

FURTHER STUDIES ON A BOVINE PLASMA MEDIUM THAT CAN BE HEAT STERILIZED FOR LACTOBACILLI

Nuevos Estudios Sobre un Medio de Cultivo de Plasma de Bovino que puede ser Esterilizado por Calor para Lactobacilos

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ABSTRACT

Preparation and efficiency of a bovine plasma medium (BPM), based on liquid or powder bovine blood plasma that can be heat sterilized, is described. Protein, amino acids, and pH of the medium were analyzed. The efficiency of the BPM was compared against a commercial medium (MRS) using *L. plantarum*, *L. casei*, *L. bulgaricus* and *L. acidophilus*. Individual effect of minerals and yeast extract were also tested. Results shown that BPM either from liquid or powder bovine plasma can be prepared by heat sterilization, remaining clear and without any turbidity if the pH is previously adjusted to 11. Final pH, protein and amino acid contents were similar when the BPM was prepared by either liquid or powder bovine plasma. When the efficiency of the new medium was compared against commercial MRS, no differences in the growth of the different Lactobacilli were observed. Mineral supplementation, was more efficient than yeast extract for Lactobacilli growth in BPM. Even without minerals and yeast extract supplementation, an important growth of Lactobacilli was observed when liquid or powder bovine plasma was used as a source of protein. It is concluded that new low cost, practical medium has been developed for the propagation of Lactobacilli.

Key words: Medium, bovine plasma, lactobacilli.

RESUMEN

En el presente trabajo se describe la preparación y eficiencia de un medio a base de plasma (BPM) líquido y en polvo que puede ser esterilizado por calor. La eficiencia del BPM fue comparado con un medio comercial (MRS) utilizando *L. plantarum*, *L. casei*, *L. bulgaricus* y *L. acidophilus*. El efecto individual de los minerales y el extracto de levadura también fue probado. Los resultados muestran que el BPM bien sea líquido o en

polvo puede ser preparado por esterilización con calor, permaneciendo claro y sin turbidez, si el pH previamente se ajusta a 11. El pH final proteína y contenidos de aminoácidos fueron similares en el BPM preparado con plasma líquido o en polvo. Cuando la eficiencia del nuevo medio fue comparado contra un medio comercial (MRS) no hubo diferencias en el crecimiento de los diferentes lactobacilos. La suplementación con minerales fue más eficiente que el extracto de levaduras para el crecimiento de los lactobacilos. Un crecimiento importante de los lactobacilos fue observado en el medio a base de plasma sin suplementar con minerales y extracto de levaduras. Se concluye que un medio nuevo, práctico de bajo costo ha sido desarrollado para la propagación de lactobacilos.

Palabras clave: Medio, plasma de bovino, lactobacilos.

INTRODUCTION

Lactobacillus strain are particularly known for their widespread application in industrial fermentation processes. The use selected Lactobacilli as food supplements has gained popularity, as consumers have recognized the desirability of fermented food and the contribution of certain intestinal Lactobacilli to human well being [23]. Some reviewed papers suggest attributes such as potential therapeutic benefit attributed to the favorable alteration in gastrointestinal microecology, prophylaxis against some types of intestinal infections, increasing the immune response, increasing tolerance to lactose containing foods, reducing the risk of colon cancer and decreasing serum cholesterol level [15,16,17,19,21]. Strains of Lactobacilli are used as starter cultures for dairy [4], meat [3], and vegetables products [9].

Lactobacilli are known to be distributed widely in nature, including, dairy products and digestive tracts of human and

animals, which are rich in amino acids and other nutrients. These favorable conditions were Lactobacilli grow may be responsible for some lesion encounter in Lactobacilli that make them incapable of synthesizing all the amino acids and that can be related with the habitat origin [20]. A medium for propagation of these bacteria must supply a variety of nutrients being the most important the amino acids. Several media have been formulated over the years for Lactobacillus propagation [7, 12, 14], being MRS most widely used for Lactobacilli cultivation [11]. Whey permeate has been used as base medium for propagating starter cultures [2, 8], however one disadvantage is the lack of sufficient amount of amino acids. To compensate the lack of amino acids nonfat dry milk solids and yeast extract were added to improve its efficiency [8].

Bovine plasma is a low cost animal subproduct that contains 7% protein with all the essential amino acid. This characteristics make it suitable to be used a source of amino acids for *Lactobacillus* propagation.

Increase in the growth of *Lactobacillus* when plasma albumin is used to enrich a medium was reported by Briggs [7] and Barboza et al. [5], however, they reported that this medium has the limitation that can not be subjected to heat-sterilization because of its tendency to coagulate, even at low concentration of bovine plasma. In other study, Barboza et al, [6] reported that a medium prepared with bovine liquid plasma can be prevented for gelation by increasing pH above 10. The objective of this work was to formulate a Lactobacilli medium using liquid and commercial powder plasma and compare this efficiency against the MRS commercial medium.

MATERIALS AND METHODS

Plasma collection

Two liters of fresh bovine blood obtained from a local slaughter house was collected in plastic clean containers with a solution of sodium citrate at 0.2% per 100 mL of blood. Then it was transported, refrigerated (at 5°C), to the laboratory where it was immediately separated in their fractions plasma and red cells by centrifugation at 2500 x g during 25 min in a centrifuge (International Model K N°69984M23).

Powder Bovine Plasma

Powder bovine plasma (PBP) used in this study was obtained from American Meat Protein (AMPC, Inc. 325 North Loop Drive, P.O. Box 645. Ames, Iowa 50010 USA).

Preparations of bovine plasma medium (BPM) using either liquid plasma or powder plasma

A bovine plasma solution (BPS) was prepared either by mixing 250 mL of liquid bovine plasma with 350mL of distilled water, or by suspending 27 g of powder bovine plasma and 0,25 g of EDTA in 600 mL of distilled water, pH was adjusted to 11 with

NaOH 1N prior to sterilization in an autoclave at 121°C for 15 min according to the methodology of Barboza et al. [6].

A solution of glucose, minerals and yeast extract (GMY) was prepared by dissolving 10 g of glucose, 6 g of sodium acetate, 1g of ammonium citrate, 3 g of KH₂PO₄, 0.05 g of MnSO₄H₂O and 5 g of yeast extract Difco Laboratories (Detroit Michigan USA) in 400 mL of distilled water, mixed and heated with frequently stirring until complete dissolution, then sterilized in autoclave under the same conditions as the diluted BPS.

To make bovine plasma medium (BPM), 600 mL of sterile BPS was mixed with 400 mL of sterile (GMY) solution. Addition of the GMY to the BPS solution lowered the pH to 6.4 approximately.

Preparation of the BPM agar

BPM agar was prepared by adding 20 g of agar to 400mL of GMY solution, mixed and boiled with frequently stirring until complete mixed and boiled with frequently stirring until complete dissolution, sterilized in autoclave at 121°C for 15 min and mixed immediately with sterile BPS solution. The solution was allowed to stand until the temperature reached 50°C and then dispensed aseptically into sterile plates.

Protein and Amino acids analysis of the BPM

Protein was analyzed using the Kjeldahl procedure AOAC [1]. Amino acids were analyzed by High-Performance Liquid Chromatography previous hydrolysis with hydrochloric acid. A Shimadzu model LC6A HPLC equipped with a FLD6A Fluorescence detector, two LC6A pumps, a SCL-6B auto injector, CTO-6A column and C-R4A Chromatopack integrator was used throughout the experiments. An Altex ultrasphere ODS, C-18, 15cm length x 4mm ID, 5 um column was used.

Two solvent systems were used. Solvent A composed by acetate buffer (0.05M), methanol and tetrahydrofurane (80:19:1). Solvent B composed by methanol and acetate buffer (80:20). A Sigma lab standard solution 50 nmol/mL amino acid concentrations was used as a reference. Precolumn derivatization of the amino acids was performed. Samples of 20ul were injected onto the column. Flow rate was 1mL/min. Fluorescence was read at 470nm with an excitation wave length of 350nm. Peak areas were used for quantitative calculations.

Bacterial strains

Microorganisms used in this research were *Lactobacillus plantarum* ATCC8014, *Lactobacillus case* ATCC7469, *Lactobacillus bulgaricus* ATCC11842, and *Lactobacillus acidophilus* ATCC4356. Culture were maintained by routine subcultures in *Lactobacillus* MRS (Man, Rogosa and Shape) broth obtained from Merck (D-61 Darmstadt) using 1% inoculate and 18h of incubation at 37°C in a Gas Pak jar with 10% CO₂, and were refrigerated (5°C) between transfer. Test cultures were transferred at least three times before being used.

Efficiency of the BPM as compared to a MRS commercial medium

The efficiency of the BPM as a culture medium, prepared with liquid or powder plasma, was compared against the MRS commercial medium. Microorganisms were grown in each medium for 18h at 37°C in a Gas Pak Jars with 10% CO₂. Lactobacilli cell numbers in growth media were estimated by plate count surface or spread method on BPM and MRS agars. Plates were incubated at 37°C for 48h in Gas Pack jar with 10 CO₂. Results were expressed as log colony-forming units per milliliters. pHs of the cultures were determined at 25°C after 18h of incubation, using a Metrohom 620 pH meter.

Effect of minerals and yeast extract

To evaluate the need for minerals and yeast extract supplementation, 600 mL of BPS prepared with liquid bovine plasma was mixed with either 400 mL of a sterilized solution of glucose and potassium dihydrogen phosphate (potassium dihydrogen phosphate was added to lower the pH to around 6.4) or 400mL of a sterilized solution of glucose, phosphate and yeast extract. The amount of glucose, phosphate, minerals and yeast extract added to the 400 mL of each the sterilized solutions was the same used to prepare the GYM solution.

Experimental Design

To compare the efficiency of the different media on the lactobacillus growth a 3x4 factorial arrangement was used with the factors being media formulations at 3 levels and 4 type of lactobacillus (*plantarum*, *casei*, *acidophilus* and *bulgaricus*). Data collected from this study were analyzed using SAS PROC GLM. Pair wise comparisons of treatment means were performed using the least significant difference (LSD) and Duncan Multiple Range Test. Procedures. Significance was determined by the F-test and differences were declared at the 5% level of probability [22].

RESULTS

Both BPM media prepared with either liquid or powder bovine plasma supported sterilization without coagulation by changing the pH to 11 before sterilization. No differences ($P>0.05$) in pH and proteins, content of both BPM were observed TABLE I. The amino acids content was expressed as mg of amino acids per mL or g of medium, the results shown the presence of all essential amino acids.

TABLE II shows mean values for *Lactobacillus* growth and final pH in the BPM and commercial MRS media. No difference in *Lactobacillus* growth was observed when BPM or commercial MRS medium was used. Final pH after 18h was between 4.1-4.2.

Results of average growth values for *L. plantarum*, *L. casei*, *L. bulgaricus* and *L. acidophilus*, in the culture media with

TABLE I
PROTEIN, pH AND AMINO ACIDS OF THE BPM MEDIUM PREPARED WITH EITHER LIQUID OR POWDER BOVINE PLASMA

| Characteristics | *BPML | **BPM ^P |
|-----------------|-----------|--------------------|
| Protein (g/100) | 2.10±0.43 | 2.18±0.57 |
| pH | 6.41±1.20 | 6.5±1.52 |
| Amino acids**** | mg/mL | mg/mL |
| Lysine | 2.86±1.53 | 2.61±0.32 |
| Leucine | 2.50±0.33 | 2.33±0.54 |
| Histidine | 1.05±0.42 | 1.20±0.67 |
| Isoleucine | 0.64±0.27 | 0.70±0.90 |
| Phenylalanine | 1.65±0.20 | 1.51±1.12 |
| Threonine | 1.93±0.30 | 1.74±1.35 |
| Valine | 2.09±0.78 | 2.25±0.56 |
| Methionine | 0.34±1.3 | 0.38±0.79 |
| Tyrosine | 1.29±1.12 | 1.41±0.66 |
| Serine | 1.62±0.45 | 1.42±0.35 |
| Glycine | 1.43±0.64 | 1.61±0.42 |
| Arginine | 2.00±0.76 | 2.13±0.50 |
| Alanine | 1.93±0.13 | 1.96±1.07 |

*BPML= Bovine plasma medium prepared with liquid plasma, **BPM^P= Bovine plasma medium prepared with powder plasma.

liquid bovine plasma as basal ingredient, with and without minerals and yeast extract supplementation are summarized in TABLE III. The least, but still remarkable growth, was obtained when culture medium was not supplemented with, either yeast extract or with minerals. Minerals supplementation was more efficient than yeast extract for Lactobacilli growth.

DISCUSSION

Results obtained are of the utmost importance because one of the limitations for using bovine plasma as a culture medium is its tendency to form gels, when subject to heat sterilization. In this case, although pH was increased up to 11 to avoid coagulation, final media pH will be around 6.4 when the rest of the ingredients were added.

Lactobacilli need an exogenous supply of essential growth factors such as minerals, vitamins and amino acids. A survey of the literature on amino acid requirements of lactic acid bacteria, revealed that glutamic acid, valine, tryptophan, isoleucine, leucine and phenylalanine are absolutely required by most species of *Lactobacillus* that have so far been examined Guirard [13], Morishita [20]; BPM is rich in protein and contains all amino acids in relative large amount. The presence of all amino acids in the BPM make it a suitable medium to the growth of microorganisms such as Lactobacilli. It is interesting to note that glutamic acid, is the most abundant amino acid found in BPM.

TABLE II

MEAN VALUES FOR *LACTOBACILLI* GROWTH AND FINAL PH IN THE BPM BASED ON LIQUID PLASMA, BPM BASED ON POWDER PLASMA AND MRS

| Microorganisms | Media | | | Final pH | | |
|-----------------------|-------|------------------|------|----------|------------------|-----|
| | BPML | BPM ^P | MRS | BPML | BPM ^P | MRS |
| <i>L. plantarum</i> | 8.49 | 8.39 | 8.50 | 4.1 | 4.1 | 4.1 |
| <i>L. casei</i> | 7.69 | 7.20 | 7.71 | 4.3 | 4.2 | 4.2 |
| <i>L. acidophilus</i> | 8.01 | 8.26 | 8.07 | 4.0 | 4.1 | 4.1 |
| <i>L. bulgaricus</i> | 7.56 | 7.78 | 7.60 | 4.1 | 4.1 | 4.2 |

BPML= Bovine plasma medium based on liquid plasma. BPM^P = Bovine plasma medium based on powder. MRS= Man Rogosa Sharpe. *LogCFU/mL.

The fact that, no differences were observed in the growth of Lactobacilli in both media (BPM and MRS) indicates the efficiency of the BPM. It contains sufficient growth factors to support propagation of Lactobacilli to a high populations or for rapid acid production. Both of which are essential to the manufacture of fermented meat, milk or vegetable products. The formulation of this media provides a necessary tool for the propagation and enumeration of this bacterial groups at relatively low cost.

The synergistic effect observed when minerals and yeast extract are used together has been reported by several researchers in other media, [10, 12, 18], The lower but still remarkable bacterial growth observed when BPM with only glucose and phosphate was used, indicates that a less expensive simple growth medium based on bovine blood plasma without the addition of minerals and yeast extract can be used for Lactobacilli propagation.

CONCLUSION

BPM contains sufficient growth factors to support propagation of Lactobacilli to a high population.

No differences were observed in the growth of Lactobacilli in both media (BPM and MRS).

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TABLE III

MEAN VALUES* FOR *LACTOBACILLI* GROWTH IN A BOVINE PLASMA MEDIUM WITHOUT THE ADDITION OF MINERALS AND YEAST EXTRACT (A) OR WITH THE ADDITION OF YEAST EXTRACT (B) OR WITH MINERALS (C)

| Microorganism | Media | | |
|-----------------------|-------------------|-------------------|-------------------|
| | A | B | C |
| <i>L. plantarum</i> | 7.44 ^b | 7.55 ^b | 8.29 ^a |
| <i>L. casei</i> | 7.23 ^c | 7.35 ^a | 7.35 ^a |
| <i>L. acidophilus</i> | 7.27 ^d | 7.56 ^b | 7.80 ^a |
| <i>L. bulgaricus</i> | 7.01 ^b | 7.10 ^b | 7.40 ^a |

*Log CFU/mL. ^{a,b,c,d}Means on a row bearing different superscripts differ significantly (P<0.05).

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