

CAMEL MILK AND MILKING

Isolation, identification and characterization of *Lactobacillus plantarum* from camel milk and its antagonist effect against diarrheal bacteria

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ABSTRACT

The main objective of this study is to define probiotic bacteria efficiency against bacterial infantile diarrhea. Lactic acid bacteria (LAB) was used as a natural resource and extracted of camel milk from southern Algeria. The isolation was followed by molecular identification using the 16S rDNA sequencing method. One hundred and fifty fecal samples were collected over 18 months from children suffering from diarrhea and aged 2 years or under. Identification results reveal the isolated lactic bacteria as *Lactobacillus plantarum*. In the other side, a total of 120 fecal samples were positive for bacterial growth, these pathogenic bacteria were identified as: *Escherichia fergusonii* (92%), *Salmonella enterica subsp. Diarizonae* (7.33%) and *Proteus mirabilis* (0.66%). The inhibitory effect study of lactic acid bacteria on pathogenic bacteria shows varying effects of *L. plantarum* in relation to the various pathogenic isolates, mentioning that the most important effect was expressed against *Escherichia fergusonii* with 22 mm. All the results allow us to classify that camel's milk is a natural source rich in lactic acid bacteria, in particular *L. plantarum*, with an inhibitory ability against the pathogenic bacteria responsible for diarrhea. Therefore *L. plantarum* produces bioactive molecules responsible for this effect.

Keywords: Antimicrobial; Diarrheal pathogens; *Lactobacillus plantarum*; Milk; Probiotic

INTRODUCTION

Three to five billion children are affected yearly by infantile diarrhea, and 1.5 to 2.5 million of them die of it. (Santosham, 2002; Dalby-Payne and Elliott, 2004). These figures make of this illness, worldwide disease-causing infants morbidity in developing countries and inflicting significant economic burden to the developed ones (Chow Chung et al., 2010).

If we have to make a ranking of the most killing infant diseases, diarrheal is placed at the forefront, just after respiratory tract infections. This unfortunate diarrheal morbidity lead position is due to infants suffering from improper feeding or rehydration care. Various pathogens such as: *Escherichia coli*, *Salmonella species*, *Klebsiella species* and *Enterobacter species*. (Ali et al., 2005; Diniz-Santos et al., 2005; Parashar et al., 2006) have been blamed, by international reports, for these cases.

In an effort to reduce the above-mentioned figures, doctors have been trying, these last years, to prescribe probiotics as alternative biotherapeutic agents against intestinal pathogenic infections, specifically: *Lactobacillus* and *Bifidobacterium*, that are sold commercially and protect against gastrointestinal pathogenic infection through various mechanisms (Thirabunyanon, 2011).

Lactobacillus spp has been praised by several reports and studies for its positive effect on mankind's intestinal flora, Crohn disease prevention and adult and infant protection against diarrhea (Pant et al., 1996).

Through this study, we would like to summarize all the work, and trials made in order to confine, define and analyze camel milk extracted *Lactobacillus plantarum* and analyze its antibacterial effect against diarrheal pathogens.

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MATERIAL AND METHODS

Mothers of 24 months old children were tasked to collect diarrheic fecal samples, put them into clean wide mouthed containers, without using any disinfectant or detergent residue and tight-fitting leak proof lids. The operation took place over 18 months in hospitals located in the following regions of Algeria: Chettia, Chlef and Algiers.

Samples were aseptically collected onto sterile universal bottles with indications of age, gender and time of collection and granted to the Microbiological laboratory of the University of Chlef, in Algeria, then air transferred to DIAGENE Laboratory in France.

The camel milk samples were collected from various regions of the south of Algeria, immediately cooled and sent to the above-mentioned labs in an ice box (4°C), then lactic acid bacteria (LAB) analyzed.

Microbiological analyses

The fecal specimens were analysed for bacterial pathogens by standard method (Abdullahi et al., 2010).

Lactic Acid Bacteria (LAB) were isolated on de Man, Rogosa and Sharpe (MRS) (Spain) agar and broth and incubated at 30°C for 24 to 48 h in order to touse and comply with identification conventional trials and properties assessment. Colonies of LAB purified by re-plating on MRS agar. From 100 isolates, 70 strains were retained. The strains were tested for Gram staining, catalase activity, and mobility. These strains were tested from the growth in NaCl, growth at different temperature and growth at different pH (Khedida et al., 2009). The strains were put into 3% glycerol and stored at -20° C.

Molecular diagnostic methods for the identification of bacteria

DNA preparation

DNA was removed from the bacteria while a fresh colony was placed into 1000 µl of sterile distilled water (10⁶ cells/ml), heated in Water Bath (Thermo Scientific™) for 10 minutes at 95°C to lyse the cells, that led to DNA release into the water. Heated tubes were placed immediately in Ice for 15 min and centrifuged to remove cell debris. The supernatant was hosted into a new tube. Quantity et quality of all extract DNA was checked by NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA) and stored at -20° C for next used (Silva et al., 2012).

PCR amplification and sequencing

Bacterial Extracted DNA was amplified with Universal Bacterial Primer for the 16S rRNA 27F (5'-AGAGTTTGTGATCCTGGCTCAG-3') and 1492R

(5'-TACGGYTACCTTGTACGACTT-3') (Eurogentec, Belgium) was used PCR amplification was carried out in a reaction mixture of 50 µl containing 1U of Go Taq hot start Taq DNA polymerase and 1X Go Taq Buffer (Promega, USA), 1.5mM MgCl₂, 0.5 µM forward primer, 0.5 µM reverse primer, and 5 µl of DNA Template.

The following PCR cycling parameters were performed: a first heating at 95°C for 6 min, then, denaturation process was performed by 35 cycles at 95°C for 30 s, then annealed at 55 °C for 45 s, extended to 72°C for 45 min, and ending by a 10 min final addition at 72 °C. PCR products were examined on agarose gels stained with 1% of Ethidium Bromide (Gel Doc XR+ System, Biorad, USA).

The amplified 16S rDNA PCR products were cleansed with Wizard® SV PCR Clean-Up System (Promega, USA), per the recommendations of the manufacturer). The purified products were sequenced by using 27F and 1492R primers by GATC Biotech (Germany).

Chromatograms of each of the forward and reverse sequences were checked using Chromas Prosoftwares (Technelysium Pty Ltd, Australia). In order to obtain the full-length sequence of each sample, forward and reverse strand sequences were combined using SeqMan software (DNASTAR) with manual adjustment.

Sequence identification were investigated by BLASTn in NCBI search to identify sequence similarity with all available 16S sequences in GenBank (National Centre for Biotechnology Information, Rockville Pike, Bethesda, MD), (<http://www.ncbi.nlm.nih.gov/>) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

In vitro inhibition test

The agar-well diffusion trial was conducted through an indicator strain overnight culture that was used to inoculate agar growth media at 45°C and poured into Petri dishes (Mallesha et al., 2012).

Inhibition zone diameter was then, measured with calipers in mm. The clear zone around the wells helped define the antimicrobial activity. Mueller-Hinton used for the interaction and the antimicrobial activities.

RESULTS AND DISCUSSION

Every year, infants and children, from developing countries, who suffered from bacterial infections die due to acute diarrhea caused by the above-mentioned infections. Clearing diarrheal disease related enteropathogens in the country is of a paramount importance to implement effective primary health care activities against

the disease (Ifeanyi Casmir et al., 2010). The result of this study shows that (*Escherichia fergusonii* (92%) to be the predominant bacteria causing diarrhea followed by *Salmonella enterica subsp. diarizonae* (7.33%) then *Proteus mirabilis* (0.66%).

The strains sequenced were identified by partial sequencing of the gene coding for the 16S rDNA. The BLAST program allowed to define the species on NCBI (<http://www.ncbi.nlm.nih.gov/>) with 99% similarity compared with 16s rRNA Genbank sequences strains. The 16S rDNA sequence analysis using GenBank and ribosomal databases (99%) allowed further asserting phylogenetic. Species were identified by sequence homology at 99% as *Escherichia fergusonii* (atKJ803903.1), *Proteus mirabilis* strain (atstrainKC456549.1), *Salmonella enterica subsp. Diarizonae* (atstrainAB273735.1) and *Lactobacillus plantarum* (DQ295035.1) (Figure 1).

Escherichia fergusonii (92%) being the most predominant the isolates in the study agrees with the result of Jackson et al. (2011) who reported isolation of *E. coli* (57.4%) from diarrheagenic stool samples from children as predominant isolate.

This also is in line with the findings of Al-Jarousha et al. (2010) who investigated the etiology of bacterial enteropathogens causing diarrhea among children and reported that Enterohemorrhagic *Escherichia coli* is the predominant pathogen isolated with 8,3%.

In the present study, the percentage of isolation of *Salmonella enterica ssp.* was 7.33 %, this rate was fully compared to previous studies done in Burkina Faso (2%) and in southeastern and eastern Korea reported by Chong et al. (1991) and in Africa reported by Bonkougou (2013).

Regarding the causes of the diarrhea episodes; they mostly occur due to maternally acquired antibodies. With regards to the time frame of these episodes, they generally, take place during the first two years of life, when the infant start to walk (Okolo et al., 2013).

100 acid bacteria strains were isolated from MRS agar. All of them grew at 30° C, were Gram positive, not-mobile and catalase negative as preliminary characteristics. The strain grows at different temperature (10 and 45°C) and it is characterized by his ability to grow at different pH (2,5 – 4,5 and 6,5). The biochemical and sugar fermentation test are presenting in Table 1.

The strain was identified by partial sequencing of the gene coding for the 16S rDNA. 16S rDNA sequence analysis

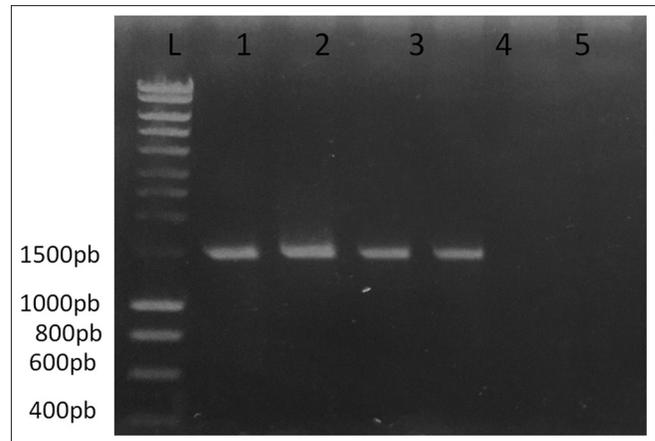


Fig 1. Representative electrophoretogram of 16s rRNA encoding region amplified by PCR from isolates. L: Ladder 1KB (Eurogentec, Belgium), 1-4: isolates, 5: control (water).

Table 1: Biochemical characteristics of *L. plantarum*

	Characteristics
Gram	+
Catalase	-
Oxidase	-
RM	+
VP	-
Nitrate	+
Reduction	
Indole	-
Galactose	-
Fructose	+
Glucose	+
Ribose	+
Sorbitol	+
Maltose	-
Xylose	+
Mannitol	+
Sucrose	+
Mannose	-
Saccharose	+
Lactose	+

using GenBank and ribosomal databases(99%) allowed to further asserting the phylogenetic *Lactobacillus plantarum* (DQ295035.1).

Antagonism effect

The role of lactic acid bacteria as antibacterial agent has been documented (Van Niel et al., 2002). *Lactobacillus plantarum* used in this study was tested for her antagonism effect. The culture was used and halos of inhibition were ranged between 6 to 22 mm against *Escherichia fergusonii*, *Proteus mirabilis* and *Salmonella enterica subsp. diarizonae*. The results revealed that the antibacterial activity of the selected strain could inhibit all tested pathogenic bacteria however at different inhibition levels (Table 2).

Table 2: Diameter of inhibition (mm) of various pathogenic organisms by *Lactobacillus plantarum*

	<i>Escherichia fergusonii</i>	<i>Proteus mirabilis</i>	<i>Salmonella enterica subsp. diarizonae</i>
<i>Lactobacillus plantarum</i>	22±0.3mm	14±0.4 mm	6±0.3 mm

**Fig 2.** Antagonism effect of *Lactobacillus plantarum* against *Escherichia fergusonii*.

The summary of the results demonstrate that inhibitive substances are being synthesized by our lactic acid bacteria strain via pathogenic bacteria. Furthermore, these strains produced inhibitive substances act differently on the pathogenic bacteria. The inhibitive substances produced by the lactic acid bacteria can be protein because we have eliminated the effect of acids (Savadogo et al., 2004).

Lactobacillus plantarum showed the most antibacterial potency to *Escherichia fergusonii* with 22 mm of diameter (figure 2), these results are in accordance with Mangell et al. findings (Mangell et al., 2002). These latter underscored the protective effect exerted by this strain in intestinal permeability against *Escherichia*.

Selvamohan and Sujitha (2010), showed that *L. plantarum* and its bacteriocins significantly controlled *E. coli* growth, but were least effective for *streptococcus* growth control. When comparing *L. plantarum* with its bacteriocins's antibacterial activity, it showed to be not affected by low temperatures, since its antibacterial activity is not altered at -20 °C.

Nissen et al. (2009) *in vitro* experiment showed that immune system responses, intestinal integrity enhancement, and intestinal metabolic activity can highly be stimulated by *L. plantarum*. In addition, epithelial cells are proven to be protected by *L. plantarum* from *E. coli*-induced damage, per Qin et al. (2009). Thus, all the above proves that *L. plantarum* has a major probiotic potential against diarrhea. Nobaek et al. (2000), also showed that abdominal pain and flatulence can be reduced by fermented foods containing *L. plantarum*.

CONCLUSION

The present study revealed that *Escherichia fergusonii* (92%) is the predominant entero-pathogen causing diarrhea followed by *Salmonella enterica subsp. diarizonae* (7.33%) and *Proteus mirabilis* (0.66%). It has shown that *L. plantarum* has inhibitory effects against the tested diarrheal micro-organisms. It could be said that organism from milk do have potential in combating children diarrhea.

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