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Proteolytic Capacity of *Lactobacillus Delbrueckii Subsp. Bulgaricus* Cil 1671 about the Betalactoglobulina Caprina

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Abstract

Cow's milk is essential in human nutrition, its consumption is restricted by allergies to milk proteins, which constitutes a serious health problem worldwide WAO [1], WHO [2]. Among the allergenic lactoproteins the Betalactoglobulin (β-Lg) is finding. In cases of allergies to cow's milk, the consumption of goat's milk is a food alternative, despite the risk of allergic reactions due to the similarity of the 95% sequences that both species share. Many investigations have focused on the hydrolysis of milk proteins as a solution to food allergy events and the use of bacterial strains generating proteases, but not all strains have this capability. Pescuma M [3] among several studies refer that the strain of Lactobacillus delbrueckii subsp. bulgarirus CRL 656, which is an acidolactic bacterium, reduces the allergenic effect of β-Lgs. Based on the capacity of this bacterium, widely used in the dairy industry, the evaluation of the proteolytic capacity of the Lactobacillus delbrueckii subsp. bulgaricus CIL 1671 isolated in goat milk on β-Lg goat, with biotechnological interest as a starter in the dairy industry.

Keywords: Lactobacillus; Milk; Goat; Betalactoglobulin

Introduction

There is a marked expression of allergic problems due to the intake of cow's milk (*Bos taurus*), Tomotake et al. [4], Garcia et al. [5], Katz et al. [6], Mousallem & Burks [7], Moreno [8], which prevent the habitual consumption of milk as a primary source of animal proteins and constitute a serious health problem worldwide, WHO [2]. These allergies are of the food type and are adverse reactions to foods of non-toxic origin, World Allergy Organization WAO [1]. They go by immunological mechanism with generation in the body of Immunoglobulins E (IgE) on contact with milk proteins (allergen), Chacón [9]. Among dairy proteins, β-Lg is the most responsible in cases of allergies, Durán P (2005), for its absence in human milk According to Park and Haenlein [10]. It has been found that goat β-Lgs are less allergenic than bovine *β*-Lgs, Kapila et al. [11] of lower proportion in LC, Sanz [12] and Ballester [13], constitutes a food alternative, Capra [14], with tolerance in 40% of sensitive patients, Chacón [9]. However, there are cases of allergies due to the intake of LC, Santos & Fernández [15], Fiocchi et al. [16] due to 95% similarity in the sequence of the βLG gene in LV and LC, [12,13], PDB Protein Data Bank. Pescuma et al. [3] indicate that the *Lactobacillus delbrueckii subsp. bulgaricus* CRL 656 hydrolyzes the allergenic sequences of *β*-Lg with reduced allergenicity and projection of use in the development of hypoallergenic dairy products. In this sense, the isolation of a strain of *Lactobacillus delbrueckii subsp. bulgaricus* from samples of milk of LC with proteolytic capacity on the goat β-Lgs caprines of biotechnological value in the dairy industry.

The Problem

The incidence of cases of food allergies derived from the consumption of proteins not only from cow's milk but also in goat's milk, this constitutes a health and food problem. Different investigations have arisen with fundamentals in the hydrolysis of the protein by different routes, among them the generation of proteases of bacterial origin. There are proteolytic bacterial strains, but not all of them have these characteristics, therefore, the isolation and identification of the proteolytic capacity of an indigenous bacterial

strain is proposed, which apart from providing improvements in the quality attributes of dairy products, such as texture and flavor, present proteolytic capacity on the problem protein. This gives the strain an appreciable biotechnological value for the dairy industry.

Justification

Goat milk is an alternative to cases of food allergies due to the consumption of cow's milk proteins, its consumption is also limited by the great similarity in the sequence of *β*-Lg, considered the most allergenic protein present in milk , and this constitutes a serious health problem worldwide. That is why there is a need to investigate and propose biotechnological strategies that reduce or inactivate the mechanism of allergenic action of this protein and obtain native bacterial ferments.

Objectives

General Objective: Isolate and identify a strain of *Lactobacillus delbrueckii subsp. bulgaricus* with proteolytic capacity on Betalactoglobulins (β-Lgs) of goat's milk (Capra hircus).

Specific Objectives

a) Isolate and phenotypically identify a strain of *Lactobacillus delbrueckii subsp. bulgaricus* from goat's milk (Capra hircus).

b) Identify at the molecular level a strain *Lactobacillus delbrueckii subsp. bulgaricus* by sequencing the 16S rDNA.

c) Determine the proteolytic activity of *Lactobacillus delbrueckii subsp. bulgaricus* on Betalactoglobulins (β-Lgs) present in goat's milk (Capra hircus).

Materials and Methods

Isolation and Phenotypical and Genotypical Identification of *Lactobacillus delbrueckii subsp. bulgaricus* CIL 1671.

Isolation and Phenotypic Identification of Lactobacillus.

Three (03) samples of goat's milk come from a mechanical milking pool of 22 mixed Alpine and Canary goats from the Caprinos Experimental Unit of the Faculty of Veterinary Sciences of the Central University of Venezuela, based in Limón, Maracay-Estado Aragua, with intensive production system for milk production, with geographical location: Latitude North 65171, Latitude East: 1136119, Time zone: 18, Datum: La Canoa (PSAD 56). The samples are analyzed under criteria of Venezuelan Standards COVENIN 903: 1993 for handling samples from farms to the laboratory. Regarding physico-chemical analysis, the guidelines in COVENIN 0983-1983 [17] and for microbiological sample management according to COVENIN 0902: 1987 are followed. The total lactic acid bacteria are isolated according to COVENIN 3006: 1993 [18], Guidelines by Evaluations of Probiotics in Food, FAO [19], Bergey's 2005 Manual, which describe morphological aspects of the characteristic colonies. The Biochemical Identification Profile follows the fermentation scheme of 5 N-acetylglucosamine (GlcNAc), glucose, lactose, maltose, sucrose and trehalose sugars confirmed by changing the color of the medium from red (pH 6.8) to yellow (pH 5.4) after incubation 24 hours at 37 ± 1 °C, with positive results for recognition of colonies of *Lactobacillus delbrueckii subsp. bulgaricus*, Tanigawa K & Watanabe K [20] a sixth sugar, mannitol, is used as a negative control (Figures 1 & 2).

Figure 1: Colonia's de *Lactobacillus Delbruck subsp. bulgaricus* CIL 1671.

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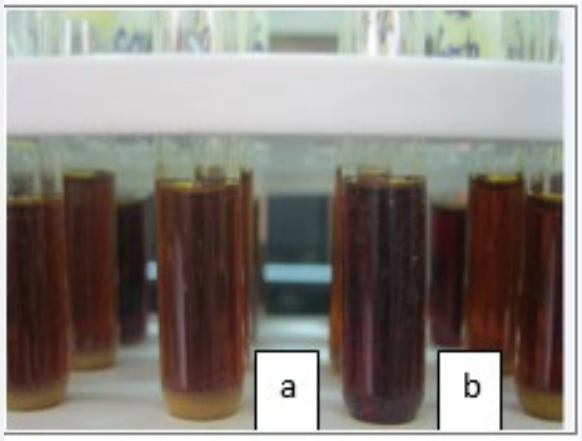

Figure 2: Sugar Fermentation Gallery proposed by Tanigawa and Watanabe, (2011), for *Lactobacillus delbrueckii subsp. bulgaricus.* a) Positive result b) Negative control.

Genotypic identification of Lactobacillus

Bacterial DNA extraction:

The activities are carried out in the ESAT-INIA Biotechnology Laboratory), the protocol of Wilson, K. (1999) is used to extract genomic DNA with the use of Phenol Chloroform. DNA concentrations are quantified on a Nano-Drop 2000.de Thermo-Scientific Spectrophotometer with results in the concentrations of 150ng of DNA / ul of average sample. Quality of DNA obtained in 1% agarose gel Buffer TE 1X with use of Sybr-Safe intercalating agent (INVITROGEN) is evaluated, the sample aliquot used is 5 ul. at a rate of 2.5 ul of sample and 2.5 ul. Loading Dye loading buffer with bromophenol purple as indicator and 1 ul. of the Benchtop molecular weight marker with 2.5 ul. of the loading buffer. A voltage of 30 V is used for approximately 1 hour. The products are displayed in a trans-illuminator (Figure 3).

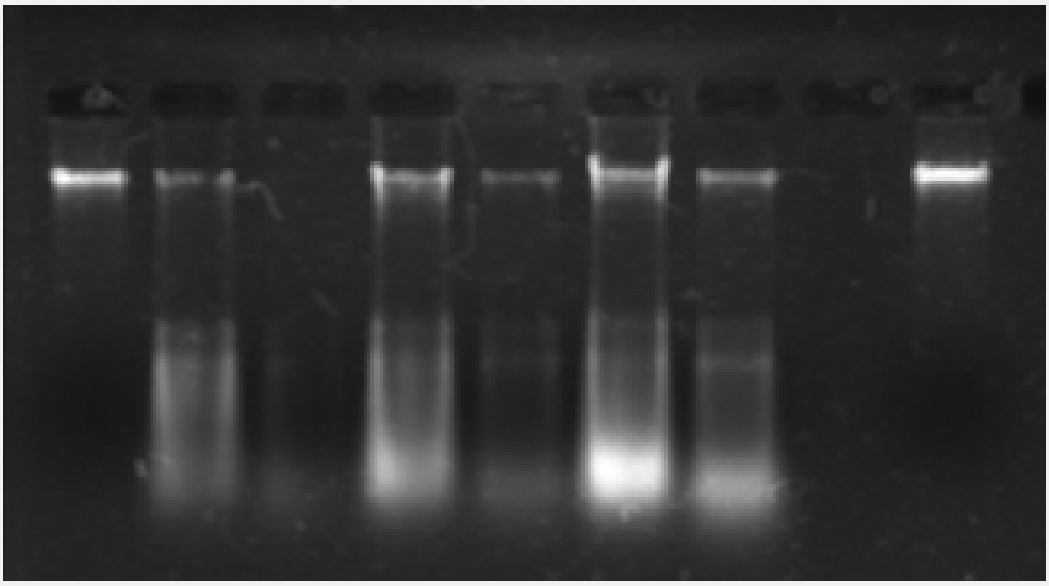

Figure 3: 1% agarose gel. MM Rails: Molecular Marker; lanes 1-5: DNA from various strains isolated in the study not phenotypically confirmatory with *Lactobacillus delbrueckii subsp. bulgaricus*; lane 6: presumptive strain of *Lactobacillus delbrueckii subsp. bulgaricus* CIL 1671.

Bacterial DNA amplification

Universal primers HAD1 and WLAB2 are used, López, et al., (2003); Súarez et al. [21], designed by Eurofins. The formulation for amplification is: 5X Buffer, 25 MgCl2, 10 mM dNTPs, 10 mM primers, 5 U Flexi GoTaq DNA Polymerase (PROMEGA) and 5ul. of diluted DNA ratio 1 DNA: 10 sterilized distilled water, for a total reaction volume of 20 ul. The 1:10 DNA ratio is since it is necessary to perform a DNA dilution profile where the dilution with the best projection of amplified DNA is 1:10 (1ul. DNA in 10ul of H_2O to ensure the concentration of amplified. The amplification profile

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used is: initial denaturation 95 °C × 5 min, followed by 35 cycles of 30 sec. at 95 °C, melting temperature of 59.3 °C × 30 sec. and 72 °C × 40 sec., with a final extension at 72 °C ×10 min. and cooling at 10 °C × 60 sec. The quality and concentration of the PCR products were examined in 2% agarose gel electrophoresis. 5 ul of the amplified are dispensed and 2 ul of loading buffer, the molecular marker used is the Benchtop 100 bp DNA Ladder of PROMEGA Corporation, with a distribution of 100 bp, from which 1 ul and 2.5 of loading buffer are dispensed.

Purification of Amplified

This procedure is performed in the Molecular Microbiology Laboratory of (IVIC), based in Los Teques, Miranda State, Venezuela. An AccuPrep® PCR Purification Kit (BIONEER) is used Cat. No. K-3034 K-3034-1) and the conditions related to the amount of DNA and primers selected are those specified by MACROGEN KOREA) concentration of 50 ng / µl 2) Minimum volume of 20µl. sample, 3) 5 ul. of primers or primers HDA1 and WLAB2 suspended in deionized water (Figure 4).

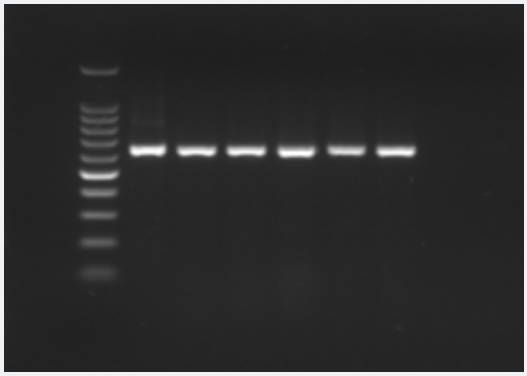

Figure 4: Evaluation of amplified 2% agarose gel, presumptive strain of *Lactobacillus delbrueckii subsp. bulgaricus*, MM: molecular marker; lane A1, A2: 1: 3 dilution; lane B1, B2: dil. 1: 5; lane C1, C2: dil. 1: 7; Lane D: 1:10.

DNA sequencing by partial sequence

The sequencing of the amplified DNA Bacterial is performed by the services of MACROGEN KOREA and is based on the identification through the partial sequencing of the 16S rRNA gene.

Alignment of the bacterial sequence obtained

The Tax Blast Report program is used, which refers to the strain as bacteria with 100% concordance, belonging to the genus Lactobacillus with 97%. Search criteria of Genbank, Nucleotide Blast of the NCBI are included the result is 100% identity for strains of *Lactobacillus delbrueckii subsp. bulgaricus* strain IMAU 32336 partial sequence of the 16S ribosomal RNA gene, *Lactobacillus delbrueckii subsp. bulgaricus* strain LB2 partial fraction of the 16S ribosomal RNA gene. The denomination of the identified strain is: *Lactobacillus delbrueckii subsp. bulgaricus* strain CIL FCV-UCV 1671, corresponding to the Center for Dairy Research of the Faculty of Veterinary Sciences of the Central University of Venezuela with internal report number in Laboratory 1671.

Evaluation of the Proteolitic Capacity of Lactobacillus delbrueckii subsp. bulgaricus CIL 1671

Obtaining Goat Milk Serum:

The serum is obtained by acid coagulation of goat's milk, with the addition of 0.1N HCL to pH 4.5-4.6, it is filtered and stored in sterile glass bottles to be cooled to 4°C until use.

Separation of β-Lg by mechanical fractionation-Molecular Excursion chromatography, [22] discontinuous and by batch (batch)

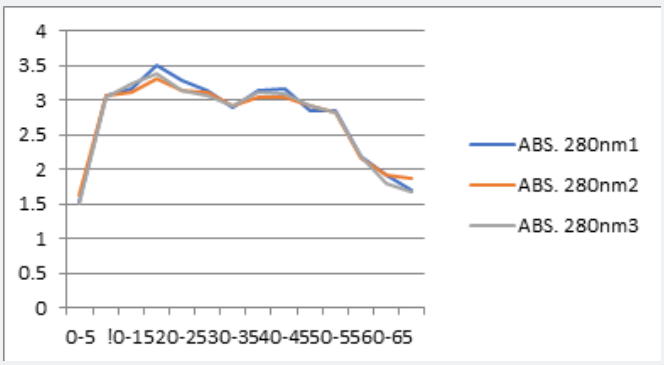

Figure 5: Absorbance values at 280nm of the filtration replicas collected in time fractions in the Sephadex G-50 column by molecular exclusion chromatography.

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A fractionation or molecular exclusion chromatographic column is used with Sephadex G-50 hydrated with phosphate buffered saline (PBS) at pH 7.4, with a constant drainage flow at 6ml / min, Bernal C [22]. Of the total serum obtained 100 ml are dispensed. of the skimmed goat milk serum in the column, the fractions are collected in time intervals of 5 minutes for approximately 2 hours (Figure 5). shows the record of high values in the corresponding time fractions between 5-10 minutes and 30-35 minutes with the formation of a peak in the curve, this tendency is manifested in each of the replicas, by likewise there is a slight increase in the fractions of time of 40-45 minutes that is repeated in each replica.

Evaluation of *β*LG separation

Sample conditioning (protein denaturation)

Aliquots of 30 ul are dispensed. of sample in a 1.5 ml Eppendorf. with addition of 30 ul. of the sun DE 1X and 10 ul. of 1N NaOH to neutralize the acidity and heat in a water bath at boiling water (100 °C) for 5 min.

Preparation of Discontinuous Polyacrylamide SDS-PAGE gel

10 ul of sample and 5 ul. of the 6-205 KD Sigma-Aldrich molecular marker are dispensed in the SDS-PAGE polyacrylamide discontinuous gel, a 10% concentrating phase and a 15% polyacrylamide separator respectively, with 1X Buffer TE. 10 V voltage for the first 10 minutes and 20 Volts for 1:30 h. (Laboratory of CIBA-FAGRO-UCV).

SDS-PAGE Polyacrylamide Gel Revelation

The revelation protocol for proteins with Coomasie R-250 blue is followed, it is recommended to place the gel in an acetic acid fixation solution to fix the proteins, the bands are visualized in a trans-illuminator. (Figure 6). Polyacrylamide discontinuous SDS-PAGE. MM Rail: Molecular Marker; lane 1: acid whey from goat's milk; lane 2: Fraction 0-5 min .; lane 3: Fraction 5-10 min; lane 4: Fraction 15-20 min .; lane 5: Fraction 25-30 min. lane 6: Fraction 35-40 min .; lane 7: Fraction 45-50min .; lane 8: Fraction 60-65 min .; lane 9: Fraction> 65 min.

Quantification of *β*-Lg Separated

The Bradford methodology is used where the absorbances at 595 nm are evaluated for bovine serum albumin at different concentrations. (Figure 2) for the calculation of Protein concentrations (Figure 6), the stoichiometric equation of V1 x N1 = V2 x N2 is applied, where the Normalities are substituted for being referred to concentrations and it is obtained that: C1 x V1 = C2 x V2, with the result of: X1 = Concentration of βLG at 0.970 nm = 0.465 µg / µl. When intercepting the 0.970 slope line, a value is projected on the axis of the abscissa corresponding to 465 value close to 500µg / ml, which is equivalent to 50 µg / µl. of βLG.

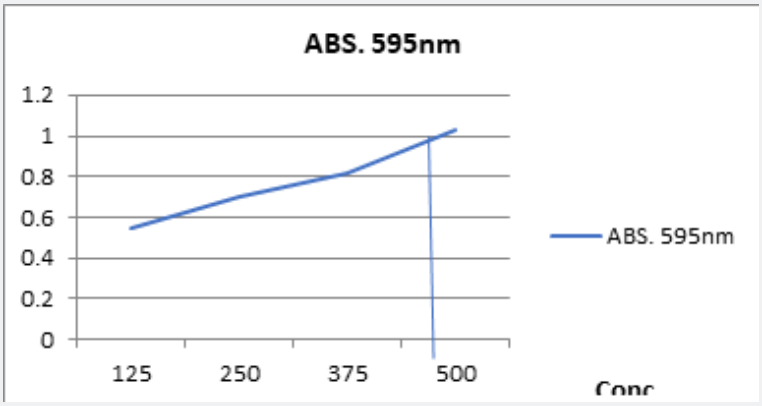

Figure 6: Fractions of serum filtrations of goat's milk vs. Polyacrylamide SDS-PAGE discontinuous gel time. MM Rail: Molecular Marker; lane 1: acid whey from goat's milk; lane 2: Fraction 0-5 min .; lane 3: Fraction 5-10 min; lane 4: Fraction 15-20 min .; lane 5: Fraction 25-30 min. lane 6: Fraction 35-40 min .; lane 7: Fraction 45-50min .; lane 8: Fraction 60-65 min .; lane 9: Fraction> 65 min.

Evaluation of the Proteolytic capacity of Lactobacillus delbrueckii subsp. bulgaricus on the β-Lg Caprina

Treatments are established for time periods 0, 3, 6, 12 hours of incubation of the system with 3 repetitions, at an incubation temperature of 37 ± 1 °C, under aerobic conditions. Changes in optical density at 595 nm, total protein content and acid values measured in lactic acid are recorded. The degree of proteolysis is evidenced by the integrity of the band of the β-Lg fraction by polyacrylamide discontinuous gel electrophoresis in the presence of Sodium Dodecyl Sulfate (SDS-PAGE) Laemmli (1970), CIBA-FAGRO-UCV, Figure 7, methodology also used by multiple investigations such as the case of Pescuma et al. [16]and Wang Y. et al., (2011). Figure 8. The bands resulting from the proteolytic effect of bacterial action on the target protein (βLG) are shown in Figure 9. A molecular marker designed by extractions of the proteins present in the Sigma-Aldrich marker and the incorporation of the purified protein as a higher molecular weight protein was used in the design of the gel represented in the graph. For this the molecular marker was mixed, 5 ul are added. of purified βLG protein, in addition 100 ul are added. of sterilized deionized water, it is subtly stirred and then dispensed in the gel 15ul. of this mixture. The degradation

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effect of the band corresponding to *β*-Lg (18.3 KDA) and the presence of multiple bands correlated with the peptides resulting from bacterial proteolysis can be seen, Figure 8. Acidity variations in the acidity are also recorded. time, absorbance at 280 nm and total colony count of *Lactobacillus delbrueckii subsp. bulgaricus* in CFU / ml in MRS agar. The acidity increases over time with different tendencies, in milk it is more pronounced than in the case of whey and purified protein. This could be related to the availability of lactose in each substrate and bacterial growth. Figure 3 Regarding the trend of the protein level curves in milk, it is observed that there is a slight increase, otherwise in the case of whey and the βLG protein that remains with a slight tendency to decrease, in contrast to what has been expressed by Conti et al., (2011), El-Gyzawi SA et al. [23]. Figure 4 In the case of bacterial growth, a good development in milk is observed, followed by serum and β-Lg., Which may be the cause of the availability of nutrients in each substrate, especially lactose (Figure 5).

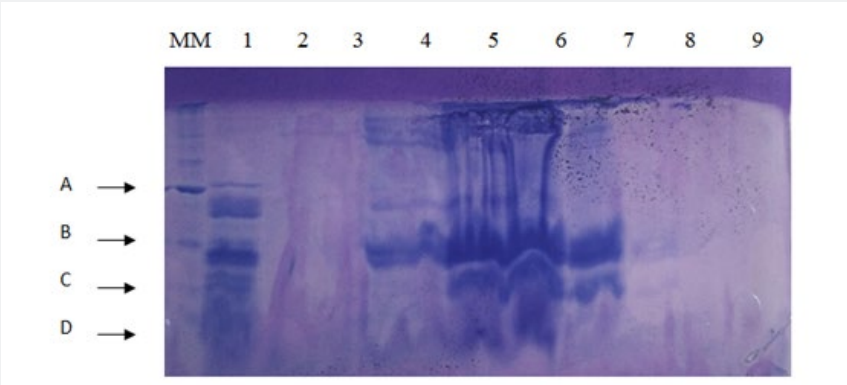

Figure 7: Absorbance curve of bovine serum albumin according to different concentrations according to the methodology described by Bradford, 1976. The curve is intercepted given the absorbance value of the βLG sample and projects a result on the axis of abscissa close to 500 µg / ml.

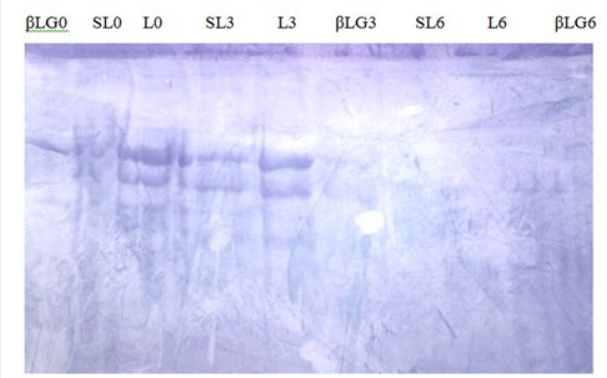

Figure 8: SDS-PAGE Polyacrylamide gel showing the proteolytic capacity of *Lactobacillus delbrueckii subsp. bulgaricus* in Fresh Goat Milk (L), Goat Milk Serum (SL), βLG isolated from goat milk serum (βLG) at different times: βLG0, L0, SL0 = Time 0 hours; βLG3, L3, SL3 = Time 3 hours of incubation; βLG6, L6 Sl6 = Time 6 hours of incubation. Incubation temperature 37 ± 1°C.

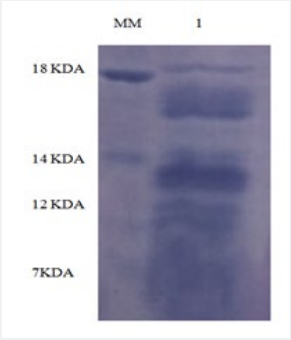

Figure 9: Fractionation of goat milk betalactoglobuline obtained by molecular weight fractionation. Comparison of molecular weight of bands obtained with the Sigma molecular weight marker of 100bp.

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Results

a) Six (6) bacterial strains were isolated from fresh goat milk samples in MRS agar and broth selective media from Hi-media Laboratories, India and RPI Research Products International Corporation respectively, with characteristics characteristic of acid-lactic bacteria. Three (03) strains of the were identified by their morphological and phenotypic characteristics as bacteria of the genus Lactobacillus. Of which at one (01) of the strains the DNA is sequenced with universal primers HAD1 and WALD2 and shows a similarity of 100% with the sequences of the *Lactobacillus delbrueckii subsp. bulgaricus* that rest in the Gene Bank database (Figures 10-12).

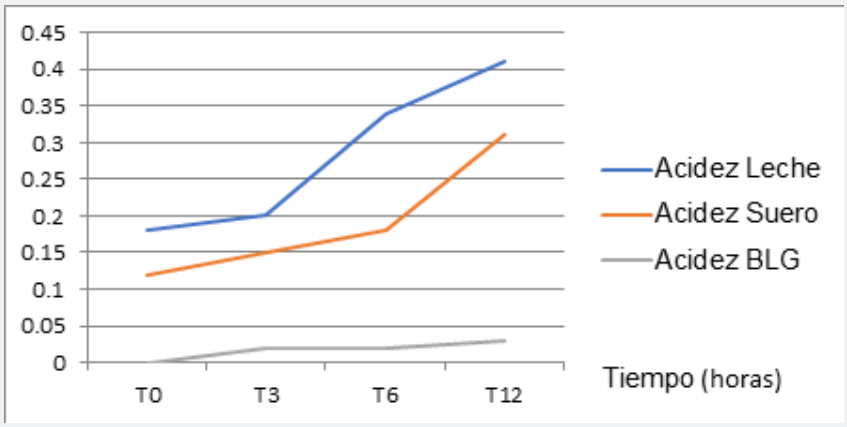

Figure 10: Total Protein Content Values vs. incubation time of *Lactobacillus delbrueckii subsp. bulgaricus* on each experimental substrate.

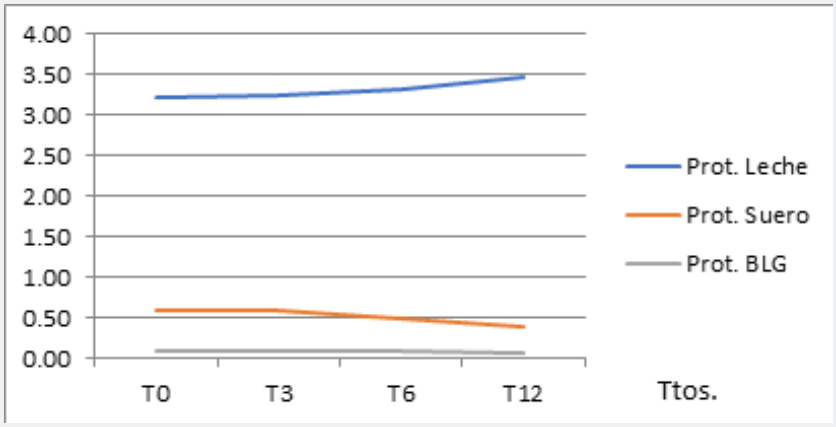

Figure 11: Total Protein Content Values vs. incubation time of *Lactobacillus delbrueckii subsp. bulgaricus* on each experimental substrate.

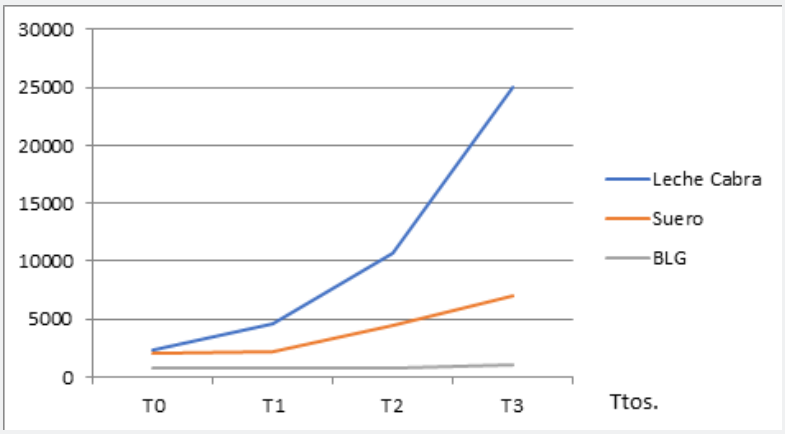

Figure 12: Colony count of *Lactobacillus delbrueckii subsp. bulgaricus* in CFU / ml. in different substrates.

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b) An effective separation of the Betalactoglobulin protein fraction from the rest of the protein components present in the fresh goat's milk whey was achieved, at a rate of 465 ug / ul% BH with the use of the mechanical-selective fractionation technique by chromatography Molecular exclusion, visualizable in discontinuous polyacrylamide gel SDS-PAGE [17-20]

c) The strain isolated and identified at the phenotypic and genotypic level as *Lactobacillus delbrueckii subsp. bulgaricus* CIL 1671. Presents proteolytic capacity on goat betalactoglobulin.

d) Peptides resulting from the proteolytic activity of *Lactobacillus delbrueckii subsp. Bulgaricus* on Betalactoglobulin isolated from fresh goat's whey are nonspecific, observable in discontinuous gels of Polyacrylamide SDS-PAGE at 15 and 20 respectively and of comparable hydrolyzate bands with low molecular weight marker Sigma-Aldrich-Germany. Porteolytic enzymes generated by Lactobacillus delbrueckii subsp. Bulgaricus are unknown as well as their cut-off sites on the β-Lg sequence. [21-25]

Conclusion

1. A strain with phenotypic and genotypic characteristics characteristic of those referred to in the Gen Bank database was isolated, with 100% identity with reported strains from other latitudes and identified as *Lactobacillus delbrueckii subsp. bulgaricus* strain CIL 1671, which proved to have proteolytic capacity over goat β-Lg.

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a) Chair of the Milk and Meat Industry. Central University of Venezuela, Faculty of Veterinary Sciences UCV-Maracay-Aragua.

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c) Agronomic Biotechnology Research Center (CIBA), Faculty of Veterinary Sciences, Central University of Venezuela, UCV-Maracay-Aragua.

d) Animal Biotechnology Laboratory. Socialist School of Tropical Agriculture (ESAT). National Institute of Agricultural Research (INIA). Maracay-Aragua

e) Experimental Microbiology Laboratory of the Venezuelan Institute of Scientific Research (IVIC). The Teques-Miranda.

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