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Scientific Research

Molecular Verification of Two Potent Bacteria Isolated from Darfiyeh Cheese: *Lactococcus lactis* Subsp. *Lactis* and *Lactobacillus plantarum*

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Abstract

Raw goat milk cheeses are known for their natural microflora linked to many biodiversity factors such as the use of raw milk. That microflora serves probiotic attribution conferring a beneficial health impact on the consumer. Darfiyeh is an artisanal raw goat milk cheese manufactured traditionally in Northern Lebanese Mountains. To emphasize its clinical significance in both digestive and immune system, and to provide health remunerations to the consumer, the cheese microbiota will be investigated. To serve that purpose, the presence of the two potent probiotic Lactic Acid Bacteria (LAB): *Lactococcus lactis* subsp. *lactis* and *Lactobacillus plantarum* will be investigated. For bacterial identification, selection and isolation: culture-dependent techniques that imply the use of laboratory media will be implemented, and culture independent techniques: Polymerase Chain Reaction (PCR) will be applied for further validation. Both bacteria were further verified as *Lactococcus lactis* subsp. *lactis* and *Lactobacillus plantarum* by implementing specie-specific primers for the qualitative PCR.

Keywords

***Lactococcus lactis* Subsp. *Lactis*, *Lactobacillus Plantarum*, Darfiyeh Cheese, Probiotic Effect**

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1. Introduction

Probiotics are receiving huge consumer, research, and industrial interest proving their capacity to enhance food functionality. Food applications for probiotics are vastly present in dairy products. Cheese is one of the most abundant dairy products that have a great capacity for delivering probiotic microorganisms into the human intestine due to its specific physic-chemical characteristics compared to other fermented milk products. Raw goat milk cheeses exist among the variety of cheeses that possess a microbial biodiversity. Among the microorganisms present in raw goat milk cheeses is Lactic Acid Bacteria (LAB) [1]. LAB is well known for their probiotic qualities. When ingested through dietary intake in adequate quantities, LAB as probiotic microorganisms provide health merits to the host.

Methods to identify the bacteria are either culture dependent or culture independent. Both methodologies have their advantages and drawbacks, and should be considered as complimentary to each other. Traditionally, the classification of LAB is based on phenotypical properties, such as their morphology, growth at different temperatures and fermentation of various carbohydrates [2]. However, classical biochemical and physiological tests are not enough for specie typing, because certain species, display similar phenotypic characteristics [2]. Their advantage is that bacteria can be collected for further study or industrial use [3]. Hence the genome-based methods are a must in identifying bacteria due to their discriminatory power [2]. Moreover, methods to identify different bacterial species within the same genera from a cheese matrix is rather challenging due to a lack of divergence between the sequences [3].

Two potent probiotic bacteria *Lactococcus lactis* subsp. *Lactis* and *Lactobacillus plantarum* reside among the microbial matrix of several raw goat milk cheeses. The probiotic effects of *Lactococcus lactis* subsp. *lactis* and *Lactobacillus plantarum* are vast and both are known for their non-pathogenicity and history of safe use [4]-[12].

Lactobacillus plantarum possesses antibacterial activity against common pathogens, antioxidative activity and hypocholesterolemic activity [11]. Another merits of *Lactobacillus plantarum* is its effectiveness in enhancing systemic immunity through increasing the number of B lymphocytes, Natural killer cells and activation of CD4+ and CD8+ T-cells, detected in a dose-response, double blind randomized clinical trial where elderly patients received 5.108 cfu/day of *Lactobacillus plantarum* in a mixture of 20 g of milk [12].

Two potent probiotic bacteria *Lactococcus lactis* subsp. *lactis* and *Lactobacillus plantarum* reside among the microbial matrix of *Darfiyeh* Cheese; a semi-hard raw goat milk cheese, ripened in goat skin and produced by artisanal technology in Northern Lebanese Mountains without any starter addition [13].

So far, information is lacking regarding the beneficial probiotic potency, in both the immune and digestive systems that *Darfiyeh* cheese might provide through *Lactococcus lactis* subsp. *lactis and Lactobacillus plantarum*.

In order to explore the probiotic potency of *Darfyieh* cheese, provided by the residing *Lactococcus lactis* subsp. *Lactis* and *Lactobacillus plantarum*, the steps would be: 1) the isolation and 2) the verification of the two bacteria by culture-dependent and culture-independent methods; 3) the application of real time PCR to reveal the initial bacterial ratios of the two LAB; 4) standardizing the ratios of the two bacteria by understanding and controlling these ratios and 5) the observation of the beneficial health contributions of the cheese in consumers.

The objective of this study was to use a combination of culture-independent and culture-dependent methods for the determination of *Lactococcus lactis* subsp. *lactis* and *Lactobacillus plantarum* in *Darfiyeh* cheese.

2. Materials and Methods

2.1. Collection of Samples

Experimental cheeses were processed at small cheese scale farming in Ehden, north Lebanon Mountains, and ripened in fresh prepared goatskins [13]. After 21 days of ripening, the goatskin was opened through the neck and representative cheeses were selected randomly and taken out.

2.2. Isolation and Biochemical Identification of LAB Isolates

Fractions of whole cheese samples (20 g interior and surface) were homogenized in 180 mL of sterile 2% (w/v) tri-sodium citrate dehydrate solution serially diluted (10^{-1} - 10^{-7}) in a sterile physiological solution and plated onto different media (PCA, MRS, Ellicker) with the intention of selecting different groups of bacteria. Total aerobic mesophilic bacteria grown on PCA medium were incubated at 30˚C for 72 h.

To determine the identity of LAB isolates, colonies were randomly picked from different agar plates, and

each colony was subcultured. The identity of Gram-positive, catalase-negative cocci and rods was determined by performing biochemical and physiological tests. Rod-shaped bacteria were tested for their ability to produce gaz and to grow in MRS broth (Biokar, Beauvais, France) at 15˚C for 7 days and at 45˚C for 2 days. Cocci were tested for their ability to produce gaz, to hydrolyse arginine and to grow in the presence of 6.5% NaCl and 10% of bile, in Elliker broth (Biokar, Beauvais, France) at 10˚C for 10 days, at 45˚C for 48 h. Carbohydrate fermentation patterns were determined using API 50 CH test strips (BioMérieux, Marcy l'Etoile, France), according to the manufacturer's instructions. The pure cultures were stored at −80˚C in M17 or MRS broth supplemented with 15% (w/v) glycerol.

2.3. Nucleic acid Extraction

2.3.1. Samples from Isolated Colonies or Broth Cultures

Bacteria were grown overnight in 10 mL Elliker or MRS broth at their optimal growth temperature. A 1 mL aliquot of each culture was centrifuged at 10,000 g (4˚C, 5 min). The supernatant was discarded and the biomass pellet was suspended in three volumes of freshly prepared lysis solution (10 mg mL^{-1} lysozyme (Sigma, Saint Quentin Fallavier, France)) diluted in Tris-EDTA buffer (Tris-HCl 50 mM, EDTA 10 mM; pH 8) and RNase solution (Sigma, 100 $mg{\cdot}mL^{-1}$), and incubated for 2 h at 30˚C.

DNA was extracted with a kit: Gene Elute TM Bacterial Genomic DNA Kit (Sigma-Aldrich, NA2110), according to the manufacture's protocol. The DNA pelletwas suspended in 30 mL of sterile distilled water and stored at −20˚C [14]. DNA was measured using the Nano drop.

2.3.2. Samples of Darfiyeh Cheeses

Extraction of genomic DNA from cheese samples was carried out [15]. Cheese samples (4 g) were dissolved in 40 mL of sterile 2% (wt/vol) trisodium citrate and homogenized by using a stomacher (Interscience, France). To each sample, 1.6 mL of 20 mg mL^{-1} pronase (Protease from *Streptomyces griseus*, EC.3.424.31, Sigma) and 80 mL of β-mercaptoethanol were added, and this was followed by 3 h of incubation at 37˚C. The bacteria were washed twice by centrifugation at 13,000 g for 10 min. The pellets were suspended first in sterile water and then in 10 mL of T1 solution (1 M sorbitol, 0.1 M EDTA; pH 8). The cells were centrifuged and suspended in 500 mL of T1 solution, transferred into Eppendorf tubes, and cooled for 10 min in ice. The cells were lysed by using glass beads (diameter, 150 - 200 mm; Sigma, Saint Quentin Fallavier, France) in the presence of T1 buffer (six cycles consisting of 30 s of vortexing at high speed and 1 min of storage in ice). After settling, the supernatant was removed and stored for 10 min in ice. DNA was extracted with phenol/chloroform/isoamyl alcohol, precipitated with isopropanol, and washed with ethanol (70%).

2.4. Classical Polymerase Chain Reaction (PCR) Amplification

For all classical PCRs, the amplification was carried out ina thermal cycler iCycler™ (Bio-Rad Laboratories, Hercules, CA, USA).The total reaction mixture (50 µL) contained: the reaction buffer 10X, template DNA, $Mgcl_2$,primers, Taq polymerase, and the mixture made up to 50 µL with sterile ultrapure water. The reaction concentrations used in both reactions were as cited in **Table 1**. The PCR conditions were those of touch down PCR after optimization, conditions are cited in **Table 2**. Following amplification, aliquots (20 µL) of the products were subjected to electrophoresisin 1.5% (w/v) agarose gels (Agarose DNA grade, Euromedex, France) in TBE buffer 0.5×. Gels were stained with Sybersafe (Invitrogen) and visualized under UV light. A 100 bp or 1000 bp DNA ladder from Invitrogen was used as a molecular size marker. PCR amplifications were performed with the primers shown in **Table 1**.

3. Results

3.1. Phenotypic Characterization of Darfiyeh LAB Isolates

Overall, 67 isolates were randomly selected from MRS and M17 agar plates for phenotypic and biochemical characterization. They were considered LAB based on their positive Gram staining, absence of catalase activity, and rod or coccal shape. Among the LAB isolates of *Darfiyeh* cheese, *Lactobacillus curvatus* (11 isolates), *Lactobacillus plantarum* (20 isolates), *Enterococcusfaecium* (10 isolates), *Enterococcusdurans* (5 isolates), *Enterococcus faecalis* (5 isolates), *Enterococcusmalodoratus* (1 isolate), *Lactococcus lactis* subsp. *lactis* (10

Table 1. Oligonucleotides used in this study and the corresponding volume of components.

	RecA Primer CCGTTTATGCGGAACACCTA TCGGGATTACCAAACATCAC Torriani *et al.* (2001)	Volume of components	*Orf-hisG* primer CTTCGTTATGATTTTACA CAATATCAACAATTCCAT Coroller *et al.* (1999)	Volume of components
10×		25 µl		25 µl
dNTP mix 10 mM		5 µl		5 µl
Forward primer		10 µl		15 µl
Reverse primer		10 µl		15 µl
25 mM $Mgcl_2$		15 µl		30 µl
Template DNA		2 µl		3 µl
Taq polymerase 5 U/µl		1.5 µl		2 µl
Water, nuclease free		175 µl		146 µl
Total volume		250 µl		250 µl

Table 2. PCR cycling conditions for RecA and Orf-hisG primers.

Primer	RecA		Orf-hisG		
Step	Temperature	Time	Temperature	Time	Cycles
Initial denaturation	94˚C	5 min	94˚C	5 min	1
Touchdown denaturation	94˚C	30 sec	94˚C	30 sec	
Touchdown annealing	67˚C to 57˚C	1 min 30 sec	53˚C to 43˚C	2 min	20
Touchdown extension	72˚C	2 min	72˚C	2 min	
Regular cycle denaturation	94˚C	30 sec	94˚C	30 sec	
Regular cycle annealing	58.5˚C	1 min 30 sec	47˚C	2 min	20
Regular cycle extension	72˚C	2 min	72˚C	2 min	
Final extension	72˚C	5 min	72˚C	5 min	1

isolates) and *Streptococcus thermophilus* (5 isolates) were identified.

3.2. Molecular Verification of *Lactobacillus plantarum* and *Lactococcus lactis* subsp. *Lactis* by Classical PCR

The proceeding PCR results (**Figure 1** and **Figure 2**) authenticated the results of the traditional microbiology methods of *lactobacillus plantarum* and *lactococcus lactis* subsp. *lactis*.

4. Discussion

To emphasize the clinical significance of *Lactococcus lactis* subsp. *lactis* and *Lactobacillus plantarum*, in both digestive and immune system, provoked the investigation of *Darfiyeh* cheese's microbiota. To serve that purpose; isolation, verification and enumeration of both LAB is a crucial preliminary step for further clinical studies on the health benefits provided by the two probiotic bacteria. In order to achieve the goals of this study, traditional microbiology and molecular biology techniques were implemented to verify the results of the culture-dependent methods.

The results obtained by the microbiology testing can be explained by the following:

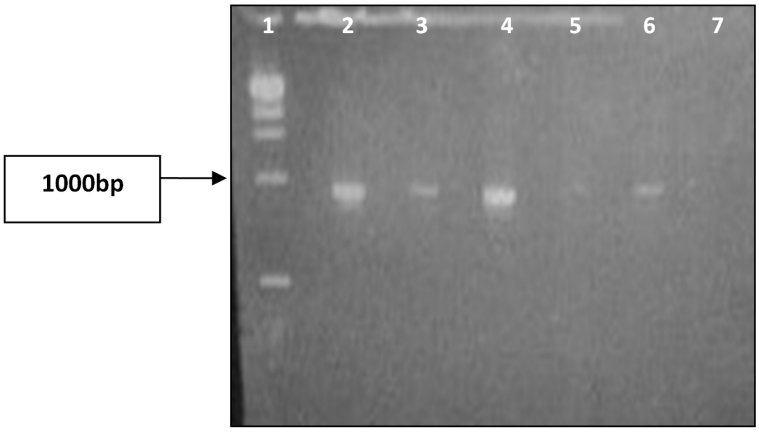

Figure 1. Species-specific PCR amplification with primers *Orf-HisG* for *lactococcus lactis* subsp. *lactis*. Lane 1: DNA molecular mass marker (100 bp from Invitrogen), Lanes 2-5 DNA extracted from isolated *Lactococcus lactis* colonies, Lane 6: Exogeneous positive control, Lane 7: Negative control.

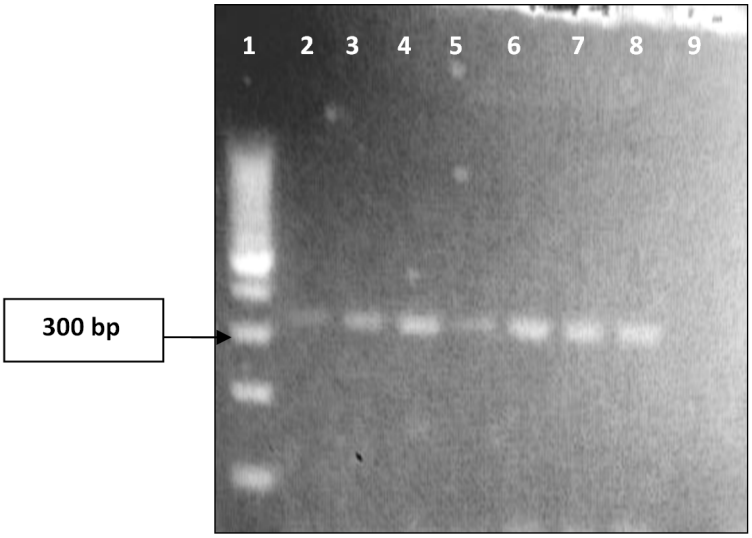

Figure 2. Species-specific PCR amplification with primers RecA gene for *Lactocobacillus plantaum*. Lane 1: 100 bp DNA molecular mass marker (1000 bpfrom Invitrogen), Lanes 2-7 DNA extracted from isolated *Lactobacillus plantarum* colonies, Lane 8: Exogeneous positive control, Lane 9: Negative control.

The growth of bacilli in Elliker and MRS broth proves that LAB has no selective media since MRS is usually a selective media for lactobacilli and Elliker for lactococci [16]. The bacterial growth on MRS, M17 and Rogosa at 30˚C, displayed growth of all dilutions; indicating that such agar media are optimal for mesophilic bacteria [3], and emphasizing the presence of such bacteria in all dilutions. MRS and M17 are also selective for thermophilic bacteria at 45˚C, however Rogosa agar is not.

The verification results of *Lactococcus lactis* subsp. *lactis* fit its identification criteria, except for the arginine hydrolysis test, implicating the possibility that the bacteria isolated could be *Lactococcu slactis* subsp. *cremoris* [17]. But, some strains of *Lactococcus lactis* subsp. *lactis* portrays phenotypically its phenotype, have been known to be *Lactococcus lactis* subsp. *Cremoris* genotypically. Recently, *L. lactis* subsp. *cremoris* showing phenotypic *L. lactis* subsp. *cremoris* were genotypically identified as *L. lactis* subsp. *lactis* [17]. The API results were not capable of 100% identifying the bacterial specie as *Lactobacillus plantarum*, although the carbohydrate fermentation results presented a high percentage of identification.

In order to confirm the findings of the culture-dependent methods, we proceeded with PCR using specie-specific primers. The findings of the culture-independent technique can be explained as the following:

The PCR amplicon of therec A gene verified the isolated species as *Lactobacillus plantarum*, the amplified 300bp band, was the confirmation result. The choice of using such primers, is because the three species; *Lactobacillus plantarum*, *Lactobacillus pentoses* and *Lactobacillus paraplantarum* are genotypically related, and share almost identical phenotypes. Their identification by traditional physiological tests is ambiguous [18]. PCR using species-specific oligonucleotides designed on the basis of phylogenetic molecular markers could be a proper approach for identification, since such markers are ubiquitous and highly conserved. However, this methodology could have its drawbacks especially when differentiating between *L. plantarum* and *L. pentosus* [18] [19], using the 16SrDNA sequences, where the two species share >99% of its identity value [19]. RecA on the other hand, could be used as a phylogenetic marker, and capable of distinguishing the three species, based on different set of primers designed. The sizes of amplicons are the eventual indicators; 318 bp for *L. plantarum*, 218 for *L. pentosus*, and 107 bp for *L. paraplantarum*.

It is noteworthy to add that the distinction of *Lactococcuslactis* subsp. *lactis* is rather challenging because, *Lactococcus lactis* subsp. *lactis and Lactococcus lactis* subsp. *cremoris*, is difficult to distinguish upon based on their perspective phenotypic criteria. Specie specific primers such as the Orf-hisG primers, differentiates the two subspecies, where the PCR product of *Lactococcus lactis* subsp. *lactis* is 933 bp. Hence, our results point out towards the specie isolated as *Lactococcus lactis* subsp. *lactis* based on the 900 bpamplicon size [20].

5. Conclusion

The correct verification of the bacterial specie was demonstrated. This is a crucial finding, because it represents the baseline of a further study, which will comprise the ingestion of *Lactococcus lactis* subsp. *lactis* and *Lactobacillus plantarum* to contemplate their probiotic effects. As determined in the study, culture-independent methods were not capable of accurately verifying the species of *Lactococcous lactis* subsp. *lactis* and *Lactobacillus plantarum*, but such techniques were vital for isolation and storage. Both LAB species were verified by specie-specific primers. Hence, the objective of isolating and correctly verifying the bacterial specie was fulfilled.

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