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ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF BOVINE WHEY PROTEINS HYDROLYSED WITH SELECTED *LACTOBACILLUS* STRAINS

***Hayet MESSAOUI[1], Salima ROUDJ[1], Nour-Eddine KARAM[1]**

[1]*Laboratory of Microorganisms Biology and Biotechnology, Department of Biotechnology, Faculty of Natural Sciences and Life, Oran1 University Ahmed Benbella, B.P. 1524, El M'Naouer, 31000 Oran, Algeria.*
mess_hayet@yahoo.fr
*Corresponding author
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Abstract: *The present study investigated the antioxidant and antibacterial properties of hydrolysates obtained from bovine whey proteins, after hydrolysis with Lactobacillus plantarum strains isolated from ewe milk and Lactobacillus brevis isolated from camel milk. The aim here was to valorize whey proteins and to constitute local collection of bacteria, with potentially interesting technological and biological features. The protein hydrolysis was determined by degree of hydrolysis (DH) and electrophoresis SDS–PAGE, and then optimized using temperature, pH, cells/whey proteins ratio and time incubation. Antioxidant activity of hydrolysates was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and nitric oxide (NO) free radicals scavenging methods. The antibacterial activity was examined by the agar well diffusion assay. The results revealed that Lb. plantarum LBBS2 and Lb. plantarum LBM2 exhibited an optimum of hydrolysis of whey proteins at pH 7, at 30 °C, with (2/1) as ratio cells/whey proteins and after 48 h of incubation. Under these conditions, the hydrolysates exhibited an antibacterial activity against Gram positive and Gram negative bacteria, including some pathogenic and spoilage microorganisms. Meanwhile, scavenging radical properties by the same hydrolysates reached a maximum after 24 h. These results suggest that Lb. plantarum LBBS2 and Lb. plantarum LBM2 could be used as new potential adjunct bacteria with interesting proteolytic activity. Moreover, milk whey which is the main by-product of food industry could be used for the production of molecules with a promising interest as antioxidants and antibacterials.*

Keywords: *Lactobacillus plantarum, milk whey, hydrolysates, degree of hydrolysis, electrophoresis SDS–PAGE.*

1. Introduction

Lactic Acid Bacteria (LAB) are widely utilized in the fermented food industry for their technological and health-promoting features, such as improving the sensorial properties of fermented products, as well as providing special characteristics to foods particularly the release of compounds with health-promoting effects [1]. LAB have an efficient proteolytic system comprised of a Cell Envelope Proteinase (CEP) which initiates protein degradation, a transport system and various intracellular peptidases. In this regard, LAB are able to degrade milk proteins [2, 3]. *Lactobacillus* spp. are greatly employed in the food industry as starter cultures for the manufacture of fermented foods and beverages or as probiotics for incorporation into functional foods [4].
Whey is the principal by-product of the food industry. It represents about 85–90% of milk volume and retains approximately 55% of milk nutrients [5]. Previously, whey was considered as important

pollutant of the dairy industry, not only due to its great organic load, but also because of its important volume [6]. In fact, continued growth in the production of dairy products, especially cheese, has seen a concomitant increase in the volume of whey worldwide (currently >200 million tonnes per year) [7]. Whey is a rich mixture of secreted proteins with wide variety of functional characteristics for biological, nutritional, and food purposes. It contains 20% of total milk proteins. Nowadays, the perception of whey as a pollutant has changed with the discovery of its functional and bioactive properties, being considered as an additional product of cheese manufacture [5]. Among the food proteins, most studies have been performed on whey proteins due to their high nutritional value, inexpensiveness, diverse functional properties and widespread application in food industry [8, 6].

Oxidative stress, the increased production of reactive oxygen species (ROS) and free radicals are incriminated in many degenerative diseases like cancer, atherosclerosis and diabetes [9]. Improvement of the body's antioxidant defences through food supplementation would appear to a practical initiative to reduce the level of oxidative stress. As obvious antioxidant feature of whey protein hydrolysates was detected in recent studies, it was good for their application in food industry as functional materials [9, 10].

Furthermore, the presence of pathogenic and spoilage microorganisms in foods, combined with new consumer interest resulting in restrictions on the use of traditional antimicrobial chemical agents, puts pressure on food manufacturers to develop new preservatives [11]. During the past decade, growing attention has been focused on the production of antimicrobial peptides derived from whey proteins in order to reduce the need for antibiotics [8, 12].

Various protein hydrolysates obtained through enzymatic catalysis demonstrate biological activities, which are usually associated with bioactive peptides. Thus, production of hydrolysates can be an interesting approach to add value to whey. In fact, the bioactive peptides are inactive while encrypted in the sequence of original protein but can be released by: a) hydrolysis by digestive enzymes, b) by proteolytic microorganisms, and c) by the action of plant or microbial proteases [13]. In this context, protein-rich by-products might be utilized to produce bioactive peptides using specific protease producing microorganisms.

To our knowledge, no study has been carried out on the antioxidant and antibacterial properties of bovine whey protein hydrolysates generated by ewe milk and camel milk *Lactobacillus* strains. Therefore, the present work was undertaken to study and compare, antioxidant and antibacterial activities of whey protein hydrolysates obtained with autochthonous *Lactobacillus* strains isolated from ewe milk and camel milk.

2. Materials and Methods

Bacterial Strains and Growth Conditions

Nine *Lactobacillus plantarum* strains (coded LBM1, LBM2, LBS1, LBSC1, LBSC2, LBBS1, LBBS2, LBBG1, LBBG2), one strain of *Lactobacillus paracasei* subsp. *paracasei* coded LBS2 isolated from ewe milk and one strain of *Lactobacillus brevis* coded CHTD27 isolated from camel milk, were supplied by the Laboratory of Microorganisms Biology

and Biotechnology, Oran1 University (Algeria). Ewe milk was collected in different regions of Algeria, and camel milk in Tindouf area (southwest, Algeria). The LAB strains were maintained in reconstituted skimmed milk (10%) at - 20 °C. Standard cultures were prepared by inoculation of de Man, Rogosa and Sharpe medium (MRS, Fluka, Saint Louis, MO) broth at pH 5.7 with the frozen stocks followed by incubation at 30 °C for 18 h.

Qualitative Determination of Proteolytic Activity

The proteolytic activity of strains was estimated using agar medium with reconstituted skimmed milk (10% w/v) at a final concentration of 3%. A loopful of lactic strain cultures (Optical Density OD_{600nm} = 1) was spotted on skimmed milk agar and incubated at 30 °C for 48 h. Proteolytic activity was detected by the occurrence of clear zones around the colonies [14].

Preparation of Whey Proteins

Whey proteins were prepared from collected cow milk as reported by Neyestani *et al.* [15] with some modifications. In brief, cream was skimmed from fresh cow milk by centrifugation (5000 g, 30 min, 4 °C) and the top lipid layer removed. The process was repeated three times. The Casein was precipitated and separated from whey by acidification with 1 N HCl at pH 4.6. The precipitate was then removed by centrifugation (5000 g, 30 min, at 4 °C). The clear straw colored whey was obtained as a supernatant and filtered through Whatman filter paper N°1 (Whatman Ltd., Maidstone, UK). Whey globulins were eliminated by precipitation with ammonium sulfate at 58% saturation (2.3 M) and at 4 °C in 24 h. The supernatant obtained from this step contained α-lactalbumin (α-Lb), β-lactoglobulin (β-Lg), bovine serum albumin (BSA) and lactoferrin (Lf). pH was adjusted to 7.0 using diluted 1 N NaOH solution. The whey proteins were dialyzed against distilled water, lyophilized, stored at - 20 °C and referred to as Cow Whey Proteins (CWP). The purity of CWP was checked using SDS-PAGE.

Preparation of Whey Protein Hydrolysates

Whey protein hydrolysates (WPH) were prepared as described by Pescuma *et al.* [16] with slight modifications. *Lactobacillus* strains were inoculated in MRS broth medium with 5 mM $CaCl_2$. The inoculum was cultured in 300 ml MRS at 30 °C for 18 h. Cells were collected by centrifugation (5000 g, 10 min, 4 °C) at the exponential growth phase (OD_{600nm}= 1), washed twice with 0.85% (w/v) saline solution supplemented with 10 mM $CaCl_2$, and suspended in 100 mM sodium phosphate (pH 7.0) with 5 mM $CaCl_2$. Cell suspensions were kept at 37 °C for 30 min for amino acid starvation; non-proliferating cells of strains were incubated with 1% CWP (w/v) in a cells/cow whey proteins ratio (C/CWP) of 2/1 (v/v). CWP were previously dissolved in 100 mM sodium phosphate (pH 7.0) supplemented with 5 mM $CaCl_2$ and heated at 80 °C for 30 min (to avoid microbial contamination of whey). The cells-whey proteins mixture was incubated for 24 h at 37 °C; samples were withdrawn and then centrifuged (5000 g, 10 min, 4 °C). WPH were analyzed by estimation of hydrolysis degree and by SDS–PAGE.

Determination of the TCA-Soluble Proteins

The trichloroacetic acid (TCA)-soluble proteins of the WPH were determined by the method of Folin-Ciocalteu [17]. An aliquot of the hydrolysates was added to an equal volume of TCA (12% w/v). The mixture was incubated for 30 min at room temperature then centrifuged (5000 g, 15 min, 4 °C). An aliquot (500 µl) of the supernatant (WPH) was added to 2500 µl of NaOH (0.2 M) and 250 µl of Folin-Ciocalteu reagent diluted twice with distilled water. The reaction mixture is stirred and left at room temperature for 10 minutes. The blue color that develops is measured spectrophotometrically (Optizen, 2120 UV, Mecasys Co., Ltd, Korea) at 750 nm against blank (mixture without hydrolysate). The results were expressed as µg of tyrosine per ml referring to a standard curve of tyrosine (0–50 µg/ml).

Determination of the Hydrolysis Degree (DH)

The degree of hydrolysis (DH) is defined as the percentage of peptide bonds cleaved. It was determined by using a modification of the method described by Boudrant and Cheftel [18] and was defined as follows, expressed as per cent:

$$DH = \frac{Dt - D0}{Dmax - D0} \times 100$$

Where Dt corresponded to the amount of TCA soluble peptides after whey protein hydrolysis expressed as tyrosine; D0 corresponded to soluble peptides of unhydrolysed whey proteins expressed as tyrosine; Dmax corresponded to the maximum amount of TCA soluble peptides expressed as tyrosine and determined after hydrolysis of the whey protein solution by HCl (6 N) at 110 °C for 24 h [19].

Electrophoresis (SDS-PAGE)

The samples from CWP hydrolysis were examined by polyacrylamide-SDS gel electrophoresis as described by Laemmli [20] using PAGE mini vertical protein electrophoresis system (Cleaver Scientific Ltd, UK). The polyacrylamide gels consisted of 17% resolving gel and 5% stacking gel. T_0, T_{24h} samples for each strain as well as sample of CWP non hydrolysed and the molecular weight marker (Protein marker standard 11-190 kDa, New England Biolabs Inc., Ipswich, MA, USA) were loaded separately at a total running time of 3 h at 120 V. The gels were fixed in a solution of 12% TCA for 15 min and then stained by using Coomassie brilliant bleue R–250 stain solution (Merck, Saint Louis, MO) for 5 h with 2–3 changes of the destaining solution.

Optimization of the Hydrolysis Conditions

Hydrolysis of CWP by the strains was conducted as described by Pescuma *et al.* [16] under different conditions of temperature, pH, C/CWP ratio and time hydrolysis.

Incubation Temperature Effect

The effect of incubation temperature was determined in 10 mM phosphate buffer (pH 7.0) in the range of 30 to 45 °C with a C/CWP ratio of 2/1 (v/v). Samples were withdrawn after 24 h and 48 h of incubation and then centrifuged (5000 g, 10 min, 4 °C). The hydrolysates have been analyzed by estimation of hydrolysis degree and by SDS–PAGE.

pH Effect

The effect of pH on the hydrolysis of CWP was examined in the pH range of 6 to 8 by using different buffers: Acetate buffer 0.2 M, phosphate buffer 0.2 M, tris (hydroxymethyl) aminomethane-HCl buffer 0.2 M. The cells-whey protein

mixtures were incubated at 30 °C with a C/CWP ratio of 2/1(v/v). Samples were pulled out after 24 h, 48 h of incubation, centrifuged (5000 g, 10 min, 4 °C) and analyzed.

C/CWP Ratio Effect

The enzymatic process was carried out at three different C/CWP ratios (1/1, 1/2 and 2/1). The relationship between the hydrolysis of CWP by the strains and the cells/whey proteins ratio was studied at 30 °C and pH 7. Samples were withdrawn after 24 h and 48 h of incubation, centrifuged (5000 g, 10 min, 4 °C) and then analyzed.

Antioxidant Activity by DPPH Radical Scavenging assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the hydrolysates was determined according to the method described by Lin *et al.* [10] with minor modifications. At first, 2744 µl of methanolic DPPH solution (0.2 mM) were added to 56 µl aliquot of hydrolysate solution or phosphate buffer (control). The mixture was shaken vigorously and kept under dark at ambient temperature for 30 min. Absorbance of each sample and control was read at 517 nm against blank by using a UV-visible spectrophotometer (Optizen, 2120 UV, Mecasys Co., Ltd, Korea). The antioxidant activity of hydrolysates was estimated by comparison with ascorbic acid which is known as natural antioxidant. The DPPH radical scavenging activity, expressed as the percentage of inhibition, was calculated by the following equation:

$$\text{DPPH scavenging activity (\%)} = \frac{AC - AS}{AC} \times 100$$

Where AC is the absorbance of the control and AS is the absorbance of the sample.

Nitric Oxide Radical Scavenging Activity

The effect of hydrolysates on nitric oxide (NO) radical scavenging was investigated by using a sodium nitroprusside (SNP) generating NO system. Briefly, 100 mM of SNP was dissolved in phosphate buffered saline (PBS) pH 7.4. The tested hydrolysates were added to SNP (10 mM) in PBS in a final volume of 2 ml and then incubated at 25 °C for 150 min. A control experiment without tested hydrolysates was conducted in an identical manner. After incubation, 1.0 ml of samples was diluted with 1.0 ml of Griess reagent (1% sulphanilamide and 0.1% naphthyletylenediamine dihydrochloride in 2% H_3PO_4) [21]. Ascorbic acid was used as a positive control. The mixtures were kept under dark at room temperature for 30 min, and then the absorbance measured at 540 nm against the corresponding blank solutions. The percentage of NO inhibition was calculated by the following equation:

$$\text{NO scavenging activity (\%)} = \frac{AC - AS}{AC} \times 100$$

Where AC is the absorbance of the control and AS is the absorbance of the sample.

Assay for Antibacterial Activity

The antibacterial activity was tested by using a well diffusion method in agar [22]. The indicator strains used to detect the antibacterial activity were *Staphylococcus aureus* 25923, *Pseudomonas aeruginosa* 27853, *Escherichia coli* 25922 (From Pasteur Institute, Algeria), ten (10) bacteria: *Salmonella typhi*, *Serratia marcescens*, *Proteus mirabilis*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Bacillus subtilis*, *Bacillus cereus*, *Enterobacter aerogenes* and *Enterococcus faecium* (isolated and identified at our laboratory). To check the antibacterial activity, target strains were

grown in nutrient broth overnight at 37 °C. The plates were further overlaid with soft nutrient agar (0.75% w/v agar) inoculated at 1% (v/v) with cultures of indicator strains adjusted to OD_{600nm}= 1. Wells of 5 mm diameter were cut with sterile well borer into these agar plates. 50 µl of WPH (T_0, T_{24h} and T_{48h} of each strain) previously filtered through 0.22 µm pore-size filters were placed into each well. Plates were stored at 4 °C for overnight to permit diffusion of samples, and then incubated at 37 °C for 24 h. The appearance of a clear halo around the wells was measured and presented as inhibition zones (mm).

Statistical Analyses:

All experiments were carried out in triplicate. The results were expressed as mean ± standard error (SE). Mean separation and significance were analyzed using the IBM SPSS® software (SPSS Statistical Software, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was carried out to discriminate among the means. Tukey and Student-Newman-Keuls procedures were used as post-hoc tests. Differences between samples were considered as significant at $P < 0.05$.

3. Results and discussion

Proteolytic Activity of Strains

The proteolytic systems of lactic acid bacteria are important means of making free amino acids and peptides from milk proteins for growth. In this study a total of 11 *Lactobacillus* strains were screened for proteolysis. The results show that all the strains exhibited proteolytic activity (Figure 1). Proteolytic activity has been reported several times for LAB, including *Enterococcus*, *Lactobacillus*, *Lactococcus*, and *Pediococcus* [23]. The *Lactobacillus* genus, produce proteases that can hydrolyze α- and β-casein thus, having a direct effect on the biochemistry of dairy products. Furthermore, the hydrolysis of proteins in milk (i.e., casein, β-Lg, and α-Lb) produces small peptides, which have been suggested to present biological activities [24].

However, the strains were different in the efficiency proteolysis. The H/C ratio varies from 1.24±0.028 (*Lb. paracasei* subsp. *paracasei* LBS2) to 2.65±0.028 (*Lb.brevis* CHTD27) and 2.71±0.042 (*Lb. plantarum* LBBS2) which represent the highest values ($p<0.05$) (Figure 1). In fact, variability in proteolysis is commonly reported for isolates of lactic acid bacteria from natural sources [25]. Each microorganism used in food fermentation has different capacities to produce enzymes that hydrolyze proteins in different conditions [26].

Based on this screening, the strains *Lb. plantarum* LBM2, *Lb. plantarum* LBBS2 from ewe milk, and *Lb.brevis* CHTD27 from camel milk which have displayed the greatest proteolytic activity among the strains tested ($p<0.05$), were further subjected to whey protein hydrolysis and whether potentially healthful bioactive peptides could be produced.

Ability of Strains to Hydrolyse Whey Proteins

The hydrolysis reaction was carried out on sodium phosphate at 37 °C, pH 7.0, with ratio C/CWP of 2/1 for 24 h.

To compare the extent of hydrolysis, whey protein hydrolysis in terms of degree of hydrolysis was determined by measuring amount of amino acids and peptides liberated, using the Folin-Ciocalteu reagent. The results showed that CWP were degraded by all the strains studied; however, the efficiency of hydrolysis was strain dependant.

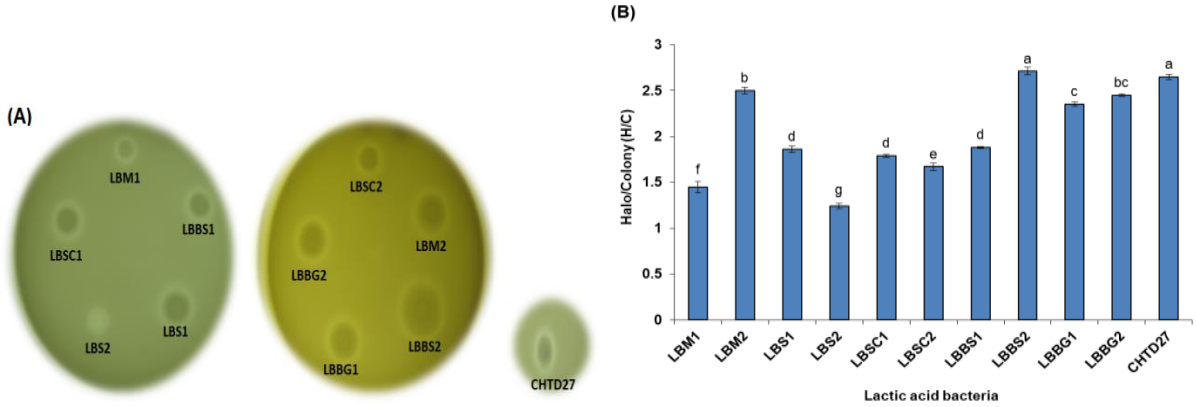

Fig. 1. Proteolytic activity of whole cells on Milk-Agar (3%) medium (A); Halo/Colony values (B) obtained at 30 °C after 48 h of incubation. Bars represent the standard error from triplicate determinations. Values with different letters (a-g) indicate results significantly different between the strains ($P < 0.05$).

As shown in Figure 2, the DH value in WPH generated by *Lb. plantarum* LBBS2 (24.92%±0.61) was significantly higher than *Lb. plantarum* LBM2 (16.51%±0.39) and *Lb. brevis* CHTD27 (10.46%±0.48) which showed the lowest rate of hydrolysis ($P<0.05$).

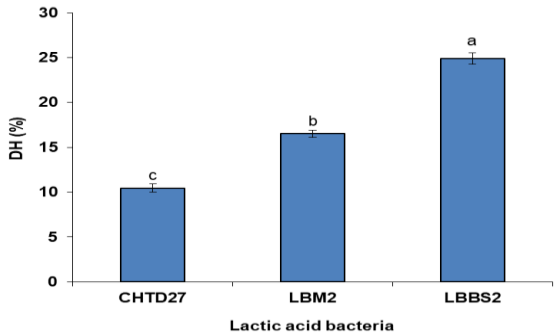

Fig 2. DH values of the hydrolysates obtained by the strains at 37 °C, pH 7 with ratio C/CWP (2/1) after 24 h of incubation. Bars represent the standard error from triplicate determinations. Values with different letters (a-c) indicate significant differences between hydrolysates ($P<0.05$).

Previous research specified that the activity of proteinases and peptidases varied among lactic acid bacteria species [27, 28]. Nevertheless, in previous research, the strain *Lb. brevis* CHTD27 from camel milk which is weakly proteolytic on whey proteins, was able to cleave to a greater extent caseins. This better performance on caseins could be due to protease specificity of this bacterium [29].

Electrophoresis SDS-PAGE revealed different peptide profiles from CWP hydrolysis according to the strain tested. The electrophoresis diagram (Figure 3) results of CWP (lane 2) showed four bands i.e. Lf (88,000 kDa), BSA (69,000 kDa), β-Lg (18,400 kDa) and α-Lb (14,300 kDa). Hydrolysis revealed a decrease in protein intensity at T_{24h} compared to T_0 for the strains *Lb. plantarum* LBM2 (lanes 5, 6) and *Lb. plantarum* LBBS2 (lanes 7, 8) but no changes were observed in the intensity of the proteins for *Lb. brevis* CHTD27 (lanes 3, 4). These data were consistent with earlier results of proteolytic activity by assessment of DH. Several studies revealed that an increase in DH of the proteins decreased their molecular size by creating small peptides [9, 24].

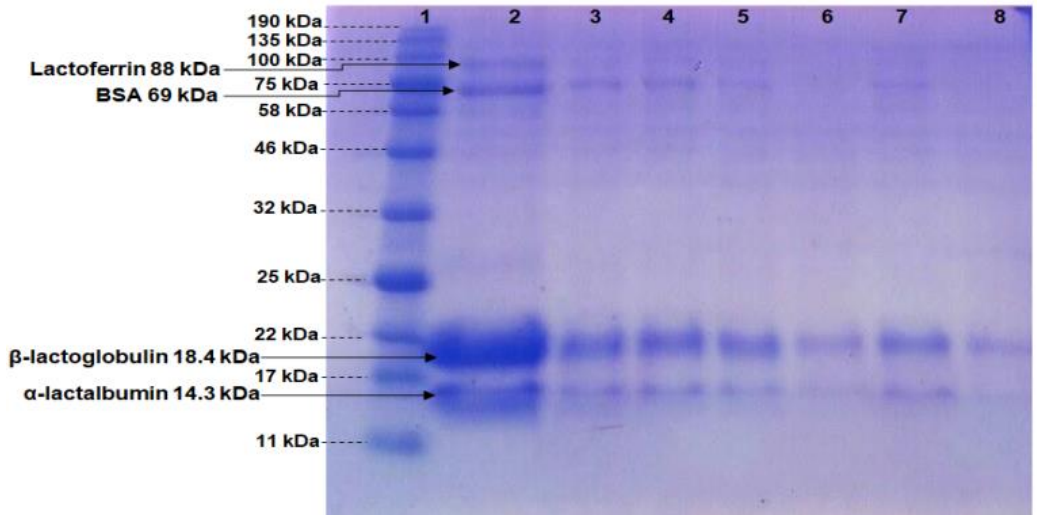

Fig 3. SDS-PAGE analysis of CWP hydrolysed by the strains at 37 °C, pH 7 with ratio C/CWP (2/1) after 24 h of incubation. (1) Molecular weight marker ; (2) Non hydrolysed CWP ; (3) and (4) Hydrolysates obtained with the strain CHTD27 at T_0, T_{24h} respectively ; (5) and (6) Hydrolysates obtained with the strain LBM2 at T_0, T_{24h} respectively ; (7) and (8) Hydrolysates obtained with the strain LBBS2 at T_0, T_{24h} respectively.

Optimization of Hydrolysis of Whey Proteins by the Strains

In order to enrich hydrolysates with potentially bioactive peptides, CWP hydrolysing ability of the strains was optimized under different conditions. Results proved that hydrolysis temperature, pH, C/CWP ratio and incubation time had a great effect on the DH of whey proteins. The strains exhibited activity at temperatures ranging from 30 °C to 40 °C with an optimum at 30 °C ($P<0.05$) (Table 1). Guo *et al.* [28] also reported an optimum temperature of 30 °C for hydrolysis of whey proteins by *Lb. helveticus* LB13. This could be explained by the fact that an overly high temperature might have caused proteinase denaturation and lowered enzyme activity. The effect of pH on the hydrolysis was examined for the pH range 6 to 8. Activity was maximal at pH 7 ($P<0.05$). Pan *et al.* [30] also optimized the proteolytic conditions of *Lb. helveticus* JCM1004 at pH 6.5–7.0. The ratio C/CWP of 2/1 revealed optimum hydrolysis of whey proteins by the strains ($P<0.05$). The increase of C/CWP ratio resulted in an increase in DH of whey proteins. The results could be due to greater hydrolysis of the proteins when more proteases were added [31]. The incubation time also influenced the degradation extent of whey proteins which was maximal after 48 h ($P<0.05$). Similar results were observed for the strains *Lb. acidophilus* CRL 636, *Lb. delbrueckii* subsp. *bulgaricus* CRL 656 and *Streptococcus thermophilus* CRL 804 when the incubation period on whey protein concentrate was extended [32].

Under optimal conditions, the DH values in whey protein hydrolysates obtained with *Lb. plantarum* LBBS2 and *Lb. plantarum* LBM2 were significantly higher than the strain *Lb. brevis* CHTD27 which showed the lowest rate of hydrolysis ($P<0.05$).

Analysis by SDS-PAGE revealed different profiles (data not shown) according to the strains and parameters studied. The peptides pattern produced from CWP hydrolysis differed among the *Lactobacillus* strains used, suggesting different protease specificities.

Table 1.

Effect of temperature, pH, ratio cells (C)/cow whey proteins (CWP) and time incubation on hydrolysis of CWP by the strains CHTD27, LBM2 and LBBS2, expressed by measurement of DH (%) (mean ± standard error, n=3).

	CHTD27		LBM2		LBBS2	
	24 h	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**
30 °C	18.01±0.47^{b}	28.66±1.42^{a}	38.33±0.56^{d}	64.71±0.75^{a}	39.80±1.04^{cd}	82.24±0.47^{a}
37 °C	10.80±0.85^{c}	19.49±0.85^{b}	17.19±1.41^{g}	38.98±0.75^{d}	34.88±0.75^{e}	51.27±0.56^{b}
40 °C	06.37±0.47^{d}	12.93±1.04^{c}	11.95±0.56^{i}	16.21±0.47^{g}	22.93±1.13^{i}	38.16±0.47^{d}
45 °C	04.08±0.47^{d}	06.71±0.37^{d}	10.80±0.75^{i}	15.06±0.94^{gh}	14.89±0.66^{k}	30.62±0.47^{f}
pH 6	00.00±0.00^{e}	00.00±0.00^{e}	00.00±0.00^{j}	00.00±0.00^{j}	00.00±0.00^{l}	00.00±0.00^{l}
pH 7	18.83±1.70^{b}	31.12±1.89^{a}	38.00±0.66^{d}	58.32±0.37^{c}	35.21±0.85^{e}	80.28±0.94^{a}
pH 7.5	00.00±0.00^{e}	00.00±0.00^{e}	21.12±0.38^{f}	33.58±1.41^{e}	27.51±0.28^{h}	41.33±0.66^{c}
pH 8	00.00±0.00^{e}	00.00±0.00^{e}	00.00±0.00^{j}	00.00±0.00^{j}	19.32±0.65^{j}	35.38±0.94^{e}
C/CWP	00.00±0.00^{e}	00.00±0.00^{e}	00.00±0.00^{j}	00.00±0.00^{j}	0.00±0.00^{l}	00.00±0.00^{l}
C/2CWP	00.00±0.00^{e}	06.38±0.85^{d}	13.42±0.85^{hi}	20.79±1.23^{f}	16.53±0.37^{k}	28.98±0.85^{fg}
2C/CWP	16.21±2.08^{b}	28.33±0.8^{a}	39.15±0.37^{d}	60.77±0.56^{b}	36.03±0.28^{e}	81.09±0.66^{a}

Different letters (a, b…) in hydrolysates of the same strain indicate significant differences at different conditions of hydrolysis ($P < 0.05$).

Results were related to the degree of hydrolysis of CWP upon hydrolysis by the strains. As depicted in Figure 4, at 30 °C, pH 7, ratio C/CWP (2/1) and at 48 h of hydrolysis, the strain *Lb. plantarum* LBBS2 exhibited the most important activity. The protein bands became lighter at T_{48h} resulting in higher hydrolysis. Furthermore, small fragments appeared with molecular weights lower than Lf and BSA. This is due to the production of some peptides obtained after CWP hydrolysis. The strain *Lb. plantarum* LBM2 showed less pronounced hydrolysis than *Lb. plantarum* LBBS2, whereas the strain *Lb.brevis* CHTD27 revealed thicker protein bands which are indicatives of lower hydrolysis. These results point to a different proteolytic behavior for the strains.

Antioxidant Properties of Whey Protein Hydrolysates (WPH)

The results indicated that all WPH reduced the DPPH radical to a yellow-coloured compound, manifestly because of the DPPH radical accepting an electron or hydrogen to become a stable molecular. However, antioxidant capacity was strain-specific and reached to maximum at 24 h of hydrolysis compared with intact whey proteins (T_0) for all the strains. As shown in Figure 5, WPH derived from *Lb. plantarum* LBBS2 resulted in significantly slightly higher antioxidant capacity (65.98%±0.11) compared to antioxidant activity of ascorbic acid (62.16%±2.69), followed by *Lb. plantarum* LBM2 (55.59%±1.30), whereas WPH from *Lb.brevis* CHTD27 exhibited the lowest

(33.99%±2.42) antioxidant capacity (P< 0.05).

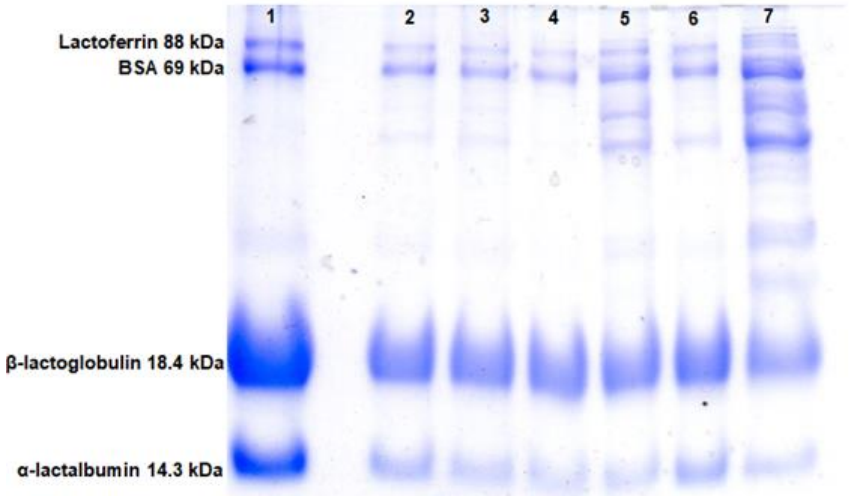

Fig. 4. SDS-PAGE analysis of CWP hydrolysed by the strains at optimal conditions: 30 °C, pH 7, with ratio C/CWP (2/1) after 48 h of incubation. (1) Non hydrolysed CWP; (2) and (3) Hydrolysates obtained with the strain CHTD27 at T_0, T_{48h} respectively; (4) and (5) Hydrolysates obtained with the strain LBM2 at T_0, T_{48h} respectively; (6) and (7) Hydrolysates obtained with the strain LBBS2 at T_0, T_{48h} respectively.

The results of NO radical scavenging assay were similar to those of DPPH radical scavenging activity. At 24 h of hydrolysis, WPH from *Lb. plantarum* LBBS2 showed significantly the highest antioxidant activity (57.41%±1.52) similar to ascorbic acid (61.71%±0.98), followed by *Lb. plantarum* LBM2 (48.29%±0.60), while WPH from *Lb.brevis* CHTD27 displayed the lowest radical scavenging properties (37.61%±1.10) (P<0.05) (Figure 5).

It was demonstrated that *Lb. plantarum* strains screened from conventional fermented food possess several functional features, including antioxidant properties [33]. The results observed in our experiments are in accordance with those reported by Lin *et al.* [10] who found that the antioxidant activity of whey protein concentrate solution was improved by enzymatic hydrolysis. This was in agreement with observations of other results that indicated a higher DPPH radical scavenging activity for an enzymatically-prepared whey protein hydrolysates than whey protein isolates [34]. The increased antioxidant activity of samples could be attributed to the hydrolysis of proteins/peptides by the proteases of *Lactobacillus* strains.

Non hydrolysed whey proteins showed a relatively low antioxidant activity. These results relating to the antioxidant activity of whey proteins are consistent with previous studies which reported that whey is essentially consisting of polar antioxidant compounds [35].

However, the radical scavenging activity of hydrolysates decreased for all strains upon further hydrolysis and was not directly connected to DH after 24 h (Figure 5). This suggests that high DH may result in further degradation of antioxidant peptides into short peptides with less activity or none [24]. These results were in agreement with the findings of Virtanen *et al.* [9] on 25 strains of LAB which showed that the radical scavenging activity is more dependent on specific proteolytic enzymes of bacterial strains than on high proteolytic

state of strains. The ability of protein hydrolysates to inhibit damaging changes caused by lipid oxidation seems to be related to the nature and the composition of the various peptide fractions produced, depending on the protease specificity [36]. Antioxidative properties of the peptides are related to their composition, structure, and hydrophobicity [6]. According to the results obtained in our work, the development of antioxidative activity was strain dependent and generally enhanced during hydrolysis but was not directly correlated with hydrolysis time [9].

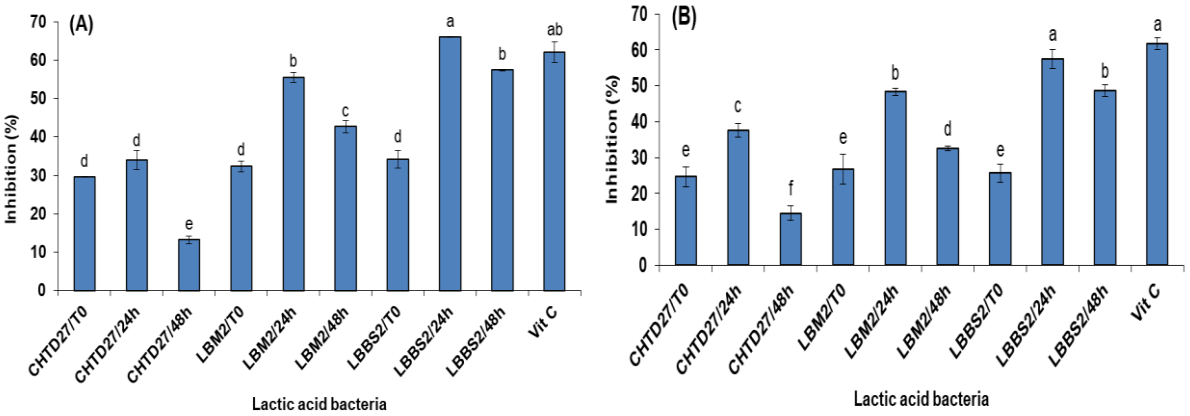

Fig. 5. Antioxidant activities of CWP hydrolysates expressed as percentage inhibition of DPPH (A), and NO (B) radicals. Error bars represent the standard error of the mean of triplicate experiments. Values with different letters (a-f) indicate results significantly different ($P < 0.05$).

Antibacterial Activity

As depicted in Table 2, no inhibitory activity was observed nor for non-hydrolysed whey proteins (T_0) nor for the hydrolysates at T_{24h} for all the strains. Nonethless, hydrolysates obtained after 48h from the strains *Lb. plantarum* LBM2 and LBBS2 exhibited growth inhibition against the target strains tested except *Bacillus subtilis* that was not affected. Salami *et al.* [8] noted that hydrolysis of cow whey proteins showed a tendency for increased antimicrobial potential when compared to non-hydrolysed whey proteins. Furthermore, the substrate specificities of CEPs determine the pools of generated peptides, which are responsible for the functional activities exerted [24]. WPH derived from the strain *Lb. plantarum* LBM2 showed antagonistic effects against 7 tested strains (2 Gram positive and 5 Gram negative), WPH from *Lb. plantarum* LBBS2 were able to inhibit 6 target microorganisms (1 Gram positive and 5 Gram negative), whereas hydrolysates obtained with *Lb. brevis* CHTD27 were unable to inhibit the growth of the thirteen target bacteria assayed. Furthermore, the highest inhibition halo zones were obtained with WPH from *Lb. plantarum* LBBS2. This could be related to its proteolytic activity which was significantly the most important ($P < 0.05$) (Table 2).

Molecular distribution profiles obtained at optimal conditions showed that hydrolysates obtained by the strains *Lb. plantarum* LBBS2 and LBM2 which exhibited antibacterial inhibition also possessed a higher proportion of peptides, in contrast with the strain *Lb. brevis* CHTD27 (Figure 4).

Table 2.

Antibacterial activity of hydrolysates obtained from the strains at t_0, t_{24h} and t_{48h} against Gram positive and Gram negative bacteria.

Microorganisms	Samples tested								
	CHTD27			LBM2			LBBS2		
	T_0	T_{24h}	T_{48h}	T_0	T_{24h}	T_{48h}	T_0	T_{24h}	T_{48h}
Gram$^+$									
Staphylococcus aureus 25923	na	na	na	na	na	na	na	na	12.95±0.086^a
Bacillus subtilis	na	na	na	na	na	na	na	na	na
Bacillus cereus	na	na	na	na	na	07.60±0.17^d	na	na	na
Enterococcus faecium	na	na	na	na	na	08.94±0.20^c	na	na	na
Gram$^-$									
Pseudomonas aeruginosa 27853	na	na	na	na	na	07.8±0.11^d	na	na	na
Acinetobacter baumanii	na	na	na	na	na	na	na	na	09.36±0.069^c
Escherichia coli 25922	na	na	na	na	na	07.86±0.054^d	na	na	na
Salmonella typhi	na	na	na	na	na	06.85±0.17^e	na	na	na
Proteus mirabilis	na	na	na	na	na	na	na	na	09.15±0.11^c
Citrobacter freundii	na	na	na	na	na	07.96±0.16^d	na	na	na
Klebsiella pneumoniae	na	na	na	na	na	na	na	na	10.71±0.23^b
Serratia marcescens	na	na	na	na	na	09.55±0.11^c	na	na	12.35±0.13^a
Enterobacter aerogenes	na	na	na	na	na	na	na	na	12.84±0.12^a

Results are expressed as the mean of the diameter of the inhibition zone in mm±standard error, n=3. Different letters (a-e) indicate significant differences between hydrolysates ($P<0.05$). na: no antibacterial activity

Thus, the pattern of antibacterial activity observed for *Lb. plantarum* LBBS2 and *Lb. plantarum* LBM2 strains could be associated with their proteolytic activity on whey proteins, since most antibacterial capacity accords with the greatest proteolytic activity and the increase of peptides production.

Pellegrini *et al.* [37] reported that proteolytic digestion of bovine β-Lg by trypsin generates four peptide fragments with bactericidal activity. Nevertheless, the most studied antimicrobial peptides are the lactoferricins derived from Lf. The antimicrobial activity of lactoferricin seems to be related with the net positive charge of the peptides. These cationic peptides kill sensitive microorganisms by increasing cell membrane permeability [6]. Indeed, the difference in amino acid sequence, charge distribution, net charge, size, and amphipathicity as well as secondary structure could be responsible for the differential behavior of antibacterial peptides against bacteria [38].

4. Conclusion

In this study, whey proteins extracted from cow milk were used in order to analyze their degradation by strains of

Lactobacillus isolated from ewe milk and camel milk. Weak hydrolysis of whey proteins was noted for the camel milk strain *Lb. brevis* CHTD27. However, interesting rate of hydrolysis was observed for two ewe milk strains *Lb. plantarum* LBBS2 and *Lb. plantarum* LBM2, suggesting the potential interest of these strains. Optimum of hydrolysis of bovine whey proteins was obtained at pH 7, 30 °C, with ratio cells/whey proteins of (2/1) and after 48 h of incubation. Hydrolysates derived from *Lb. plantarum* LBBS2 and *Lb. plantarum* LBM2 exhibited antioxidant and antibacterial activities suggesting that proteases of these strains have the ability to release bioactive peptides from cow whey proteins. At optimal conditions, hydrolysates from the strains *Lb. plantarum* LBBS2 and *Lb. plantarum* LBM2 have displayed a large activity spectrum towards different Gram positive and Gram negative bacteria. However, the highest antioxidant activity was obtained from these same hydrolysates at 24 h. Based on these interesting properties, whey proteins could be used for the production of molecules with a promising interest as antibacterials and antioxidants. The strains *Lb. plantarum* LBBS2 and *Lb. plantarum* LBM2 could represent promising candidates to be used as potential food biopreservatives. Meanwhile, additional studies are needed to prove the safety of these strains, to identify and characterize peptides produced and nutraceutical properties.

5. References

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