Chapter 26

Biotechnological Aspects in the Selection of the Probiotic Capacity of Strains

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Additional information is available at the end of the chapter

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1. Introduction

Several genera of bacteria and yeast have been reported as probiotics. The most used are of the genus *Lactobacillus, Bifidobacteruin and Saccharomyces.* Although the benefits of its use have been widely reported, the selection of probiotic strains with effective capacity has been a complex process that must take into account efficacy and safety conditions. In this way, the selection of strains can be divided into three stages:

1. Selection and characterization of strains
2. Capacity Assessment In vitro probiotic
3. Capacity Assessment In vivo probiotic

Strain selection includes sources of screening, identification, assessing growth conditions of biomass such as growth kinetics, substrates, pH and temperature allowing calculation appropriate kinetic parameters for comparing strains in order to establish the feasibility of industrial scale production. Also take into account the conditions of preservation and maintenance of microorganisms in stock collections to ensure genetic and metabolic stability of selected strains [1].

Some of the effects reported *in vitro* probiotics are the production of enzymes, vitamins and amino acids, adherence capacity, the antagonistic effect on pathogenic microorganisms, tolerance to bile salts, production of bacteriocins, resistance to gastric juices, the reduction of cholesterol levels and immune system stimulation among others. In general, probiotic characteristics depend on many aspects that usually does not have a single strain, it is often necessary to include characteristics of several strains in a single product.

A probiotic is a preparation or a product that contains viable microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host provoking beneficial effects to that host's health [2]. In general, the probiotic

characteristics depend on multiple aspects, which are generally not specific to a single strain. Some of the probiotic effects reported *in vitro* are the production of enzymes, vitamins, and amino acids, the capacity of adherence, the antagonistic effect on pathogenic microorganisms, tolerance to bile salts, production of bacteriocins, resistance to gastric juices, reduction of cholesterol levels, and stimulation of the immune system among others [3].

Lactic acid bacteria (LAB) belong to a group of bacteria that ferment sugars like glucose and lactose to produce lactic acid. This is important because it generates a decrease of pH and, hence, the inhibition of pathogenic and alteration microorganisms. Within this group, the existence of aerobic and anaerobic microorganisms and facultative anaerobes is recognized. The most representative LAB genre that have been used as probiotics are: *Lactobacillus*, *Leuconostoc*, *Streptococcus, Bifidobacterium,* and *Pediococcus* [4].

Lactic acid bacteria have effects that have been widely reported like the capacity to produce bacteriocins, which have antimicrobial activity against pathogens *like Listeria monocytogenes, Escherichia coli, and Salmonella* among others [5].

Likewise, the role of Lactic acid bacteria has been evaluated in food allergies, specifically in milk proteins where it has been suggested that probiotics have immunoregulatory characteristics in pathologies where the immune system [6], is implied like atopic dermatitis [6,7], genitourinary tract infections [9,10], colon cancer prevention, and reduction of colonization by *Helycobacter pylori* among others [11,12].

Probiotics, especially those contained in fermented milk, play a very important role in the prevention and treatment of diarrhea, given that they produce local intestinal and systemic effects that aid in preventing and reducing post-antibiotic therapy intestinal infections.

Several mechanisms exist by which a microorganism presents interaction against others; basically, three forms exist:

1. Competition for space,
2. Competition for nutrients,
3. Production of antimicrobial compounds attributed to the accumulation of products of fermentation processes like lactic acid, hydrogen peroxide, and bacteriocins.

Regarding yeasts, the probiotic capacity of *S. cerevisiae var boulardii* has been broadly studied; however, little is known about its action mechanism, given that research has focused on other microorganisms of greater use, mainly those from the group of the lactic acid bacteria previously mentioned [13] . This yeast has been reported as a supplement in the diets of monogastric animals like poultry, indicating that its use as a probiotic reduces some enteropathogens, produces favorable changes in the intestinal mucosa, and improves the productive behavior with rations low in protein [14]. It has also been recognized for promoting growth, increasing the production of vitamin B, helping in weight gain, improving the digestion of some foods, stimulating the immune system, improving the assimilation of nutrients, and correcting the microbial population balance.

In evaluating the probiotic capacity of strains it is important to verify their tolerance to the conditions of the gastrointestinal tract, recreating the intestinal conditions in *in-vitro* tests; thereafter, the effect should be evaluated *in vivo* [15].

2. Selection and screening of strains

A reliable probiotic product requires correct identification of the bacterial species used and a statement on the label of the species actually present. This is important because quite often the identity of the microorganisms recovered does not always correspond to the information indicated on the product label [16].

The first step for the selection of a strain with probiotic capacity is the determination of its taxonomic classification, which can give an indication of the origin, habitat, and physiology of the strain. The classification of probiotics is based on comparing the highly conserved molecules, *i.e.*, genes encoding ribosomal RNA (rRNA). Main progress in molecular biology methods has permitted sequencing the 16s and 23s rRNA subunits and, consequently, the generation of data bases of sequences of desired probiotic strains. Additionally, strains currently closely related have been distinguished by using methods based on molecular biology like plasmid profile, restriction enzyme analysis, ribotyping, random amplified DNA, and pulsed electrophoresis [17].

Once the taxonomic identification has taken place, a screening process is carried out by evaluating some physiological aspects or criteria like: [16]

- Fermentation of carbohydrates and enzymatic activity
- Adhesion to intestinal sites or areas that leads to colonization and favors equilibrium of intestinal microbiota, aids in intestinal permeability, inflammation relief, and strengthening of the barrier.
- Production of metabolites with antimicrobial activity and/or with effects at epithelial level, which help to strengthen the barrier and regulate bowel movements.
- Production of cytokines that reduce the risk of developing inflammation and generate a protection against deviations in the intestinal immune response.
- Evaluation of the link to specific toxins like mycotoxins, cyanotoxins, heavy metals, and other diet and water contaminants. This leads to the protection of the intestinal integrity and reduction of the risk of induced deviations.
- Characterization of the quorum sensing, based on detection and reaction against deviations in the diversity of intestinal tract microbiota, which favors the equilibrium of intestinal microbiota and immune response.
- Safety properties like production of anti-inflammatory cytokines contrary to pro-inflammatory cytokines and absence of antibiotic resistance genes.
- Tolerance to gastrointestinal conditions like stability at acidic pH, and tolerance to bile.

Additionally, in 2003, the FAO established some desirable key criteria for the selection of probiotics like: [12,18,19]

- Safety criteria: origin, pathogenicity, and infectivity, virulence factors (toxicity, metabolic activity, and intrinsic properties)
- Technological criteria: genetically stable strains, long-term viability of processing and storage, good sensory properties, phage resistance, and large-scale production.
- Functional criterion: tolerance to gastric juices and acids, tolerance to bile, adhesion to the surface of the intestinal mucosa, and effects on health validated and documented.
- Desired physiological criteria: immunomodulation, antagonistic activity to gastrointestinal pathogens, anti-mutagenic and anti-carcinogenic properties, and metabolism of cholesterol and lactose.

3. Conservation of strains

Freeze – drying is commonly used for the long – term preservation and storage of microorganisms in stock collections as well as for the production of starter cultures for the food industry. The choice of an appropriate suspending medium is of primary importance to increase the survival rate of the lactic acid bacteria (LAB) and yeasts during and after freeze – drying although the success of the process also depends on several factors such as growth phase, extent of drying, rehydratation, suspension medium, cruoprotectors, and so forth. During freezing or freeze – drying, cellular damage may occur, resulting in a mixed population containing unharmed cells and dead cells as well as those sublethally injured. Damage may not lead directly to death since in a suitable environment the injured cells may repair and regain normal functions.

LAB and yeasts can also be preserved for short – term storage. The techniques may be:

3.1. Short term storage

For daily or weekly use. Rich undefined media such as MRS broth (polypeptone 10g; meat extract 10g; yeast extract 10g; glucose 20g, ammonium citrate 2g; sodium acetate 5g; MgSO47H20 0,2g; MnSO44H2O 0,05g; KH2PO4 2g; Tween 80 1mL; the pH is adjusted to 6.4 ± 0.2 before autoclaving) [20] LAPTg broth (yeast extract 10g; universal peptone 10g; tryptone 16g; glucose 10g; Tween 80 1m; the pH is adjusted to 6.6 before autoclaving), [21] M17 broth (phytone peptone 5g; polypeptone 5g; yeast extract 5g; beef extract 2.5g; lactose 5g; acorbic acid 0.5g; β – disodium glycerophosphate 19g; MgSO47H20 1mL; the pH is adjusted to 7.1 before autoclaving) [22], or Elliker broth (tryptone 20g; yeast extract 5g; gelation 2.5g; dextrose 5g; lactose 5g; sucrose 5g; sodium chloride 4g; sodium acetate 1.5g; ascorbic acid 0.5g; the pH is adjusted to 6.8 before autoclaving) [23] are commonly used for LAB. For the storage of yeasts, rich undefined media such as YPG (yeast extract 10g; peptone 20g; glucose 20g), YGC (yeast extract 5g; glucose 20g; chloramphenicol 0.1g).

3.2. Storage on liquid medium

Tubes of any of the broth media, as described previously. Inoculum: bacterial cells, grown for 16 h in any of the media described to approximately 10^8 – 10^9 CFU/mL or McFarland´s tube No. 3

3.3. Long – term storage

Where inmediate acces is less important, but maintenance of the characteristics of the species and the strains is the primary objective.

3.4. Lyophilization

Cultures grown in any of the cultures media describe previously, for 16 h (overnight) at 37°C. In the case of thermophilic species the optimum incubation temperature may be in the range 39 – 41°C.

- Prepare outer vials by placing a small amount of silica gel granules (6 – 16 mesh) in the vial to cover about half of the bottom. Add a small cotton wad to cushion the inner vial and heat at 100° C overnight. The silica gel should be dark blue after heating; this serves as a moisture indicator during storage. Place vials in a dry box (<10% relative humidity) to cool.
- Aseptically, mix equal amount of inoculum (washed) and suspending medium in a sterile tube or bottle.
- Inoculation of the inner vial: Six drops of the mixture (0.2mL) are transferred to the bottom of each vial with a sterile Pasteur pipet.
- Replace the cotton plug and trim it so the cotton is even with the rim of the vial. Place de inner vial in a pan, in racks, or in boxes in a freezer at – 60°C to – 70°C and let the sample freeze for 1 – 2 h.
- Chamber – type freeze – dryer: The plates of the freeze dryers should be frozen as well. Let the condenser cool at – 60°C to – 70°C about 30 – 45 min and then place the frozen inner vials on the plates. Evacuate the system to below 30 μmHg.
- Start the process in the afternoon and allow to run about 18 h. The system is monitored by a thermistor vacuum gage. When the vacuum sensor is placed between the product and the condenser, it will show an increase in pressure as drying occurs. However, when drying is complete, the pressure should return to below 30 μmHg.
- When the cycle is complete, close the vacuum line between the chamber containing the plates with the dried samples and the condenser. Open the valve on the inlet port to admit air, allowing pressure in the cabinet to reach atmospheric pressure.
- Insert the inner vials into the outer vials. Tamp at ¼ inch plug of glass fiber paper above the cotton – plugged inner vial. Heat the outer vial in an air/gas torch, rotating the vial and keeping the flame just above the glass fiber paper until the glass begins to constrict. Pull the top of the vial slowly with forceps until the constriction is a narrow capillary tube. Cools the vials in a dry cabinet.
- Attach each vial to a port of a manifold. Each port has a single – holed rubber stopper that fits the open end of the vial. Evacuate the system to less than 50 μmHg. Seal the vials at the capillary using a double – flame air/gas torch.
- Store vials at 2 – 8°C. To open the vials, heat the tip of the outer vial in a flame, then squirt a few drops of water on the hot tip to crack the glass. Strike with a file or pencil to remove the tip. Remove the fiber paper insulation and the inner vial. Use forceps to

gently remove the cotton plug and rehydratate with 0.3 – 0.4mL of appropriate broth medium. When suspended, transfer the content to 5 – 6mL of broth and incubate ate the selected temperature for 16 – 18 h.

3.5. Freezing

- Inoculum: washed bacterial cells obtained by centrifugation of cultures grown for 16 h in any of the media described, and takedn to half of the initial volume (approximately 10^8 – 10^9 CFU/mL or McFarland´s tube No. 3) with sterile distilled water.
- Inoculation into NFM: Harvest and wash once by centrifugation the cells rom a 10mL overnight culture. Resuspend the cell pellet into 1 – 2 mL 10% NFM supplemented with 1% (w/v) glucose, 0.5% (w/v) yeast extract, and 10% (v/v) glycerol (final concentration) and store in a domestic freezer (- 20°C to – 30°C) or even better, at – 60°C to – 70°C.
- Inoculation into glycerol solution: Take an aliquot of the washed pellet and make up to a glycerol concentration of 15 – 50%.
- Transfer the mixture of the sterile cryovials, freeze, and store as described previously.
- Routine transfers are made by scraping a little of the culture from the surface of the frozen medium and transferring to fresh medium.
- Survival is for several years, cultures stored at – 70°C surviving longer than those kept at – 20°C.
- For thawing, place the cryovials at room temperature or in water bath at 37°C and inoculate tubes containing 5 – 10mL of the proper liquid medium. Incubate the tubes at the selected temperature for 16 – 18 h. Make at least two or three transfers in fresh medium before using.

3.6. Storage under liquid nitrogen

- Inoculum: bacterial cells, grown at the selected temperature for 16 h in any of the liquid media described, to a cell density (approximately 10^8 – 10^9 CFU/mL or McFarland´s tube No. 3).
- Mix equal quantities of inoculum (washed previously) and the glycerol 95% (v/v) solution (or other cryoprotectant) in a sterile tube, so that the final concentration of glycerol is 10% (v/v). Transfer 1mL of the mixture to each of the ampules.
- Freeze the preparations in a domestic freezer or cooling bath, to – 30°C, at a rate of about 5°C/min and allow to dehydratate for 2h.
- Transfer the frozen ampules, without thawing, to the liquid – nitrogen refrigerator.
- Maintain the level of liquid nitrogen to where the ampules are completely submerged.
- Cultures are revived by rapid thawing in a water bath at 37°C.

4. Culture media for biomass production

One of the biotechnological aspects of biomass production implies the design or selection of the culture medium. For the selection phase of strains, commercial culture media may be

used that favor growth of the biomass and rapid development of the exponential phase of the microorganism being evaluated. For said purpose, conditions must be established for the bioreactor operation, such as: temperature, oxygenation, agitation, volume and ideal carbon source to reach high concentrations of biomass (10^{12}- 10^{14}).

After standardizing the production process in the commercial culture medium, evaluation of economic substrates must be carried out in the greater-scale production phase. For the production of yeasts with probiotic capacity, substrates have been evaluated with sugar cane molasses, which contributes necessary nutrients for growth and production of the strain under study [15]. These molasses have compounds that favor development of biomass like high contents of carbohydrates (sucrose, glucose, and fructose), proteins, fats, calcium, phosphorus, amino acids, and vitamins among others. Sugar cane molasses can be satisfactorily used as substrate; however [24], analyzed that for more demanding microorganisms it is necessary to supplement with certain free amino acids or ammonium sulfate that serve as a source of nitrogen and suggested controlling pH for the media with sugar cane molasses become excellent substrates for microbial fermentations.

5. Growth kinetics

5.1. Production of inoculum

This stage seeks to diminish the adaptation phase of the microorganism in fermentation. For this, initially, an enriched culture medium must be prepared for the microorganism sought to be evaluated; for lactic acid bacteria an MRS broth [20] is used and for yeasts an YGC broth. Thereafter, the contents of a vial are added onto an agar plate, and then this is incubated at the necessary temperature and time for the growth of the characteristic colonies. During this stage of the process the macro and microscopic characteristics of the strain are evaluated. Then, a cell suspension in saline solution 0.85% (p/v) is conducted until obtaining a concentration corresponding to an absorbance of 0.5 to 540 nm for LAB or 620 nm for yeasts. This suspension is added to the culture medium and it is incubated at the optimal growth temperature of the microorganism with constant agitation at 150 rpm, during 12 hours [15].

6. Discontinuous fermentation

Discontinuous fermentation seeks to produce a high concentration of microorganisms in exponential phase; this must be quantified through specific techniques like spectrophotometry and dry weight or plate counts; likewise, the consumption of the substrate must be quantified during the time of fermentation. A volume corresponding to 10% (v/v) of inoculum must be added to the sterile culture medium. The conditions of the culture must be kept at 150 rpm, 30 ºC during a maximum of 20 hours. Samplings are made every two hours to determine the concentration of biomass and concentration of residual substrate. Once the culture conditions have been established, discontinuous fermentations will be carried out at bioreactor scale [15].

The culture in the bioreactor must keep the same conditions of inoculation preparation, agitation, aeration, temperature, and time established during the previous stage.

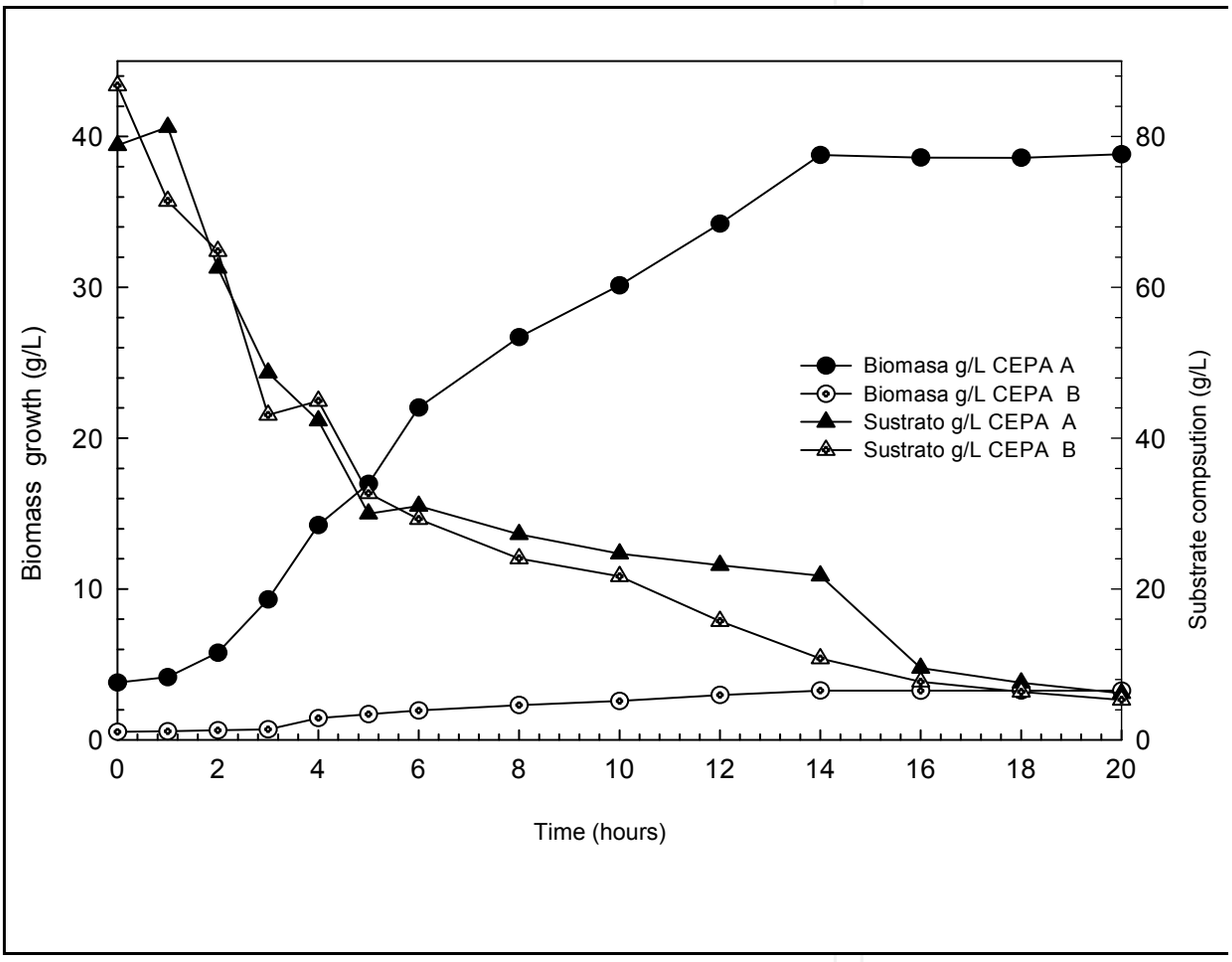

Figure 1. shows the growth kinetics results obtained by Ortiz *et al.,* at bioreactor level with a concentration of 20% (p/v) of sugar cane molasses in which is noted increased concentration of *S. cerevisiae* biomass (strain A), during 14 hours, compared to the control strain (Strain B) at Erlenmeyer level [15].

The data obtained are generally evaluated with the calculation of kinetic parameters that permit comparing the behavior of strains and operating conditions. The biomass concentration obtained along the fermentation process are logarithmically transformed according to formula 1 with the prior elaboration of the biomass pattern curve (g/L).

$$LN(\frac{X}{X_{0)}}) \tag{1}$$

Where:

X_0 represents the biomass (g/L) at the time 0 of the process (once inoculated).

X represents the biomass (g/L) during each of the hours of the process.

In addition, kinetic parameters are calculated like biomass/substrate yield Y(x/s) (g/g) (Formula 2); specific growth rate, *mx* (h-1) (Formula 3); and time of duplication, *td* (h) (Formula 4),

$$Y_{\left(x/s\right)} = \frac{dx}{ds} \tag{2}$$

$$\mu_{(x)} = \frac{1}{x}\frac{dx}{dt} \tag{3}$$

$$td = \frac{Ln(2)}{\mu_{(x)}} \tag{4}$$

7. *in-vitro* tests to evaluate probiotic capacity

7.1. Tolerance to bile salts

Resistance to bile salts is a mechanism involving membrane proteins bound to ATP, which permit efficiently transporting bile acids. The presence of vesicles in yeasts has been found, similar to those found in mammals that can internalize salts, for their later degradation through catabolic enzymes [15,21].

Another mechanism by which yeast is resistant to high concentrations of bile salts is the accumulation of polyols and glycerol as elements to regulate cell osmotic pressure with the external environment.

To evaluate tolerance to bile salts, an adequate culture medium is prepared for the microorganism to be evaluated and it is supplemented with bile salts (Bile Oxgall Difco®) to obtain different concentrations (0.05, 0.1, 0.15, 0.2, 0.25, and 0.3% (p/v)). Thereafter, it is inoculated with a previously obtained suspension of the microorganism equivalent to 10^8

cells/ml. The samples are incubated under ideal conditions for each microorganism. Upon completing the incubation period, the biomass is quantified via the plate count technique [15,25].

7.2. Tolerance to ph

Tolerance to pH may be due to two types of Na^+/H^+ antiporters in yeast; Nha1p, found in the plasma membrane and Nhx1p, which is located in the pre-vacuolar/endosomal compartment. These proteins catalyze the exchange of monovalent cations (Na^+ or K^+) and H^+ through the membranes, so that they regulate the concentrations of cations and pH at organelle and cytoplasmic levels [26,27]. Another of the possible regulation mechanisms is an ATPase located in the cytoplasmic membrane; it can create an electrochemical proton gradient that leads to the secondary transport of solutes and which is implied in keeping pH close to neutral [28]. The capacity to withstand pH ranges and concentrations of bile salts was demonstrated in combination with the capacity to grow at 37 °C, ensuring that these were selection criteria to evaluate the probiotic potential of strains [29].

Tolerance to pH was assessed adjusting the culture medium to different pH ranges (2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0) with concentrated HCl. Each tube was inoculated with a suspension of the microorganism to be evaluated at a previously obtained concentration of 10^8 cells/mL. The samples were incubated at ideal conditions for each microorganism. Once done with the incubation period, plate counts were carried out via the plate count technique [25].

7.3. Determination of resistance to gastric juices

Another test that shows the probiotic capacity of a strain is resistance to gastric juices. The gastric juice secreted has a pH ~2.0 and a concentration of salts ~ 0.5% (p/v) along with catabolic enzymes [30].

Tolerance to gastric juices was evaluated by preparing artificial gastric juice, for which NaCl (2 g/l) and pepsin (3.2 g/l) were added, adjusting to final pH from 2.0 - 2.3 with concentrated HCl. As control, artificial gastric juice was adjusted to neutral pH 6.5 – 7.0 with NaOH *5N.* Sterilization was conducted through filtration with 0.22-μm membrane. The artificial gastric juice and the control were inoculated with a suspension of the microorganism at a concentration of 10^8 cells/ml; these were incubated at 30 ºC, taking samples at different times (0, 1, 2, 3, 4, and 24 hours). Plate counts were carried out in each sampling [25].

7.4. Reduction of cholesterol in the presence of bile salts

Cholesterol reduction is a desired characteristic, given that for humans the condition of hypercholesterolemia or increased levels of cholesterol in blood is considered the greatest

risk for the development of heart disease; and in animals lower presence of cholesterol generates high-quality meats and of great demand, given that they are fat free. The administration of probiotics has demonstrated that they can notably reduce cholesterol levels [31,32].

Cholesterol does not destabilize or precipitate in the medium due to its conjugation with bile salts, which is why it is possible that the microorganisms assimilate the cholesterol present in the medium to incorporate it to its cell membrane. Studies suggest that yeasts exposed to culture medium enriched with cholesterol were more difficult to lyse after being subjected to sonication than yeasts that did not grow in the medium enriched with cholesterol, which indicates a possible morphological change in the wall or in the cell membrane, given that upon the microorganism incorporating this sterol onto its structure, it becomes more resistant to cell lysis, compared to those not incorporating it [29].

It is important to add bile salts to the culture medium with added cholesterol to extract samples to elaborate the pattern curve. This is because the bile salts are present in the organism during activities of lipid emulsion, solubilization, and absorption in the intestine [33]. To evaluate the reduction of cholesterol, a culture medium was prepared supplemented with bile salts (Bile Oxgall Difco®). Thereafter, 224.2 μg/ml of Lipids Cholesterol Rich (Sigma ®) were added. This medium was inoculated with 1% (v/v) of the suspension of the microorganism to be evaluated at a concentration of 10^8 cells/mL. The mixture was incubated for 12 hours at the adequate temperature according to the microorganism.

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The medium was centrifuged at 8000 x g for 15 minutes, 3 ml of ethanol at 95% (v/v) were added to the supernatant, followed by 2 ml of potassium hydroxide at 50% (v/v). Afterwards, the samples were heated to 60 ºC for 10 minutes, then 5 ml of hexane and 3 ml of distilled water were added agitating in vortex after adding each component. From the aqueous phase (hexane layer) 2.5 ml were transferred onto a tube; this was evaporated in a furnace at 60 ºC. The residue formed was resuspended in 4 ml of *0 – phthalaldehyde*. After remaining at rest at room temperature for 10 minutes, 2 ml of concentrated sulfuric acid were added. Finally, absorbance at 550 nm was measured against the target reagent with prior elaboration of a pattern curve with a concentrated solution of 130 μg of cholesterol/mL.

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7.5. Adherence test

One of the important criteria for a probiotic strain is the ability to adhere to the mucous surface of the gastrointestinal tract, given that "*in vivo*" probiotic microorganisms adhere to enterocytes avoiding possible strains from effecting cell adherence as pathogenicity mechanism. Exclusion through the competition for adhesion sites and for substrate is one of the action mechanisms of yeasts used as probiotics.

Cells can be used from the Caco-2 cell line from adenocarcinoma of human colon, which develops characteristics of mature enterocytes and provides a uniform population of differentiated cells, which can be used under conditions defined to quantify adhering microorganisms. According to the study in which adherence tests were conducted of Caco-2 cells with several strains of *Lactobacilli*, it was determined that strains presenting an adherence count below 40 microorganisms in the 20 fields counted at random were considered as non-adhering, between 41 and 100 microorganisms as adhering and over 100 microorganisms as strongly adhering [34].

The Caco-2 cell line must be grown at 37 ºC in an environment with 5% CO_2 by using the minimum essential medium (GIBCO ®) until observing a monolayer. Then, the cells were washed three times with sterile PBS (pH 7.0 ± 0.2). A total of 5 ml of culture was taken from the strains previously grown at culture conditions; then, they were centrifuged and washed with sterile PBS (pH 7.0 ± 0.2) and resuspended in minimum essential medium.

The Caco-2 cells were inoculated with 0.8 ml of the culture of the previously treated microorganism. The mixture was incubated at 37 °C during 90 minutes in an environment with 5% CO_2, and then four washes were carried out with sterile PBS (pH 7.0 ± 0.2). This was followed by Wright's staining, which was observed in the inverted microscope

counting the number of microorganisms adhered to the Caco-2 cells in 20 random microscopic fields. Adherence capacity is expressed as the number of microorganisms adhered to 100 Caco-2 cells [34].

Figure 2 and 3 shows the behavior of two strains that adhered to the Caco-2 cell line, where it is observed that the strain of study isolated from sugar cane molasses (strain A) had greater adhesion than the control strain (strain B) [15].

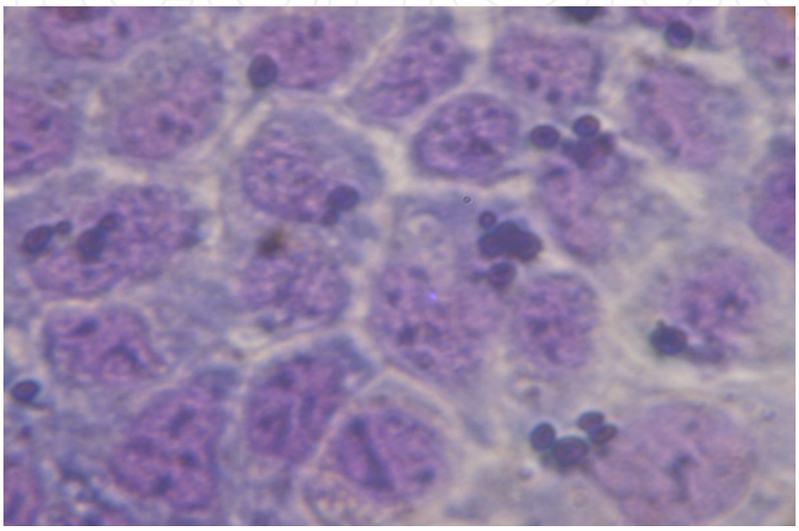

Figure 2. Inverted microscope adherence analysis of *Saccharomyces cerevisiae* (strain A) to Caco-2 cells with Wright's staining.

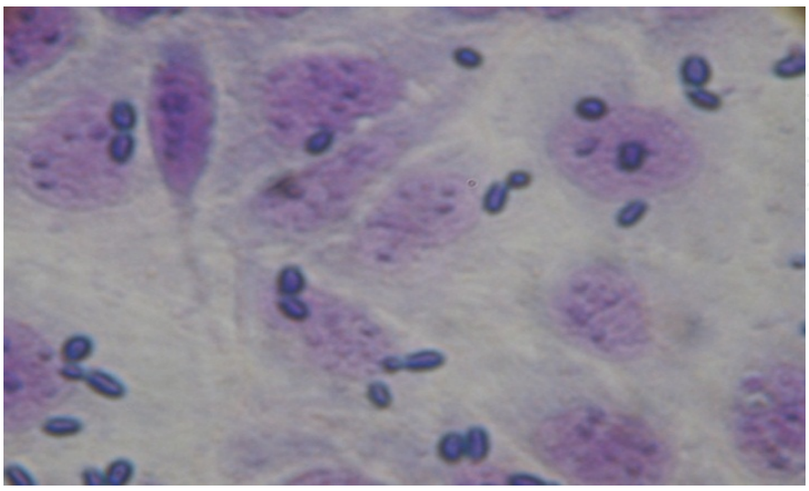

Figure 3. Inverted microscope adherence analysis of *S. cerevisiae* var. *boulardii* (strain B) to Caco-2 cells with Wright's staining.

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