Global Advanced Research Journal of Agricultural Science (ISSN: 2315-5094) Vol. 3(12) pp. 423-432, December, 2014 Special Anniversary Review Issue.
Available online http://garj.org/garjas/index.htm

Full Length Research Papers

Characterization of a new isolate of *Lactobacillus brevis* WD19 from Algerian goat milk with proteolytic activity

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Accepted 04 December, 2014

A collection of 60 isolates was obtained from 15 samples of Algerian goat milk. Five isolates (2 cocci and 3 bacilli) were able to hydrolyze milk proteins in UHT skim milk, but to different extents. The highest proteolytic isolate was selected and identified as *Lactobacillus brevis* by 16S rDNA sequencing (it was, therefore, named *Lactobacillus brevis* WD19). Experiments regarding bacterial growth, pH reduction capability and lactic acid production of LB19 were carried out by milk fermentation at 45 °C for 24 h. A significant increase in lactic acid production ($p < 0.001$) was observed for fermented milk samples (51.6 ± 1.4 ° D), followed by a pH decrease (5.11 ± 0.4) and a significant increase in bacterial numbers (5.10^{10} UFC/mL), which corresponded to high number of functions α-NH_2 (90.78 ± 9.62 µM/mg), when compared to the results observed for controls (19.4 ± 0.4 ° D, pH 6.68 ± 0.1, 8.24 ± 0.39 µM/mg). The characterization of proteolytic activity of the selected strain, performed using Na-caseinate and whey proteins in non-proliferative cell system, revealed that the optimum conditions for the proteolysis were achieved at 37-45 ºC and pH 7.2. The tests performed with protease inhibitors demonstrated that *L. brevis* WD19 produces both serine and metalloproteinases. The results presented in this manuscript suggest that *L. brevis* WD19 could be used in the manufacture of hypoallergenic fermented dairy products.

Keywords: *Lactobacillus brevis*, proteolytic activity, goat milk, fermented milk

INTRODUCTION

Lactic acid bacteria (LAB) have been used in food fermentations from Neolithic times (Gereková et al., 2011). LAB are a group of Gram-positive bacteria, non-sporulating, carbohydrate-fermenting lactic acid producing, acid tolerant from non-aerobic habitat and catalase negative. LAB found naturally in food are present also in large numbers in the normal human and animal gastrointestinal microbiota (Sgouras et al., 2004; Dalié et al., 2010). The use of LAB, including *Lactobacillus* spp. such as *Lactobacillus casei, Lactobacillus acidophilus,* and *Bifidobacterium* spp. is becoming very popular in dairy industries, due to their health beneficial effects. Besides, the proteolytic activity of some LAB strains can contribute to the sensorial characteristics of fermented dairy products.

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The structural components of the proteolytic system of LAB can be divided into three groups on the basis of their functions: (i) proteinases, which split caseins to peptides; (ii) peptidases, hydrolyzing peptides and (iii) transport systems that translocate the breakdown products across the cytoplasmic membrane (Kunji et al., 1996). Proteinases help to reduce the allergic properties of milk and milk products for infants, what can lead to a severe nutritional problem of protein-energy deficiency (Yuan, 2009; Hadji Sfaxi et al., 2012). Further degradation to amino acids is mediated by a set of peptidases (Gilbert et al., 1997). *Lactobacillus brevis*, *Lactobacillus cellobiosus*, *Lactobacillus fermentum* and *Lactobacillus plantarum* (Mugula et al., 2003) harbor similar proteinases. Specific proteases have also been detected in dairy products fermented by *Lactobacillus brevis* and *Lactobacillus buchneri* (Sumner et al., 1985; Rahman et al., 2009). The bacterial species *Lactobacillus brevis* is qualified by the European Food Safety Authority to have Qualified Presumption of Safety (QPS) status (Aquilina et al., 2011). Therefore, the study of new proteolytic *Lactobacillus* species can contribute to reduce the problem of milk allergy.

In this context, the present study was undertaken to investigate the proteolytic activity of *Lactobacillus brevis* WD19, isolated from Algerian goat milk, in order to evaluate its potential for application in the production of fermented hypoallergenic dairy product. The characterization of the proteolytic activity was performed by the investigation of the type of proteinases produced and the optimum conditions of pH and temperature.

MATERIALS AND METHODS

Samples collection

Thirty-five samples of goat milk were obtained from different regions of west Algeria. Samples (250 mL) were collected in sterile bottles and kept under low temperature, using an ice-cooled box, to be brought to the laboratory. To favor better growth of the bacteria, one milliliter of each sample was mixed with nine milliliters of sterile peptone physiological saline solution (5 g peptone, 8.5 g NaCl, 1000 mL distilled water, pH 7.0). The homogenate was serially diluted and the appropriate dilutions were surface plated on MRS agar (De Man et al., 1960). Plates were then incubated at 37 °C for 48 h. Colonies presenting different morphologies were randomly selected from each plate and re inoculated in MRS broth, incubated at 37 °C and checked for purity on MRS agar. Selected isolates were evaluated by catalase test, Gram staining, and cell morphology. The Gram-positive isolates, presenting negative results for catalase production and bacilli morphology were considered as LAB, selected for further tests and lyophilized for long time storage.

Amplification and sequencing of 16S rDNA

DNA was extracted using the commercial kit Bacterial DNA (Omega bio-tek, USA), according to the manufacturer recommendations and was used as a template for 16S rDNA gene amplification. The universal primers fD1 (5'AGAGTTTGATCCTGGCTCAG3') and rD1 (5'TAAGGAGGTGATCCAGGC3') were used (Weisburg et al., 1991). DNA amplifications were performed in DNA thermal cycler model (Techno, Barloworld scientific, Cambridge, UK). Mix reaction contained: PCR buffer (20 mM Tris–HCl, 50 mM KCl, pH 8.4), 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 1 U Taq DNA polymerase (Qiagen GmbH, Hilden, Germany), 1 mM of each primer and 40 ng DNA in a final volume of 50 µL. PCR amplifications were performed under the following conditions: first denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 56 °C for 1.15 min and DNA extension at 72 °C for 1.15 min. A final extension step was performed at 72 °C for 5 min. Amplicons were analyzed on 1% (w/v) agarose gel with ethidium bromide (0.5 mg/mL) in 0.5 X TAE (40 mM Tris–acetate, 1 mM EDTA) buffer, pH 8.2–8.4, for 30 min at 100 V and made visible by UV trans-illumination. DNA sequencing was carried out by MilleGen sequencing service (Labège, France).

Measurement of milk acidification through fermentation

The acidity developed in milk, throughout the fermentation with the selected strain was assessed by titration and pH measurement, after 0 h and 24 h of fermentation. The titration was performed using sodium hydroxide (N/9) in the presence of phenolphthalein (1%), as an indicator, and was expressed in Dornic/liter (Accolas et al. 1977).The pH was measured by using a digital pH meter (Dreiech, West Germany), standardized with pH 4 and 7 buffers before use.

Bacterial enumeration

In order to investigate the capability of *L. brevis* to grow in the substrate used for fermentation, the microorganism, inoculated in UHT skim milk, was enumerated by plating suitable dilutions on MRS agar medium (pH 5.4) and incubation at 37 °C for 48 h, according to De Man et al. (1960).

Measurement of the released α-NH_2 groups

The α-NH_2 terminal groups were determined after 0 h and 24 h of fermentation according to Doi et al. (1981).

Proteolytic activities assessed on Na-caseinate and denatured whey protein fractions, in non-proliferative cell system

Proteolytic enzymes production was induced by growing the strain on milk citrate agar (MCA) plates containing 4.4% (w/v) skim milk powder, 0.8% (w/v) Na-citrate, 0.1% (w/v) yeast extract, 0.5% (w/v) glucose and 1.5% (w/v) agar, as previously described (Fira et al., 2001). The plates were incubated for 48 h at 37 °C prior to cell collection. Collected fresh cells were re-suspended in 100 mM Na-phosphate buffer (pH 6.8) and brought to final optical density (DO_{600}) of 20, just before application. The cell suspensions were mixed with substrate dissolved in the same buffer at a 1:1 volume ratio and incubated at 37 °C. Na-caseinate (12 mg/mL) or denatured whey proteins (5 mg/mL, heated at 85 °C for 20 min) were used as substrates. The final mixtures were incubated for different periods (3, 6, 9, and 24 h) at 37 °C. Controls were prepared by incubating equivalent protein fractions solutions for the same periods, without adding cells. At the end of incubation periods, samples were taken and cells were pelleted by centrifugation (10 min at 8000 *g*, 5 °C). The clear supernatants were mixed with solubilization buffer at 1:1 volume ratio and heated at 100 °C for 3 min. Protein hydrolysis was analyzed by SDS–PAGE according to Laemmli (1970).

Effect of pH and temperature on proteolytic activity

The cell suspensions (OD_{600} = 20), in 100 mM Na-phosphate buffer at different pH values (5.4, 5.7, 6.0, 6.5, and 7.2) were mixed with substrate dissolved in the same buffer at a 1:1 volume ratio and incubated for 24 h at 37 °C. Na-caseinate (12 mg/mL) or denatured whey proteins (5 mg/mL) were used as substrates. A control was performed similarly without adding the bacterial cells. Protein hydrolysis and peptides formation were analyzed by SDS-PAGE. Similarly, to test the influence of temperature on proteolytic activity, the reaction mixtures containing cells and Na-caseinate were incubated in 100 mM Na-phosphate buffer (pH 7.2) for 24 h at different temperatures (30, 37 and 45 °C). Controls were prepared in the same conditions but without bacterial cells.

Effect of inhibitors

Cell suspensions obtained in 100 mM Na-phosphate buffer (pH 7.2), as previously described, were incubated for 2 h at 37 °C in the presence of different protease inhibitors [10 mM final concentration of ethylenediaminetetraacetic acid (EDTA), iodoacetic acid or phenylmethyl-sulfonyl fluoride (PMSF)], prior to addition of 12 mg/mL Na-caseinate or 5 mg/mL denatured whey proteins. Substrate solutions without cells and substrate cells mixture without inhibitor were used as controls. Protein hydrolysis and peptides formation were analyzed by SDS–PAGE.

Assessment of resistance to antibiotics

Susceptibility of *Lactobacillus brevis* WD19 to antibiotics was assessed against ampicillin (≤ 4 µg/mL), penicillin (≤ 4 µg/mL), gentamicin (≤ 2 µg/mL), kanamycin (≤ 512 µg/mL), vancomycin (≤ 1 µg/mL) and tetracycline (≤ 32 µg/mL) by disk diffusion method (Andrews, 2001). The Minimum Inhibitory Concentration (MIC) breakpoint values, as described by the European Food Safety Authority (EFSA, 2008), were recorded and the strain was qualified as resistant, intermediate or sensitive to antibiotics.

RESULTS AND DISCUSSION

Screening for proteolytic activity in UHT skim milk

From the 368 isolates obtained from Algerian goat milk, 60 isolates were found to be Gram-positive and catalase-negative (LAB). These isolates were inoculated into UHT skim milk and incubated for 24 h at 37 °C to evaluate their proteolytic activities. SDS-PAGE profile showed that 5 isolates (2 cocci and 3 bacilli) were able to hydrolyze milk proteins. Therefore, the proteolytic isolate with the highest activity was selected (data not shown). The 16S rDNA amplification and sequencing indicated that the selected isolate presented 99% identity with the 16S rDNA sequences reported for *Lactobacillus brevis* strains in GenBank database, such as *Lactobacillus brevis* NBRC 3345 (accession number: AB680070.1) and *Lactobacillus brevis* PB117 (accession number: JN792496.1). Therefore, the isolate was named *Lactobacillus brevis* WD19.

Measurement of titrable acidity in milk

Results have shown that pH of milk decreased with increasing acid production during incubation time. After 24 h of incubation at 37 °C, highest acidity (51.6 °D) and lowest pH (5.11) (Figure 1) were observed for *Lactobacillus brevis* WD19 compared to fermented milk at 0 h (24 °D; pH 6.4) and sterilized milk (19.2 °D; pH 6.68) (P<0.001). These results agreed with those of Chekroun et al. (2011) and Pereira de Souza et al. (2013).

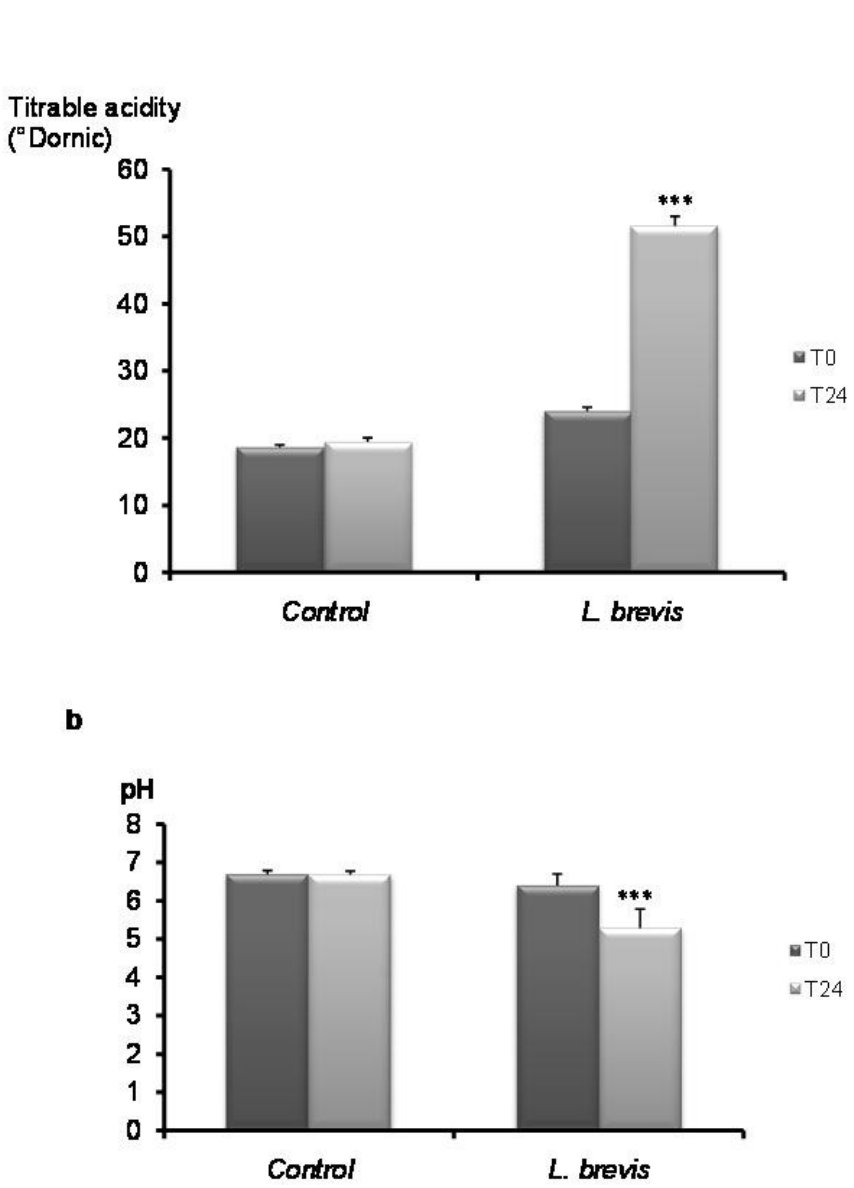

Figure 1. Production of acid lactic (degree Dornic) (a) and pH evolution (b) of *Lactobacillus brevis* WD19 in sterile skim milk during 24 h at 45°C.

Results are mean ± SE per group. The statistical significance of comparisons between *Lactobacillus brevis* at T24 and *Lactobacillus brevis* at T0 was assessed by using *Student t test* (***$p < 0.001$).

Growth of bacteria

The results regarding the enumeration of *L. brevis* WD19 revealed that the microorganism was able to grow in UHT skim milk, reaching significant numbers (1.5×10^{10}), which suggested its capability to ferment the substrate and produce important metabolites, such as lactic acid and proteolytic enzymes (Figure 2). These results agree with those of Dib et al. (2012), which showed that other *Lactobacillus* species, such as *L. plantarum* and *L. paracasei*, can grow significantly in milk, which enables their application for the production of commercials products.

Measurement of the released α-NH_2 groups

Measurement of the released α-NH_2 groups of fermented cow milk (Figure 3) shows that after 24 h, *L. brevis* WD19 degraded proteins as demonstrated by the increase in α-NH_2 groups (90.78 ± 9.62 µM/mg) compared

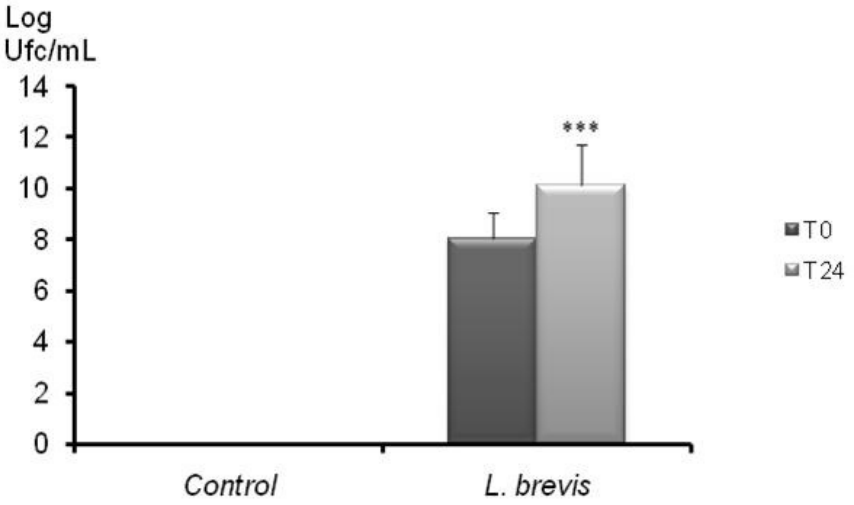

Figure 2. Growth of *Lactobacillus brevis* WD19 in MRS agar after the end of fermentation.
Results are mean ± SE per group. The statistical significance of comparisons between the groups was assessed by using the ANOVA test.
***P<0.001, *Lactobacillus brevis* at T24h vs *Lactobacillus brevis* at T0 and control at T24 (sterile skim milk)

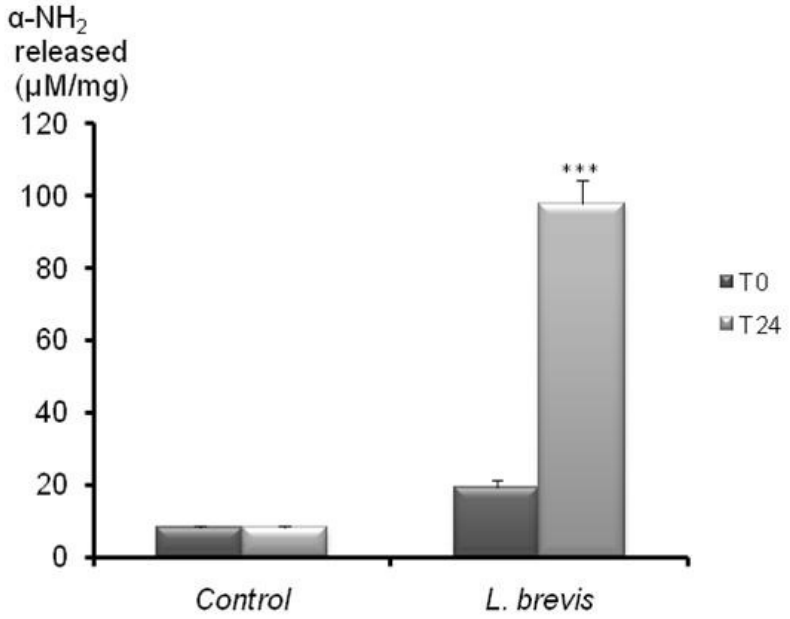

Figure 3. α-NH_2 groups (µmol/mg) of fermented milk with *Lactobacillus brevis* WD19 at 45 °C for 24 h as compared to control incubated without strain.
Results are mean ± SE per group. The statistical significance of comparisons between the groups was assessed by using the ANOVA test.
***P<0.001, *Lactobacillus brevis* at T24h vs *Lactobacillus brevis* at T0 and control at T24 (sterile skim milk)

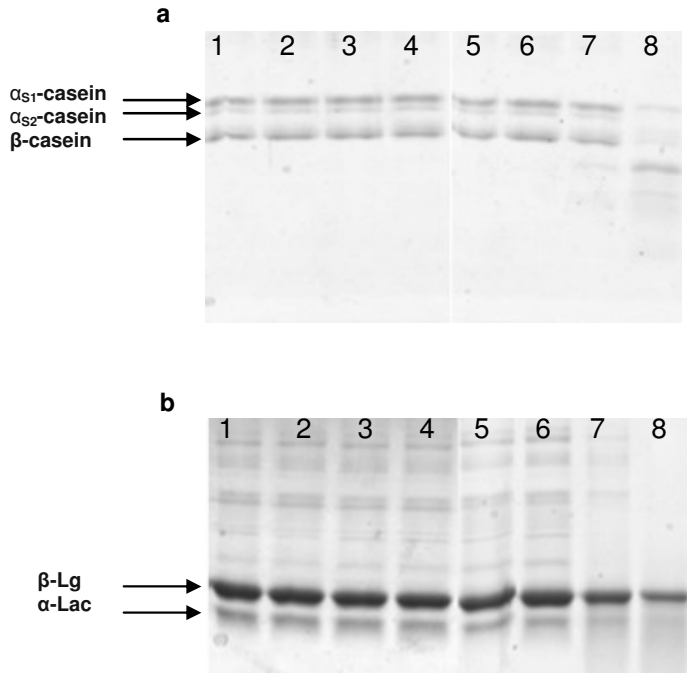

Figure 4. SDS-PAGE profiles of Na-caseinate (a) and denatured whey proteins (b) incubated at 37 °C for different times with *Lactobacillus brevis* WD19 as compared to control incubated without strain. Lanes 1, 2, 3 and 4: control samples, substrate incubated in the absence of strains; lanes 5, 6, 7 and 8: substrate incubated in the presence of *Lactobacillus brevis* WD19, for 3, 6, 9 and 24h.

to fermented milk at T0 (19.23 ± 2.14 µM/mg) and UHT skim milk (control) (8.24 ± 0.39 µM/mg) (P<0.001). Similar data were reported by Chekroun et al. (2011) on fermentation of cow's milk with the association of *Lactobacillus* and *Bifidobacterium*, which showed high number of α-NH_2 groups (103.32 ± 12.81 µM/mg).

Proteolytic activity on Na-caseinate and denatured whey proteins

After induction of proteinase production on MCA media, by incubation at 37 ºC for 48 h, bacterial cells were collected and their ability to hydrolyze different fractions of milk proteins was assessed at pH 6.8. Solutions of Na-caseinate in 100 mM Na-phosphate buffer pH (6.8) were combined with the bacterial cell suspensions at a 1:1 (v/v) ratio. As shown in Figure 4a, the extent of proteolysis increased with the time of incubation, reaching a maximum after 24 h. Similar data were reported by El-Ghaish et al. (2010) on *Lactobacillus fermentum* isolates collected from Egyptian Ras cheese, which showed proteolytic activity on β-casein (85% hydrolysis), α_{S1}-casein (68% hydrolysis) and α_{S2}-casein (47% hydrolysis).

In another set of experiments, the suspensions of *Lactobacillus brevis* WD19 were combined with solution of denatured whey proteins at a 1:1 (v/v) ratio. SDS-PAGE of the cell free supernatant generated in the presence of whey protein was analyzed, showing that the proteolysis started after 9 h of incubation and reached a maximum after 24 h of incubation (Figure 4b). According to the literature, proteolytic activity of *Lactobacillus* spp. has been associated with cell wall proteinases, as in the case of *Lactococcus* spp. (Kunji et al., 1996). Similar observations were reported previously (Pescuma et al., 2007).

Effect of pH on proteolytic activity

After 24 h of incubation, hydrolysis of Na-caseinate (Figure 5a) and denatured whey proteins (Figure 5b) was observed by SDS-PAGE. The hydrolysis was maximal at pH 7.2. Moreover, the extent of hydrolysis at basic pH was higher than that in acidic pH (5.4–6.0). These results are in agreement with those of Hadji Sfaxi et al. (2012) and El-Ghaish et al. (2010).

Effect of temperature on proteolytic activity

The optimum temperature for *Lactobacillus brevis* WD19 proteolytic activity was studied at pH 7.2. The cells of the strain were incubated with Na-caseinate and denatured

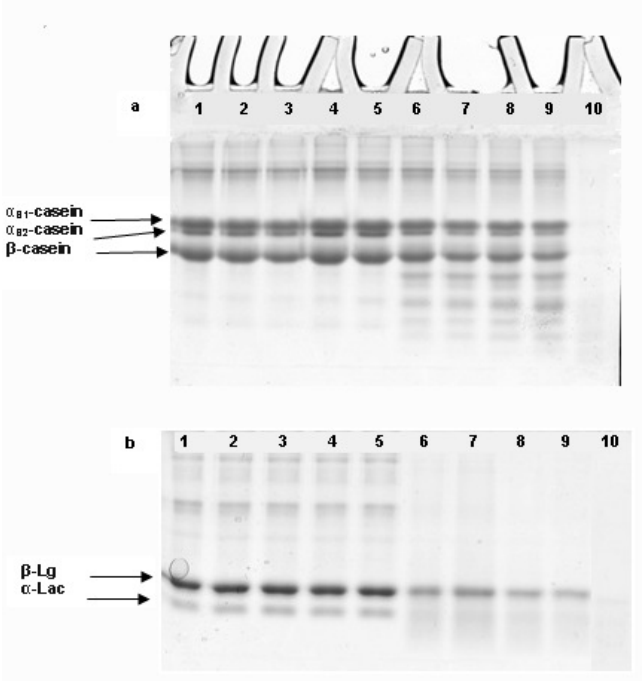

Figure 5. SDS-PAGE profile of Na-caseinate (a) and denatured whey proteins (b) hydrolyzed by *Lactobacillus brevis* WD19 at different pH values after 24 h incubation at 37 °C. Lanes 1, 2, 3, 4 and 5: control samples, substrate incubated in the absence of strains at pH 5.4, 5.7, 6.0, 6.5 and 7.2, respectively; lanes 6, 7, 8, 9 and 10: substrate treated with *Lactobacillus brevis* at pH 5.4, 5.7, 6.0, 6.5 and 7.2, respectively.

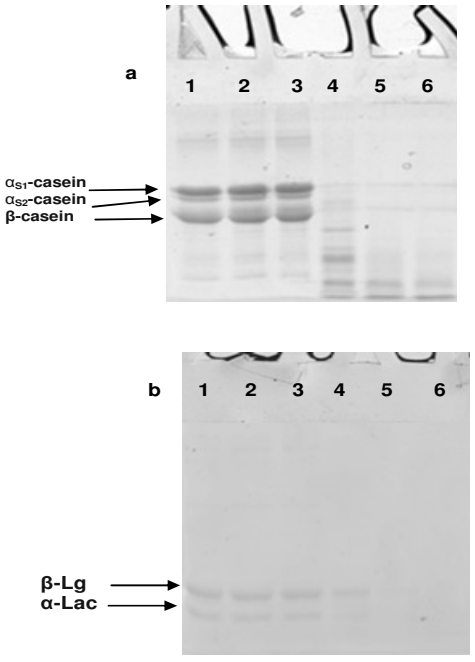

Figure 6. SDS-PAGE profile of Na-caseinate (a) and denatured whey proteins (b) hydrolyzed by *Lactobacillus brevis* WD19 at different temperatures after 24 h incubation at pH 7.2. Lanes 1, 2, and 3: control samples, substrate incubated in the absence of strains at 30 ºC, 37 ºC and 45 ºC, respectively; lanes 4, 5 and 6: substrate treated with *Lactobacillus brevis* at 30 ºC, 37 ºC and 45 ºC, respectively.

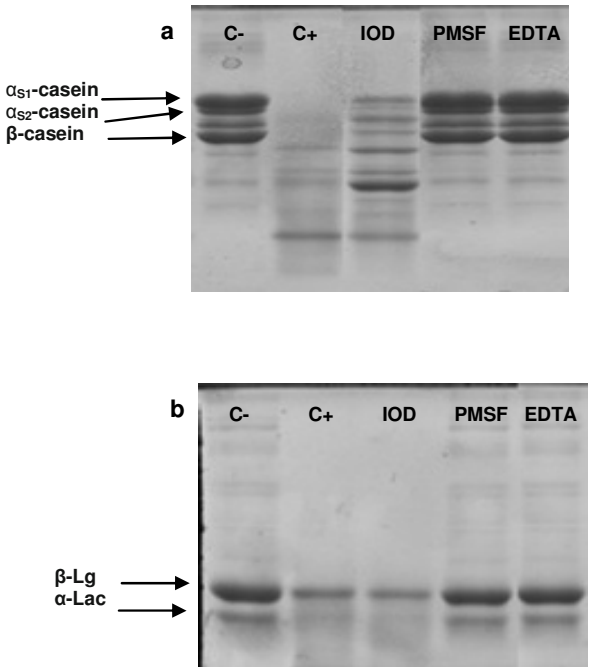

Figure 7. Effect of protease inhibitors on the proteolytic activity of *Lactobacillus brevis* WD19. (a): Na-caseinate; (b): denatured whey proteins; C-: Na-caseinate and denatured whey proteins alone; C+: sodium caseinate and denatured whey proteins treated by *Lactobacillus brevis* WD19 cells in the absence of inhibitors; IOD: in presence of iodoacetic acid, PMSF: in presence of phenylmethyl-sulfonyl fluoride, EDTA: in presence of ethylenediaminetetraacetic acid.

whey proteins, at different temperatures (30, 37 and 45 °C). As shown in Figure 6, the proteolytic activity was important at 30 °C and reached its maximum at 37 and 45 °C. Similar results were obtained by other authors (Ahmadova et al., 2011; El-Ghaish et al., 2010; Hadji Sfaxi et al., 2012).

Effect of inhibitors on protease activity

Proteolytic activity was strongly inhibited in the presence of EDTA and PMSF, indicating the presence of metallo-proteases and serine-proteases, respectively (Figure 7). In contrast, no inhibition of proteolytic activity was observed in the presence of iodoacetic acid, indicating the absence of cysteine-proteinases. These results remain in agreement with those of Hadji Sfaxi et al. (2012) and Pereira de Souza et al. (2013). It can be suggested that the active proteases produced by *Lactobacillus brevis* WD19 are metallo-proteases and serine-proteases.

Assessment of resistance to antibiotics

Danielsen and Wind (2003) suggested that MIC can be used as a microbiological breakpoint when screening *Lactobacillus* strains for transferable resistance genes. The antibiotic resistance genes can be transferred to other bacteria, so it is necessary to evaluate the safety of strains before its application in foods.

The susceptibility of *Lactobacillus brevis* WD19 to antibiotics was established according to breakpoints. The strain has been found susceptible to penicillin and ampicillin (cell wall synthesis inhibitor) (Table 1). Similar observations were reported previously (Hadji Sfaxi et al., 2012). *Lactobacillus brevis* WD19 was also susceptible to chloramphenicol (protein synthesis inhibitors) and to tetracycline (Hadji Sfaxi et al., 2012; Klare et al., 2007).

In contrast, *Lactobacillus brevis* WD19 presented resistance against gentamicin (aminoglycosides) (Nawaz et al., 2010). In addition, Lactobacilli are usually

Table 1. Antibiotic resistance profile of *Lactobacillus brevis* DW19

Antibiotics	MIC breakpoints (mg/mL)	Status
Ampicillin	2	S
Gentamicin	32	R
Penicillin	4	S
Tetracycline	4	S
Chloramphenicol	4	S

MIC, Minimum inhibitory concentration breakpoint according to EFSA (2008)
R, resistant; S, sensitive

susceptible to ampicillin and chloramphenicol (Katla et al., 2001; Ammor et al., 2007).

CONCLUSIONS

The obtained results demonstrate the proteolytic activity of new strain *Lactobacillus brevis* WD19, isolated from Algerian goat milk. Its potential to hydrolyze main allergenic milk proteins suggests that *Lactobacillus brevis* WD19 could be applied for the production of hypoallergenic dairy products or the in the process of extensive hydrolysis of milk proteins to small peptides, which can be used to make hypoallergenic dairy proteins to be applied in commercial milk products.

ACKNOWLEDGMENTS

We would like to express our gratitude for the support of this work in the frame of TASSILI project #10MDU 792 “Alimentation de l’enfant allergique: Allergénicité des protéines du lait bovin après traitements technologiques et étude du rôle prophylactique des laits hypoallergéniques chez le modèle animal d’allergie et les enfants à risque” and for Algerian Ministry of higher Education and Scientific Research (DR-RSDT) for its financial support.

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