as possible to 1500 bp which is the length of the 16S-rRNA gene and hypervariable region mainly the V3 and V4 region. PCR conditions were donewiththe thermal cycler (StepOnePlus™ Real-Time PCR System, Germany). They were carried out as following: pre-denaturation at 95°C for 5min followed by 33 cycles each of 95°C for 30 sec, 55°C for 45sec, 72°C for 30 sec and in the end final elongation at 72 °C for 10min.

Table 2. Primer sequence and obtained reference studies

Name	Primer Sequence	References
27F	5'-AGAGTTTGATCCTGGCTCAG-3'	[7]
1492R	5'-CGGTTACCTTGTTACGACTT-3'	[8]
1492R2	5'-CTTGTGCGGGCCCCCGTCAATTC-3'	[7]
BA338F	5'-ACTCC TACGG GAGGC AG-3'	[9]
BA805R	5'-GACTA CCAGG GTATC TAATC C -3'	
BA516F	5'-TGCCA GCAGC CGCGG TAATA C-3'	

3. RESULTS AND DISCUSSION

Probiotics such as *Lactobacillus* and *Bifidobacterium* are lenient when cultivated. They are easy to grow and can sustain for a long time at the temperatures such as 4°C. All these properties showed benefits when applied to a differentiation medium such as HHD agar. With this agar it was possible to successfully separate the bacteria based on the number of products they can produce. From here DNA was isolated with the kit that was described in the section Materials and Methods. DNA purity and concentration was high enough to continue to the PCR step in which different DNAs were successfully amplified. The Figure 1 shows clearly the difference in the band size after amplification. Size of those bands were as expected, 1500 bp and 1000 of the gene.

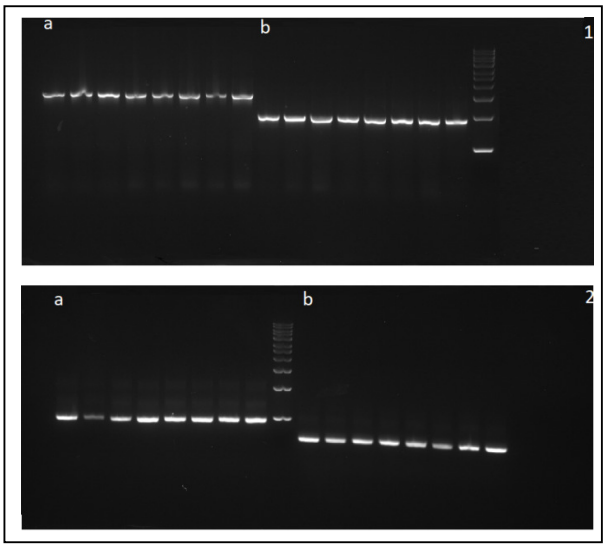

Figure 1: PCR products after amplification. 1a) represents the whole gene segment with the 1500 bp region. 1b) display the 1000 bp amplificon. 2a) represents the 500 bp amplificon while the 2b) depicts the 350 bp region.

4. CONCLUSION

The amplified DNAs after purification can be sent to the sequencing. The sequence will provide more information about the bacteria itself and it will prove that using the HHD medium and universal primers represents a reliable source when it comes to identification of bacteria by combining the PCR and bioinformatic analysis.

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