UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO: Identification and Characterization of microbial flora in the processes of storage, extraction and clarification of sugar cane juice at "Ingenio Azucarero Del Norte".

Trabajo de integración curricular presentado como requisito para la obtención del título de Bióloga.

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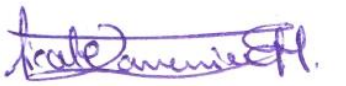

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Dedicatoria

A mi padre Edgar Espinosa, que a diario me enseña que cuando se hacen las cosas que a uno verdaderamente le gustan, no existen sacrificios.

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Con todo el cariño,

Nicole Doménica Espinosa Hidalgo

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Nicole Doménica Espinosa Hidalgo

Resumen

El “Ingenio Azucarero del Norte” es una pequeña pero longeva productora de azúcar en Imbabura – Ecuador. Con el objetivo de potenciar sus resultados industriales y mejorar la rentabilidad de sus productos, busca investigar diferentes microorganismos con potencial biotecnológico, especialmente bacterias, que se desarrollan naturalmente en el almacenamiento, extracción, y clarificación del jugo de caña de azúcar. Esta idea surge a partir de que el ingenio azucarero presenta retos en la producción de azúcar debido a la presencia de bacterias, que pueden llegar a ser contaminantes, en las tuberías de producción y en los biorreactores. Para solventar esta problemática, es necesario conocer la diversidad microbiana asociada a los jugos de caña y por ende a la maquinaria de producción. Por lo tanto, el objetivo principal de este estudio es identificar y caracterizar los microbios presentes en los primeros pasos de la producción de azúcar. Adicionalmente, esta investigación también está dirigida a encontrar bacterias con potencial para producir metabolitos a partir de los cuales se puede obtener un beneficio económico extra.

Para alcanzar el objetivo planteado, se utilizaron técnicas microbiológicas y moleculares tales como preparación de medios, desarrollo de cultivos bacterianos, tinción gram, y secuenciación de ADN, con lo que se obtuvieron e identificaron seis especies bacterianas diferentes. Estas fueron: *Leuconostoc mesenteroides, Lactiplantibacillus plantarum, Leuconostoc holzapfelii, Gluconobacter oxydans, Lactococcus lactis,* y *Kurthia gibsonii.*

Una vez que las bacterias fueron identificadas, se realizó una fermentación preliminar donde el sustrato fue melaza, un subproducto de la producción del azúcar. Esto se realizó para buscar la o las bacterias capaces de fermentar melaza y por ende producir metabolitos. Tras 48 horas de fermentación, las bacterias con potencial industrial de producción de proteínas y ácido láctico (lactato) fueron *G. oxydans* y *L. plantarum* respectivamente. Estas bacterias presentaron los valores más altos de concentración de los metabolitos mencionados contrario a *K. gibsonii* que no presentó un buen desempeño.

Palabras clave: Caña de azúcar, aislamiento, *Leuconostoc mesenteroides, Lactiplantibacillus plantarum, Leuconostoc holzapfelii, Gluconobacter oxydans, Lactococcus lactis, Kurthia gibsonii,* fermentación.

Abstract

The “Ingenio Azucarero del Norte” is a small but long-lived sugar producer in Imbabura – Ecuador. To enhance its industrial results and improve the cost-effectiveness of its products, the sugar mill wants to investigate different microorganisms, especially biotechnologically attractive bacteria, which thrive on the storage, extraction, and clarification of sugarcane juice. This idea arises from the fact that the sugar mill presents challenges on sugar production due to the appearance of bacteria on its production pipelines and bioreactors as contaminants. Therefore, to deal with this issue, it is needed to have an idea and knowledge about the microbial diversity associated with cane juices and the way to sugar production machinery. The primary purpose of this study is to identify and characterize microbial diversity at the initial steps of sugar production. In addition, this research could lead to finding bacteria with the potential to produce metabolites that can be used as profitable products.

Different microbiological and molecular techniques that include media preparation, bacterial culturing, gram staining, and DNA sequencing, were developed, and six different bacterial species were obtained and identified. These species were *Leuconostoc mesenteroides, Lactiplantibacillus plantarum, Leuconostoc holzapfelii, Gluconobacter oxydans, Lactococcus lactis,* and *Kurthia gibsonii*.

Once the bacteria were identified, a preliminary fermentation was carried out with molasses as a substrate. Molasses is a sugar production by-product. This preliminary fermentation was done to look for the bacteria capable of fermenting molasses and therefore producing metabolites. After 48 hours of fermentation, bacteria with the industrial potential for the production of proteins and lactic acid (lactate) were *G. oxydans* and *L. plantarum*, respectively. These bacteria presented the highest concentration values of the mentioned metabolites contrary to *K. gibsonii* which did not perform well.

Key words: Sugarcane, isolation, *Leuconostoc mesenteroides, Lactiplantibacillus plantarum, Leuconostoc holzapfelii, Gluconobacter oxydans, Lactococcus lactis, Kurthia gibsonii,* fermentation.

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1. INTRODUCTION

Sugarcane *Saccharum spp*. is used by the sugar industry as raw material for sugar production. However, the yield of this process is diminished by post-harvest sucrose deterioration in sugarcane which is ligated to invasion, proliferation, and growth of microorganisms in the sugarcane stalk (Cuervo Mulet *et al.*, 2010; Misra *et al.*, 2020). Microorganisms thrive on sugarcane stalk and live in sugarcane juice due to the physical-chemical properties (richness in sugar and minerals) that convert it into a suitable substrate for microorganisms development (Cuervo Mulet *et al.*, 2010; Misra *et al.*, 2017).

Microbes associated with sugarcane comprise a vast diversity where the commonest is *Leuconostoc mesenteroides,* followed by genera *Bacillus, Lactobacillus,* and *Enterococcus.* These bacteria have been associated with sucrose consumption and conversion to polysaccharides such as dextrans (Daza *et al.*, 2019). High dextran production in sugar processing affects sugar production because it causes processing difficulties like filter blinding by the polysaccharides, increment in juices acidity and viscosity, and crystal elongation due to the reduction of evaporation and crystallization rates (Saye and Cawley, 1994; Moore and Botha, 2014). Hence, knowledge about the microbial flora associated with sugarcane could provide the sugar industry with better control and management regarding those aspects.

1.1. Problem Statement

The sugar industry is conscious of the sucrose losses and the adverse metabolite accumulation caused by microbial flora (Daza *et al.*, 2019). These conditions affect sugar production and reduce incomes associated with this product. Consequently, identifying the species that reside on the first steps of sugar production could contribute to control exacerbated microbes' growth. Additionally, it could be beneficial to have an idea of the by-products obtained from natural microbial metabolism from which profitable uses or applications can be proposed because, to be realistic, the complete eradication of bacteria is chimeric.

Fermentation, carried out with the same bacteria that thrives on the sugar mill, aiming to improve the nutritional value or develop active biological compounds from

either by-products or residues can be considered an exciting proposal (Plessas *et al.*, 2020). This bioprocess can carry benefits in making the most resource utilization, acquire new sources of economic profits, and use the bacteria (which produce losses in sugar mills) on a profitable bioprocess.

Therefore, the isolation and characterization of microbial flora from the process of storage, extraction, and clarification of sugarcane juice at Ingenio Azucarero del Norte is proposed. So, once the bacteria have been identified, a preliminary study of their industrial potential in molasses fermentation in terms of both protein and lactic acid production will be raised.

1.2. Objectives

1.2.1. General

- To identify and characterize microbial flora in the first steps of sugarcane production at Ingenio Azucarero del Norte.

2.1.2 Specific

- To develop pure bacteria isolates.
- To identify different bacteria isolates.
- To propose an industrial potential associated with the sugar-associated bacteria in protein and lactate production through preliminary molasses fermentation.

2. THEORETICAL BACKGROUND

2.1. Sugar Industry

The sugar industry forms part of the economically productive industries in the country. To show this, in 2018, the contribution of sugar industry production represented 0.05% of the Ecuadorian GDP[1]. According to Federación Nacional de Azucareros (FENAZUCAR), in Ecuador, there are more than 110.000 hectares of

[1] GDP: Gross Domestic Product which means the sum of all produced goods and services in the national territory over a year (Fernando, 2020).

sugarcane from which 85.000 hectares are for sugar production performed by sugar mills from Guayaquil, Loja, and Imbabura (Sánchez *et al.*, 2020).

Talking specifically of Imbabura, its first sugar mill was established in 1908 due to the favorable geographical and climate conditions for sugarcane crops. Time after, in 1985 the "Empresa de Economía Mixta Ingenio Azucareo del Norte" was set up and over time it has established as the most important agro-industrial company in Imbabura and Carchi provinces. This industry offers jobs for the country's northern residents and cultivates 4.600 hectares of sugarcane (Ingenio Azucarero del Norte, 2019).

2.2. Sugarcane

Sugarcane is a worldwide important crop for feeding and industry because it can provide the third highest quantity of plant calories consumed in the human diet (Sánchez *et al.*, 2020) and performs an efficient conversion of solar energy into harvestable chemical energy as sucrose and biomass. This last fact makes sugarcane to be recognized as one of the world´s most productive crops (Moore and Botha, 2014).

In general terms, sugarcane is a sucrose-storing, perennial, large, and tropical grassy plant that belongs to the Poaceae family. It proliferates under high conditions of sunlight and temperature and requires significant amounts of water. Sugarcane sucrose concentration is enhanced by plant ripening and cold temperatures regimen where respiration decrease (Moore and Botha, 2014; Mwambete and Mpenda, 2019). Along with this, is worth mentioning that the primary sugarcane species destined for sugar production crops is *Saccharum officinarum*, which has thick stalks, high sucrose, and low fiber content. Yet, currently used sugarcane plants are complex hybrids (Moore and Botha, 2014).

Sugarcane anatomy and morphology shape its specialized ability to accumulate sucrose. It is assembled by continuous internodes that contain cellular structures peculiar for sucrose transfer and storage (Moore and Botha, 2014). Even though sucrose biosynthesis in sugarcane is not entirely identified, some studies suggest that sucrose is a photosynthate produced by photosynthesis in the mesophyll cells of leaves and then translocated to the stem sugar storage areas. To reach this last step, the sucrose produced in the mesophyll is loaded through the plasmodesmata into phloem sieve-tube elements by active transport (Rice University, no date; Moore and Botha, 2014).

Sucrose concentration is accruing on storage vacuoles of culm parenchyma cells, and also it can be found on symplastic and apoplastic compartments (Figure 1). As well as that, a report concludes that the cell wall free space harbors 12% of sucrose. Specifically, filled apoplasts can harbor 20% of the stored sucrose. The apoplastic storage of this disaccharide suggests an evolutionary mechanism that produces scattered, unlignified, and specialized storage parenchyma cells that allow both high sucrose concentration storage without producing high solute and turgor potentials and the impossibility of sucrose entrance to the xylem stream due to the barriers in the cell wall of the bundle sheath (Moore and Botha, 2014).

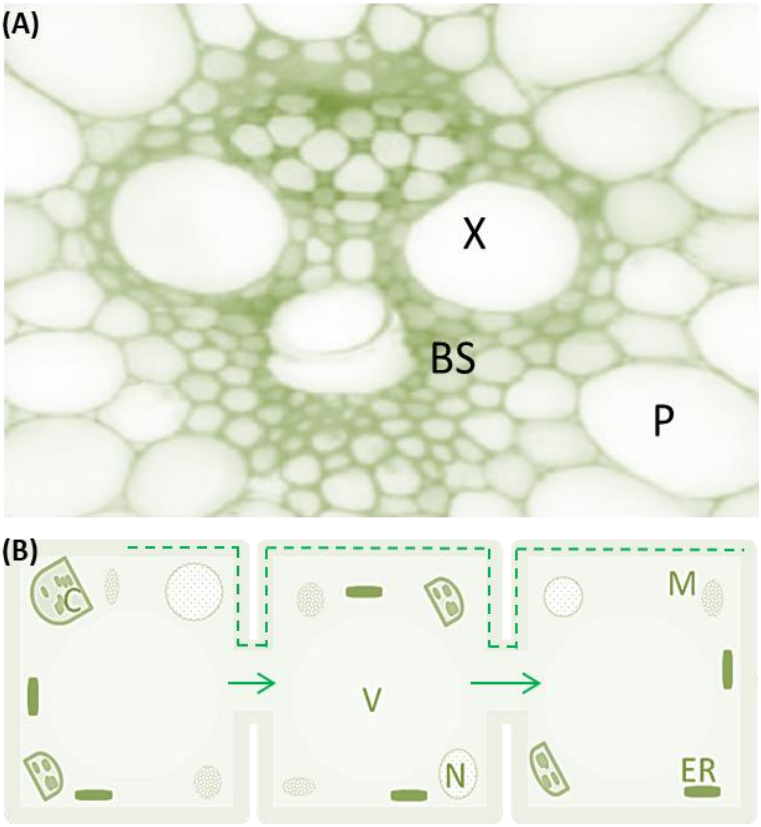


Figure 1: Anatomy of sugarcane tissues. A. Culm sugarcane tissues. X: Xylem, BS: Bundle sheath, P: Parenchyma cell (Moore and Botha, 2014) . **B.** Scheme of symplastic and apoplastic routes of parenchyma cells where sucrose can be stored. C: Chloroplast, N: Nucleus, ER: Endoplasmic reticulum, M: Mitochondria, ----- : Apoplastic route, →: Symplastic route.

2.3. Sucrose Deterioration

Sucrose decline occurs naturally in postharvest through chemical, enzymatic, and microbial degradation. The enzymatic and microbial degradation are the most worrying

because they respectively represent 6% and 93% of sucrose losses while chemical only represents 1% of sucrose diminishment (Moore and Botha, 2014).

Enzymatic sucrose deterioration involves both invertases that hydrolyze sucrose to glucose and fructose and sucrose-phosphate synthase that forms sucrose through the synthesis of sucrose-6-phosphate (Moore and Botha, 2014; Dunford, 2015). These enzymes are present in sugarcane tissues and imply that the more they are, the more the plant´s tendency to be subject to this post-harvest sucrose deterioration. Besides, downregulation of either invertase or pyrophosphate-dependent phosphofructokinase contributes to enhancing sucrose accumulation.

Likewise, microbial sucrose deterioration occurs post-harvesting and takes place both on sugarcane stalk transport and on the lag time on mill yards before milling. Another important point of this kind of sucrose diminishment is from milling until filtration on sugar mills due to the favorable conditions as temperatures below 70°C, low soluble solids, and pH ranging from 5.2 to 7.0 that allow the development and proliferation of microorganisms (Daza *et al.*, 2019).

In humid and warm areas sucrose deterioration is produced by *L. mesenteroides*, whereas, in dry conditions, it is caused by fungi (Moore and Botha, 2014). Bacteria enter the cane stalk sugar-rich region through the cut ends or cracks resulted from the mishandled harvesting, which inactivates phenol oxidase, a protective enzyme that acts as a protective and antimicrobial shield for cane stalk (Misra *et al.*, 2020).

2.4. Sugarcane and Microbes

Sugarcane by itself harbors enormous microbial diversity, from where the commonest is Lactic Acid Bacteria (LAB) (Cuervo Mulet *et al.*, 2010). LAB are gram-positive bacteria where cocci as *Lactococcus, Vagococcus*, *Leuconostoc, Pediococcus, Aerococcus, Tetragnococcis, Streptococcus,* and rods as *Lactobacillus, Carnobacterium, Bifidobacterium*, can be found (De Vuyst and Vandamme, 1994). LAB are widely distributed among different plant niches due to their high adaptation ability (Plessas *et al.*, 2020).

On the other hand, endophytic bacteria of the genera *Gluconacetobacter, Azospirillum, Burkholderia, Herbaspirillum, Klebsiella, Burkholderia, Pantoea,*

Pseudomonas and *Microbactrium* are also found on sugarcane. These diazotrophic[2] bacteria reside on the rhizosphere and intracellular spaces in shoot and root tissues (Moore and Botha, 2014).

Bacterial association with sugarcane could involve benefits and prejudices. As well as there are bacteria that can fix nitrogen, produce growth hormones such as indoleacetic acid, and synthesize antibacterial or/and antifungal metabolites; other bacteria can be pathogens (latent or active), or individuals that contribute to sucrose deterioration (Moore and Botha, 2014; Misra *et al.*, 2020).

2.5. Bacterial Isolation

In order to study a specific bacterial species, it is needed to know about its morphology and physiological characteristics. These are aspects that can be easily observed on a pure culture plate. It is worth mentioning that most of the samples collected from the environment harbor a huge microorganism's diversity.

To achieve a pure culture, where only one kind of microorganism is present, it is convenient to make dilutions of the stock sample and proceed with culturing and sub-culturing specific colonies. Sub-culturing is the microorganisms transference from a stock or original culture to a fresh nutritive medium (Varghese and Joy, 2014; Aakanchha, Richa and Sourabh, 2020). Various and subsequent sub-culturing of specific colonies lead to achieve a pure stock.

Steak Plate Method and Pour Plate Method are the main procedures to obtain pure cultures where bacterial morphology and theirs physiological features can be seen. The first one is the most used and its principle is to spread the sample along the surface of the culture plate so the bacteria can form individual colonies (Truckee Meadows Community College, no date). The second one allows bacterial colony formation within the medium and on the surface. This is practical for bacterial counting, but can be difficult for sub-culturing the colonies that grow in the media (Tankeshwar, 2016).

For general isolation, a general medium can be used. Non-specific media contains only the nutrients required for bacteria survival and growth (Ruangpan and Tendencia,

[2] Diazotrophic refers to organisms with the metabolic activity to fix atmospheric nitrogen into a biologically useful form such as ammonia (Peretó, 2011).

2004). Even though certain media are categorized as selective, it could allow the growth of other individuals. An example of this is MRS Broth, which is used for isolation and culture of *Lactobacillus* from different types of materials. However, its low selectivity grade is evident due to its rich nutrient base that also allows *Pediococcus* and *Leuconostoc* growth (Merck, no date). The selective grade of a particular media can be controlled by physical, chemical, and environmental conditions (Varghese and Joy, 2014).

2.5.1. Bacterial Identification

Despite being used for the first time in 1884, gram staining is currently used to differentiate bacteria based on its cell wall and cell membrane permeability (Smith and Hussey, 2005; Thairu, Usman and Nasir, 2014). This staining classifies bacteria in Gram-positive or Gram-negative (Thairu, Usman and Nasir, 2014). Gram staining is based on a series of stains and discoloration of a prepared smeared sample. Bacteria with a thick cell wall (90% peptidoglycan) stain purple and are categorized as gram-positive; whereas, bacteria with a thin cell wall (10% peptidoglycan) stain pink and are gram-negative (Smith and Hussey, 2005; Thairu, Usman and Nasir, 2014).

In addition, as a staining procedure, it creates a contrast between the bacteria and its background and allows seeing through an optic microscope the phenotypic characteristics of the bacteria cells. This information is not enough to identify bacteria (Smith and Hussey, 2005; Aakanchha, Richa and Sourabh, 2020).

Certainly, gram staining is not the best procedure to identify bacteria, but due to technological advances, accurate techniques for bacterial identification have been developed. Molecular analysis through ribosomal DNA gene sequencing is considering the most reliable bacterial identification method. This technique is developed by sequencing the highly conserved gene that encodes for the 16S rRNA. This region allows to identify bacterial species that are not too closely related (Tortora, G. J.; Funke, B. R.; Case, 2019).

2.6. Preliminary Study for Industrial Potential

As mentioned above, LAB is the most typical reported bacteria group associated with sugarcane, yet this does not imply that they are the only ones. Even though LAB

carries industrial interest for improving the nutritional value of food matrices, biopreservation, probiotic capacity, and the biological isolation of active compounds for functional food formulation. It is worth considering that these capacities can also be developed by microorganisms such as yeast, molds, or even by different bacteria types (Ossa, Vanegas and Badillo, 2010; Plessas *et al.*, 2020).

As it is known, agro-industrial processes produce a diversity of wastes and by-products prone to be spontaneously fermented by the microbiota naturally present (Moore and Botha, 2014). To take advantage of this, the idea is to ferment with the previously isolated bacteria the sugar production by-products to valorize them and get profits from it. To carry this, the literature suggests considering direct and controlled fermentation to obtain a profitable final product (Plessas *et al.*, 2020; Torres *et al.*, 2020). Moreover, to develop and enhance other bacteria performance, their fermentative capacity can be tasted over non-lactic media (Ossa, Vanegas and Badillo, 2010). This can extend bacterial applicability.

2.6.1. Molasses

Molasses is a viscous and dense dark liquid obtained from sugar production. It can contain salts, vitamins, minerals, and lower sugar content whose extraction is not economically profitable. This sugar production by-product is obtained from the centrifugation of the second boiling process of sugarcane juice (Procaña, no date; Krans, 2019). Molasses also known as “honey-like viscous syrup” could have 4% of protein and vitamins and 50% of sucrose prone to be hydrolyzed to glucose and fructose (Qureshi, 2009; Nikodinovic-Runic *et al.*, 2013). It is vital to notice that molasses composition can vary according to the feedstock, production batch, and season.

Among the sugar industry by-products, molasses is one of the most valuables. An estimation suggests that 10 tons of sucrose and 4 tons of molasses can be produced per approximately 100 tons of processed cane (Nikodinovic-Runic *et al.*, 2013). Commonly, this by-product is used as an animal feed supplement, soil fertilizer, feedstock for ethanol production, and even as a flavoring agent in some foods (Ossa, Vanegas and Badillo, 2010; Nikodinovic-Runic *et al.*, 2013).

3. METHODOLOGY

3.1. Materials

Glass material
Petri dishes
Falcon tubes
Inoculation loops
Syringe
Syringe filter (RC 0.22 µm)
Micropipettes
Parafilm
Cryotubes

3.1.1. Equipment

Thermometer
pH-meter
Precision weight balance
Incubator
Laminar flow cabinet
Shaker incubator
Autoclave
Stirring hot plate
Optic microscope
Spectrophotometer
Fridge
Deep freezer
Alcohol lamp
Centrifuge
Polarimeter

3.1.2. Reagents

Alcohol 70% (V/V)
Saline solution 0.85% (W/V)
Glycerol 99% (V/V)
Sterile water (Type II)
MRS Broth TM MEDIA
Agar agar powder TM MEDIA
Sucrose ACS grade TM MEDIA
Buffered Peptonated Water TM MEDIA
Staining reagents (Crystal violet, Iodine)
Decolorizing agent (Ethanol-acetone)
Counter stain (Safranin)
Bradford reagent
Lactic acid 88% (W/W)
$FeCl_3 \cdot 6H_2O$

3.2. Methods

3.2.1. Study Area

Sampling was carried out in November 2020 along with the first steps of sugarcane production at Ingenio Azucarero del Norte. This factory is geographically located at 0° 28´ 52.93" N and 78° 05´ 52.15" W, in Imbabura province, Ecuador.

3.2.2. Laboratory

Ingenio Azucarero del Norte has three laboratories where different procedures were carried out. Material and media preparation and sterilization, development of Bradford test, and cryopreservation were developed at the Biotechnology lab. Isolation, incubation, gram staining, and preliminary fermentation were performed at the Microbiology lab. Finally, molasses preparation, pH measurement, and plate storage take place at the Principal lab.

For bacteria molecular identification, the service of IDgen molecular lab was utilized.

Finally, the Yachay Tech laboratory was used to take photographs of gram staining and for the spectrophotometric determination of lactic acid.

3.2.3. Sampling

For this study, the Conveyor belt ("marmaja" in Spanish), Trommel mill, Primary juice, Filtered juice, and Filter cake ("cachaza") were set up as sampling points. At each point, 50mL of the sample juice was collected (by duplicated) on falcon tubes previously rinsed with the respective sample. Falcon tubes were labeled with the sample's temperature and code and then put into a rack to carry to the laboratory to measure its pH before the bacterial isolation.

It is essential to mention that the samples were cane scraps on the first two points, so to taking the sample it was needed to squeeze them. Then, the following two points were liquids so the sampling was done without complication. Finally, for the mud´s nature of the third point sample, this was collected as it, so then it was diluted with sterile water in the laboratory.

3.2.4. Culture Media Preparation

This procedure used a prepared selective culture media composed of MRS broth (De Man, Rogosa, and Sharpe broth), agar 15% (W/V), and sucrose 10% (W/V). The culture media preparation started by measuring with a graduated cylinder the amount of sterile water required being prepared and pouring its half on the media bottle placed with a stirrer over the stirring hot plate. Then, solid reagents (MRS broth, agar, and sucrose) were weighted one by one with a precision balance and added to the rest of the water into the media bottle. Once all the elements were completely mixed, the stirrer was removed and the bottle was taken to the autoclave for sterilization. When this process finished, media was taken to the laminar flow cabinet to be a bit cooled and dispensed on Petri dishes. Media solidify after 10 minutes approximately.

3.2.5. Bacterial Isolation

To start, the laminar flow cabinet should be clean and must contain a beaker with alcohol and an alcohol lamp or burner. Alcohol lamp serves to maintain a barren environment and, with alcohol, sterilize the inoculation steel loop.

Since the samples have high microbial density, a 1:10 dilution was prepared with sterilized and cooled buffered peptonated water. Both, a carefully shaken test tube with the diluted sample and the Petri dish with solidified media and correctly labeled with the inoculation date and sample´s code, were placed inside the laminar flow to perform the isolation procedure per each sample by the following (Figure 2):

1. A sterilized inoculation loop was introduced in the test tube, moved in circles three times, removed, and carried to the plate to spread back and forth. The test tube has to be closed because it is no longer use.
2. After the spread, the wire loop must be sterilized on the flame until red hot, cooled (by sinking it on the plate) and then spread again by dragging material from the previous extending. This step was repeated twice so the final plate has four spreads.
3. The plate was sealed with parafilm and placed face down on an incubator at 35°C for at least 24 hours. Yet, an optimum incubation period was 48 hours because it allowed noticing different colony morphologies because they achieve good size.

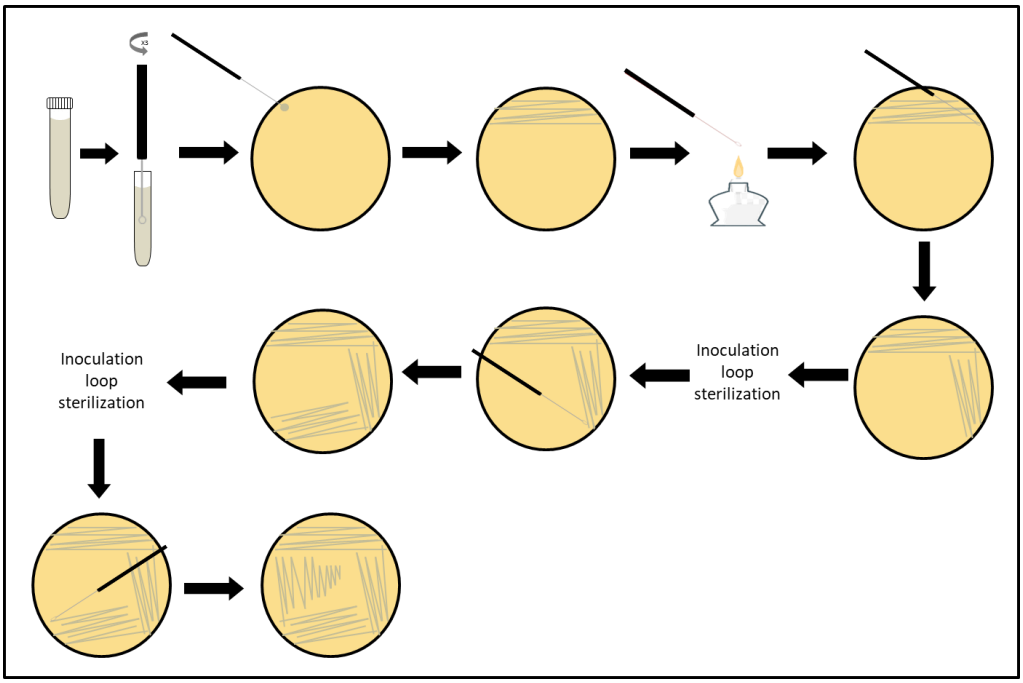


Figure 2. Isolation procedure. The figure represents the steps followed to carry out bacterial isolation beginning with taking the sample from the test tubes until its full streaking.

This procedure (Figure 2) was used at the beginning to isolate bacteria present in the samples, but then it served to sub-culturing until obtaining pure colonies. For sub-culturing, instead of taking the sample directly from the test tube, it was collected from the plate where colonies with different morphologies were seen. Different features of each isolated were recorded in a lab notebook for its posterior classification by following the aspects on "Observing Bacteria in a Petri Dish" (Society, no date) and "Microbiology Laboratory Manual" (Varghese and Joy, 2014). Contaminated plates or those that were saturated with many colonies were discarded, while those used for subcultures were stored at 4°C in the fridge.

3.2.6. Gram Staining

The gram staining procedure was developed as a checkup to seek the purity of cultures among isolations. Single colonies that at the naked eye appeared different and those that were pretty similar were subjected to this procedure to identify if they are composed of similar bacteria or not. After selected the plates that contained showy and distinct colonies, clean slides were labeled with the plate´s code. Plates and slides were placed into the laminar flow cabinet and the procedure per each colony was accomplished by the following:

1. Few drops of sterile saline solution were placed over the slide with a sterile inoculation loop. After this, the wire loop must be sterilized on the flame and let cool.
2. The plate with the interesting colony was opened. With the sterile and cool inoculation loop the chosen colony was picked up and mixed with the saline solution on the slide. The wire loop was sterilized again and placed in alcohol.
3. The resulted smear was heat-fixed before staining. The staining was performed by following the "Gram Stain Protocols" (Smith and Hussey, 2005).
4. A cover slide was placed over the staining and dried slide. The used plate was sealed with parafilm and stored at 4°C on the fridge.

After the gram staining process, slides were carried to the optic microscope to observe the colony cell phenotypic features and recognize if the smeared colony was pure or not. In the lab notebook, these features were reported. Gram staining allows identifying plates where different microorganisms form a single colony or those where yeast has been grown. Colonies assembled by different microorganisms were subjected to sub-culturing until obtaining pure and not contaminated colonies. Contrary, plates where were colonies formed by yeast were discarded because this organism was not interesting for this study.

3.2.7. Cryopreservation

Once it was guaranteed that a petri dish contains a single culture and the gram staining showed unique features, the colony was ready to be cryopreserved. For this, it was required a large bacterial population; so to proliferate bacteria, broth media had to be prepared. The broth media was the isolation media without agar. An operating volume of 30mL of MRS broth with sucrose was prepared on a 250mL Erlenmeyer. This flask had to be autoclaved, cooled, and placed inside the laminar flow cabinet.

The single colony plate was opened to broth inoculation, and with a plastic sterile inoculation loop, some colonies were collected and placed on the Erlenmeyer matrix. After this, the loop and the plate were discarded while the flask was sealed and placed on the shaker incubator for 24 hours at 35°C with 110 rpm. Then, cryopreservation continued as per the protocol "Instructivo General Conservación Cepas por Congelamiento en Glycerol" (Universidad Nacional de Colombia, 2002). Finally, the obtained cryovials were stored at -80°C on a deep freezer.

3.2.8. Bacterial Identification

After bacterial cultures were isolated, purified, and stored, a plastic petri dish with the pure exemplar was prepared and carried to Quito for its molecular identification at IDgen Laboratorio de Diagnóstico Molecular.

3.2.9. Preliminary Study for Industrial Potential

3.2.9.1. Inoculum Preparation

For the fermentation procedure, a cryotube with the pure bacteria was reconstituted by following the protocol "Instructivo General Conservación Cepas por Congelamiento en Glycerol" (Universidad Nacional de Colombia, 2002). As a result of this procedure, after 24 hours in the shaker incubator at 35°C and 110 rpm, a volume of 40mL of medium (MRS Broth and Sucrose) with 1mL of the reconstituted bacteria was obtained. From this overnight culture, 25mL were used as inoculum for molasses fermentation.

3.2.9.2. Molasses Preparation

Molasses was collected, and its brix degree was measured with a polarimeter and the result was 85.40°. Brix degree refers to the amount of water-soluble solids especially sugar in fruit juices or aqueous solution. Simply, it is the sugar content of a solution (Stands, no date; Türkmen and Eksi, 2011). For this study, Brix degrees were reduced with type II water until reach a value that fits in the range where bacterial growth occurs. In previous studies performed by the sugar mill laboratory, this range was established between 10-15°Brix. Therefore, for this preliminary fermentative study, 12.87 °Brix was applied.

Once the molasses achieved the desired °Brix, it was sterilized and supplemented with some reagents (Urea, $(NH_4)_2SO_4$, NaCl, K_2HPO_4, $MgSO_4 \cdot H_2O$, $FeSO_4 \cdot H_2O$, $MnSO_4 \cdot H_2O$, Biotin, and Thiamine) to enrich the media for ensuring bacterial survival. Then, enriched molasses went for another sterilization process and a volume of 225mL was filtered with a syringe filter (RC 0.22µm) [3]on a 500mL Erlenmeyer. A total of seven Erlenmeyer flasks with enriched molasses at pH 6.71 were prepared, one for the control and the other six for each of the pure isolated bacteria.

[3] RC corresponds to the type of filter membrane (Regenerated Cellulose) while the 0.22µm is the pore size.

3.2.9.3. Molasses Fermentation

For fermentation, an operating volume of 250mL was prepared on a 500mL Erlenmeyer. As mentioned before, seven flasks were prepared. The control was 250mL net of prepared molasses, and the other six were composed of 225mL of prepared molasses and 25mL of pure bacteria culture. Then, the flasks were sealed with parafilm and incubated for 48 hours on a shaker incubator under 35°C and 110 rpm (Annex 9.7).

This process was a batch fermentation because the inoculum and the substrate were placed at time zero and were not removed until the end of fermentation (Plessas *et al.*, 2020).

3.2.9.4. Fermentation Residues

At the end of molasses fermentation (48 hours), the fermented solution of each one of the seven flasks was transferred from the Erlenmeyer to a 50mL sterile falcon tube. These were centrifuged for 15 minutes at 4.400 rpm before being leaked with a syringe filter (RC 0.22µm) in other sterile tubes. These obtained tubes were labeled, sealed, and stored at 4°C until lactic acid analysis.

3.2.10. Protein Quantification

After 24 and 48 hours of fermentation, an aliquot of 10mL of each solution was taken and prepared on test tubes for Bradford test. Eventually, to accomplish this test, the protocol described on "Total Protein of Crude Extracts and Quantification the Native *Bacillus thuringiensis* Strains Isolated from Boyacá and Cundinamarca" was followed (Torres, Hernández and Peréz, 2013). The measurements were performed with a spectrophotometer at 595nm.

3.2.11. Lactic Acid Analysis

Lactic acid was measured as lactate production through a colorimetric test with $FeCl_3$ on a spectrophotometer at 390nm (Borshchevskaya *et al.*, 2016). This procedure was done according to the protocol on "Spectrophotometric Determination of Lactic Acid" where the colorimetric reaction is described as: $3C_3H_6O_3 + FeCl_3 \cdot 6H_2O \rightarrow (C_3H_5O_3)_3Fe + 6H_2O + 3HCl$ (Borshchevskaya *et al.*, 2016; Torres and Gómez, 2019). The detected analyte at 390nm is the product of the reaction between lactic acid and

iron (III) chloride (0.2% W/V) that produce iron (III) lactate, a yellowish-green solution. This method detects L- and D- lactic acid in complex mixtures (Borshchevskaya *et al.*, 2016).

4. RESULTS

4.1. Bacterial Identification

In general terms, a bacteria isolate was obtained from each sampling point. However, for primary juice and filter cake, two bacterial isolates were obtained. This was contrary to the filtered juice, where no one was found. This does not imply that the sampling point was bacteria-free, but because isolated organisms were similar to those previously found from another sampling point or due cultures allow yeast growth. Features of the sampling points are on Figure 3.

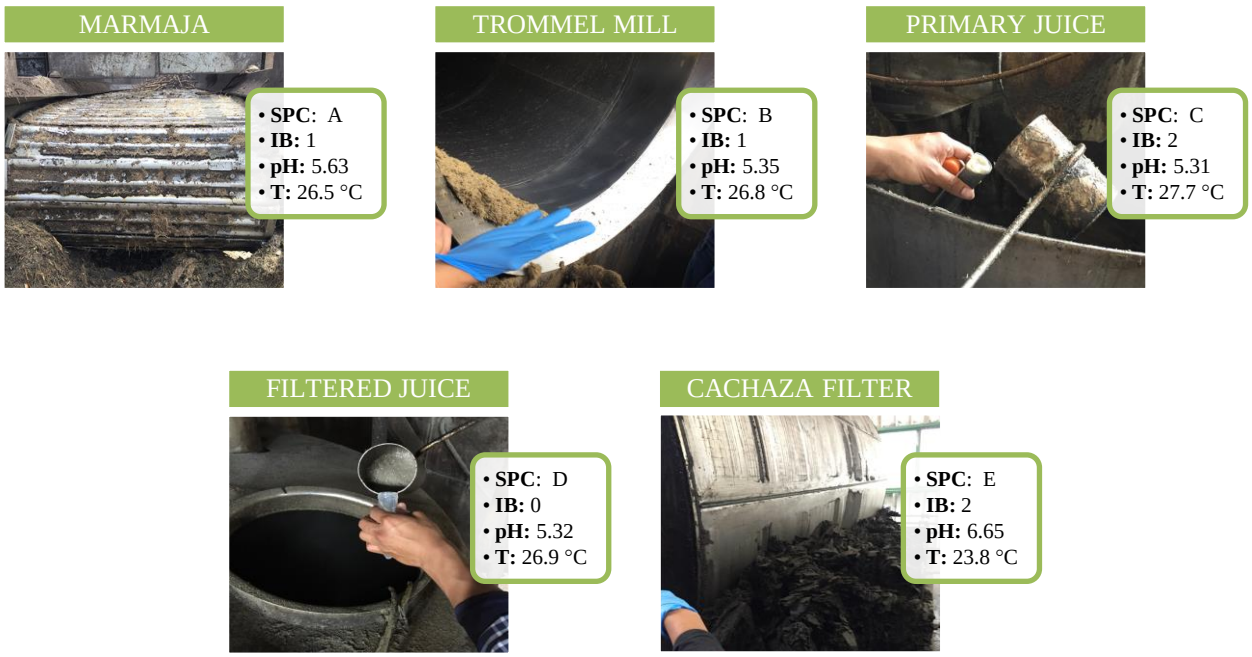


Figure 3. Sampling points features. The figure shows samples nature, SPC: Sampling Point Code, IB: Isolated Bacteria (number), pH, and T: Temperature.

After isolation, subcultures, and gram staining, six different bacterium cultures were obtained (Table 1). Their general classification is shown in Table 2, while each

bacterium's features are in Tables 3 to 8. Once the isolates were pure, they were carried to the IDgen laboratory for molecular identification. Here, DNA evaluation was performed and the giving results fit on a range between 97 to 99.4 percent of DNA quality (Table 1). That is to say that the DNA obtained from the bacterial isolates was high-quality DNA because the isolates were well-prepared. The importance of this achievement is because this DNA is used to amplify and sequence the DNA encoding for the 16S rRNA gene, which is used for bacteria identification, so it allowed obtaining accurate identification.

DNA amplification was developed through the Polymerase Chain Reaction (PCR) using universal primers 27F/1492R (Lane, 1991). As a result of this procedure, amplicons (16S rDNA region) nearly to 1500bp were obtained (Annexes 1- 6) and used for sequencing both DNA strands by Sanger technology. After the sequencing, assembling a consensus sequence for each microorganism was performed. With this, there was a search on the GenBank nucleotide database of The National Center for Biotechnology Information (NCBI), and the isolates were identified at species level.

Table 1. Molecular features of isolates. DNA quality percentage, bacteria identification, and GenBank (nucleotide) accession number.

SAMPLING POINT CODE	ISOLATE CODE	QUALITY of DNA (%)	ORGANISM	ACCESION NUMBER
A	A1	99.4	*Leuconostoc mesenteroides*	CP015247.1
B	B1	98.8	*Lactiplantibacillus plantarum*	MH544641.1
C	C11	98.2	*Leuconostoc holzapfelii*	LC519862.1
C	JP	97	*Gluconobacter oxydans*	NR 118196.1
E	Filtro C	98.1	*Lactococcus lactis*	CP065737.1
E	E22	98.8	*Kurthia gibsonii*	NR 118298.1

Table 2. Bacterium general classification.

BACTERIUM	BACTERIAL GROUP[a]	TEMPERATURE AT ISOLATION (°C)	pH AT ISOLATION	FEATURES OF BACTERIAL COLONIES	SAMPLING POINT
L. mesenteroides	LAB	26.5	5.63	Granular clots	Marmaja (cane scraps)
L. plantarum	LAB	26.8	5.35	Turn yellowish over time	Trommel Mill (cane scraps)
L. holzapfelii	LAB	27.7	5.31	Colonies with internal air space	Primary Juice
G. oxydans	AAB			Mucous-rich	
L. lactis	LAB	23.8	6.65	Milky appearance	Cachaza Filter (mud)
K. gibsonii	---			Gelatin liquefy	

[a] LAB: Lactic Acid Bacteria
AAB: Acetic Acid Bacteria

Table 3. *Leuconostoc mesenteroides* **features.**

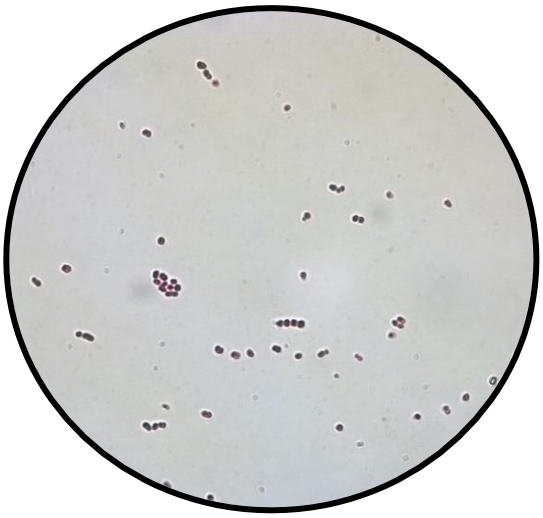
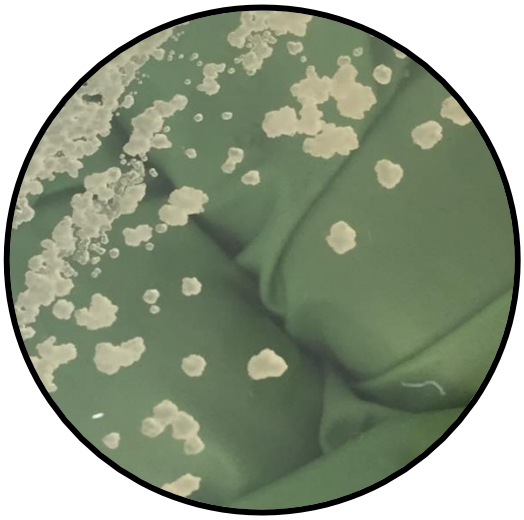
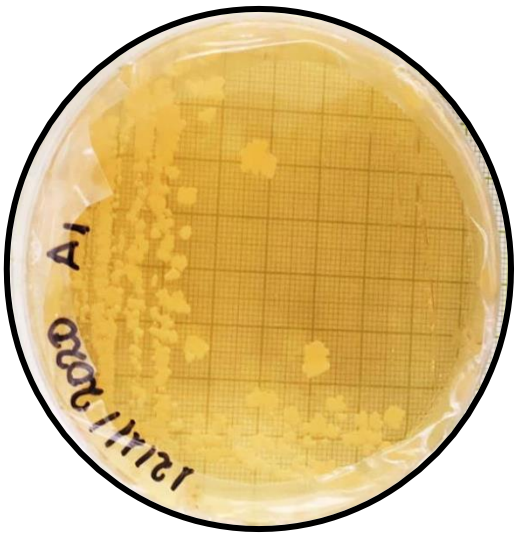
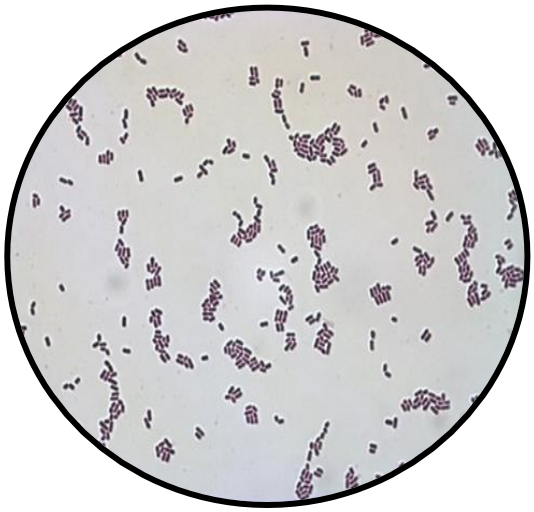
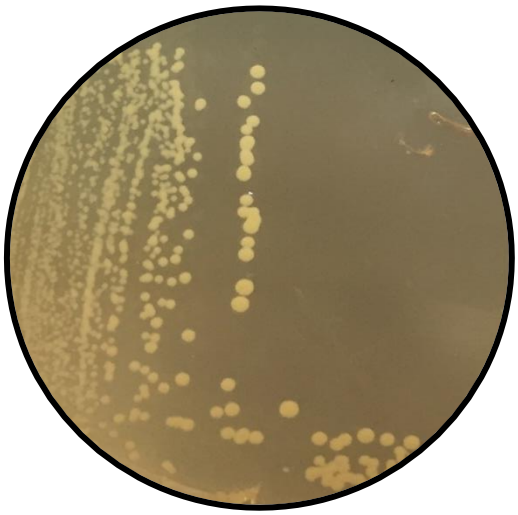
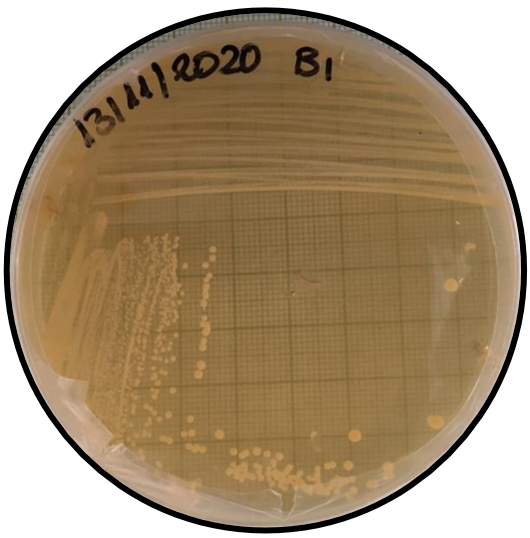
<table>
<tr><th colspan="7">*Leuconostoc mesenteroides* PHENOTYPIC FEATURES</th></tr>
<tr><th colspan="3">Microscopic</th><th colspan="4">Macroscopic</th></tr>
<tr><th>Gram Staining</th><th>Morphology</th><th>Zoom (X)</th><th>Color</th><th>Opacity</th><th>Shape</th><th>Elevation</th></tr>
<tr><td>Positive</td><td>Spheres</td><td>100</td><td>White</td><td>Opaque</td><td>Irregular</td><td>Granular</td></tr>
<tr><td colspan="3">  </td><td colspan="4">  </td></tr>
<tr><td colspan="7">Colony detailed characteristics</td></tr>
<tr><td colspan="7">   </td></tr>
<tr><td colspan="7">Individual colonies are small; they achieve a size of 1mm approximately.
As the growth continues, they tend to form big clusters.
The highlighting feature is their granular appearance.</td></tr>
</table>

Table 4. *Lactiplantibacillus plantarum* **features.**

<table>
<tr><th colspan="7">*Lactiplantibacillus plantarum* PHENOTYPIC FEATURES</th></tr>
<tr><th colspan="3">Microscopic</th><th colspan="4">Macroscopic</th></tr>
<tr><th>Gram Staining</th><th>Morphology</th><th>Zoom (X)</th><th>Color</th><th>Opacity</th><th>Shape</th><th>Elevation</th></tr>
<tr><td>Positive</td><td>Rods</td><td>100</td><td>White</td><td>Opaque</td><td>Circular</td><td>Convex</td></tr>
<tr><td colspan="3">  </td><td colspan="4">  </td></tr>
<tr><td colspan="7">Colony detailed characteristics</td></tr>
<tr><td colspan="7"> 

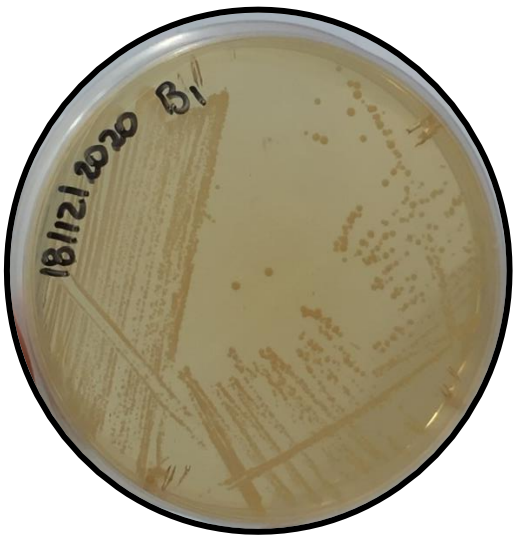
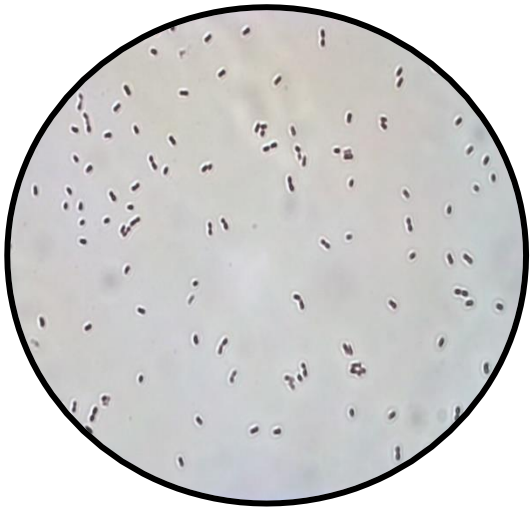
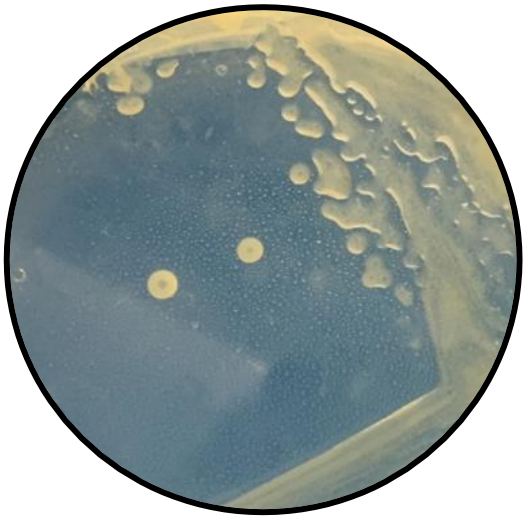

</td></tr>
<tr><td colspan="7">Bacterial morphology is small rods with rounded ends.
Colonies are round and not too big, they diameter reach a maximum of 2 mm.
Fresh colonies were white, but they tend to turn yellowish through time.</td></tr>
</table>

Table 5. *Leuconostoc holzapfelii* **features.**

<table>
<tr><th colspan="7">*Leuconostoc holzapfelii* PHENOTYPIC FEATURES</th></tr>
<tr><th colspan="3">Microscopic</th><th colspan="4">Macroscopic</th></tr>
<tr><th>Gram Staining</th><th>Morphology</th><th>Zoom (X)</th><th>Color</th><th>Opacity</th><th>Shape</th><th>Elevation</th></tr>
<tr><td>Positive</td><td>Ellipsoidal pairs</td><td>100</td><td>---</td><td>Translucent</td><td>Circular</td><td>Convex</td></tr>
<tr><td colspan="3">  </td><td colspan="4">  </td></tr>
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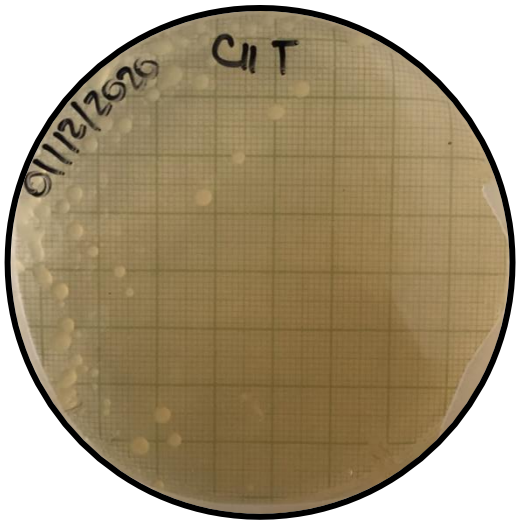
<tr><td colspan="7"> 

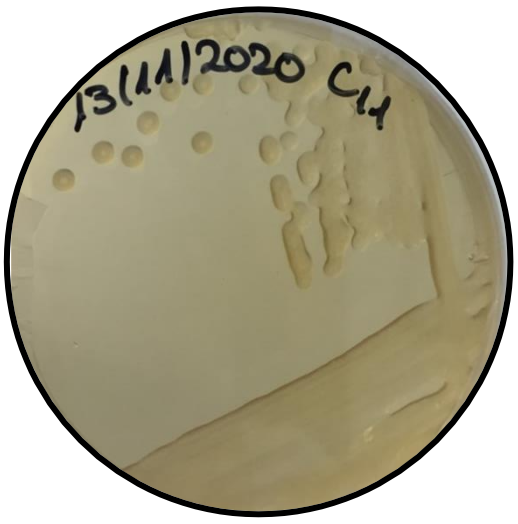
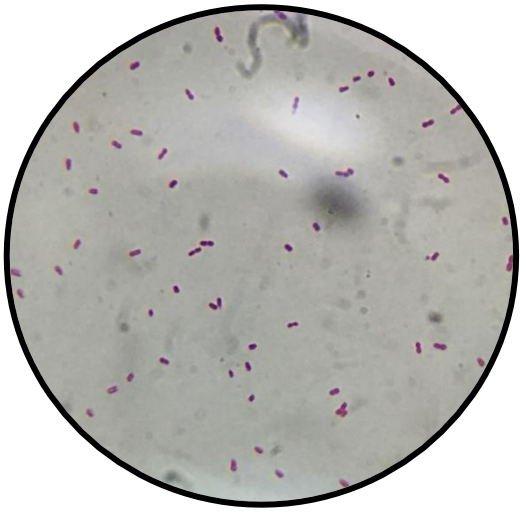
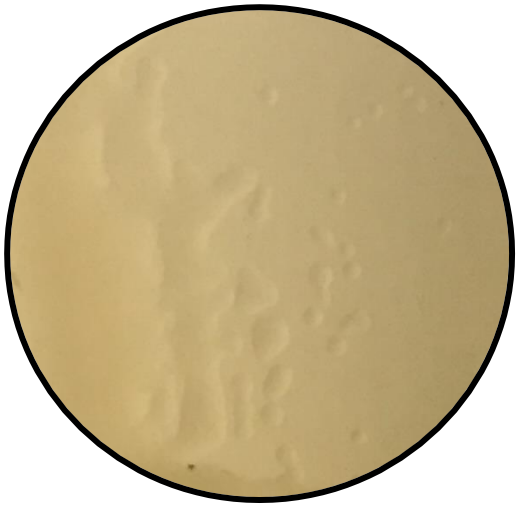
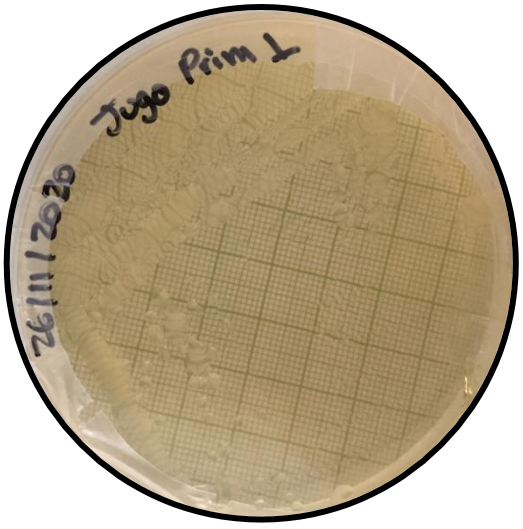

</td></tr>
<tr><td colspan="7">Among the first 24 hours, colonies reach a diameter of 1 mm and can grow until 3mm approximately.
Mucous-rich colonies.
The relevant feature is their internal air space.</td></tr>
</table>

Table 6. *Gluconobacter oxydans* **features.**

<table>
<tr><th colspan="7">*Gluconobacter oxydans* PHENOTYPIC FEATURES</th></tr>
<tr><th colspan="3">Microscopic</th><th colspan="4">Macroscopic</th></tr>
<tr><th>Gram Staining</th><th>Morphology</th><th>Zoom (X)</th><th>Color</th><th>Opacity</th><th>Shape</th><th>Elevation</th></tr>
<tr><td>Negative</td><td>Paired spheres</td><td>100</td><td>---</td><td>Translucent</td><td>Circular</td><td>Raised</td></tr>
<tr><td colspan="3">  </td><td colspan="4">  </td></tr>
<tr><td colspan="7">Colony detailed characteristics</td></tr>
<tr><td colspan="7"> 


</td></tr>
<tr><td colspan="7">Mucous-rich colonies.
Colonies' consistency is viscous.
Small colonies tend to present diameter of 1mm.</td></tr>
</table>

Table 7. *Lactococcus lactis* **features.**

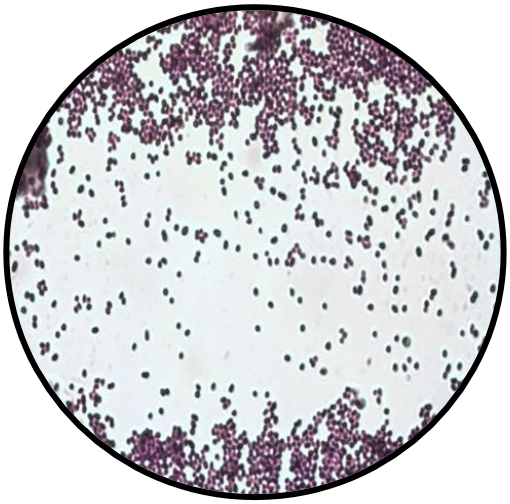
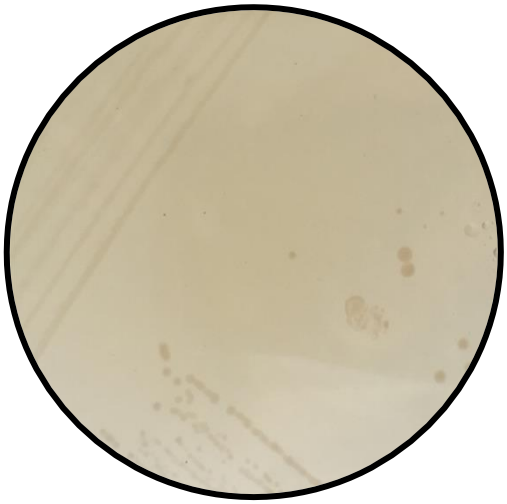
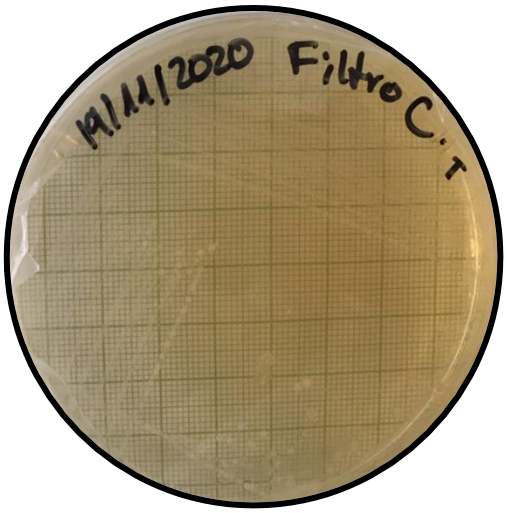
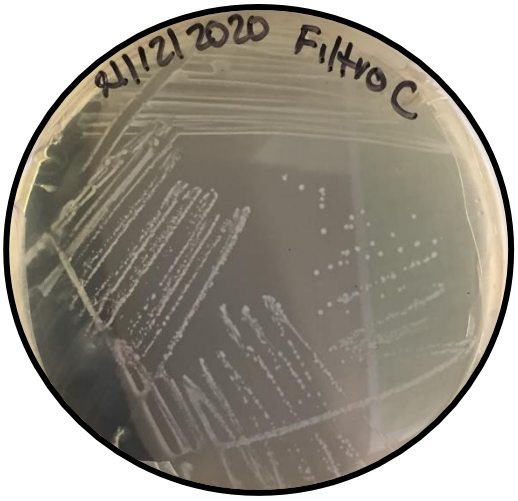
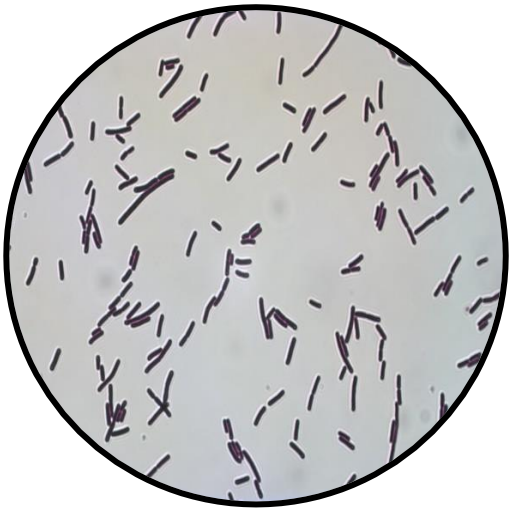
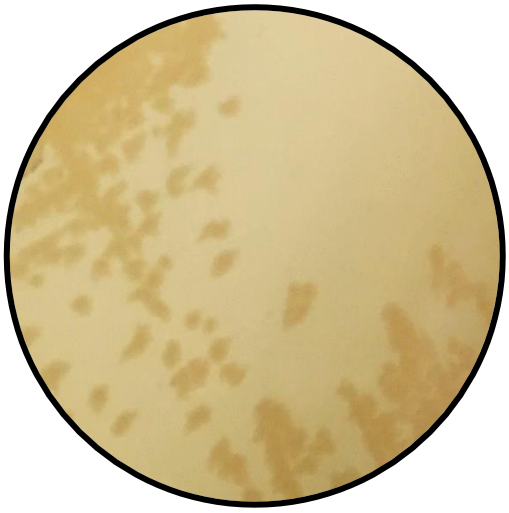
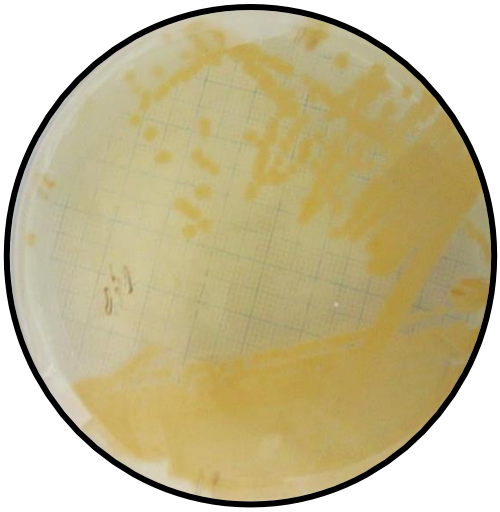
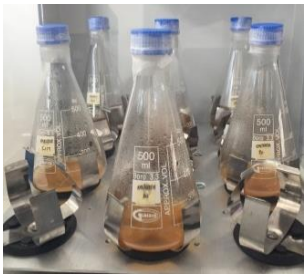
<table>
<tr><th colspan="7">*Lactococcus lactis* PHENOTYPIC FEATURES</th></tr>
<tr><th colspan="3">Microscopic</th><th colspan="4">Macroscopic</th></tr>
<tr><th>Gram Staining</th><th>Morphology</th><th>Zoom (X)</th><th>Color</th><th>Opacity</th><th>Shape</th><th>Elevation</th></tr>
<tr><td>Positive</td><td>Spheres</td><td>100</td><td>White</td><td>Opaque</td><td>Punctiform</td><td>Flat</td></tr>
<tr><td colspan="3">  </td><td colspan="4">  </td></tr>
<tr><td colspan="7">Colony detailed characteristics</td></tr>
<tr><td colspan="3">  </td><td colspan="4">  </td></tr>
<tr><td colspan="7">Colonies are too small. Its diameter often is less than 1mm, so they are categorized as punctiform. Colonies consistency appears as milky.</td></tr>
</table>

Table 8. *Kurthia gibsonii* **features.**

<table>
<tr><th colspan="7">*Kurthia gibsonii* PHENOTYPIC FEATURES</th></tr>
<tr><th colspan="3">Microscopic</th><th colspan="4">Macroscopic</th></tr>
<tr><th>Gram Staining</th><th>Morphology</th><th>Zoom (X)</th><th>Color</th><th>Opacity</th><th>Shape</th><th>Elevation</th></tr>
<tr><td>Positive</td><td>Elongated rods</td><td>100</td><td>Yellowish</td><td>Opaque</td><td>Irregular</td><td>Flat</td></tr>
<tr><td colspan="3">  </td><td colspan="4">  </td></tr>
<tr><td colspan="7">Colony detailed characteristics</td></tr>
<tr><td colspan="7">   </td></tr>
<tr><td colspan="7">Colonies do not have a regular shape. They look like flat filiform.
Colonies after 24 hours reach a size of 2mm approximately.</td></tr>
</table>

4.2. Preliminary Industrial Potential

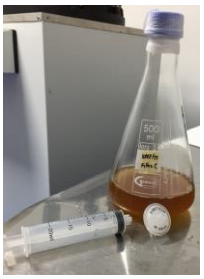


Pure bacteria culture for fermentation.

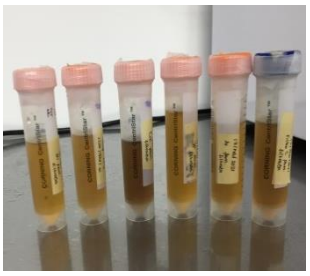
Molasses fermentation after 24 hours.

Molasses fermentation after 48 hours.

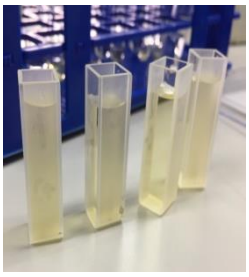
Spectrophotometric protein detection. Bradford test



Material for filtration.



Fermentation product filtered and centrifuged.



Spectrophotometric lactic acid detection with $FeCl_3 \cdot 6H_2O$

Figure 4. Scheme of the proposed preliminary molasses fermentation. This scheme is to perform the preliminary study for the bacterial industrial potential in terms of protein and lactic acid production.

4.2.1. Protein Production

The enriched molasses fermentation was performed with the obtained native bacteria. This let directing the study to seek bacteria's industrial potential for protein production. To assess this, the fermentation product of each bacterium was subjected to a Bradford test to quantify its net protein production.

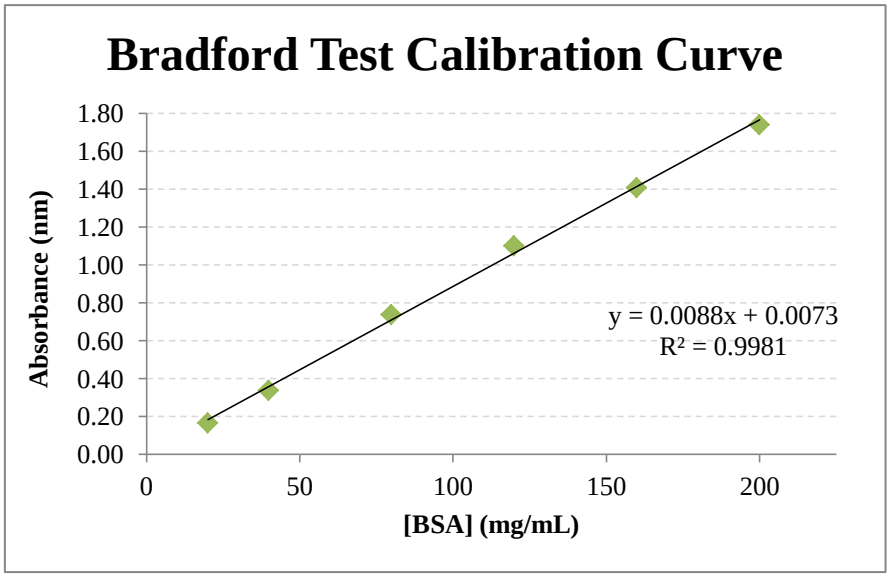


Figure 5. Bradford´s Calibration Curve at 595nm. The figure also has the equation of the calibration curve where y, is absorbance at 595nm; x, is protein concentration; and R^2, is the linear correlation coefficient.

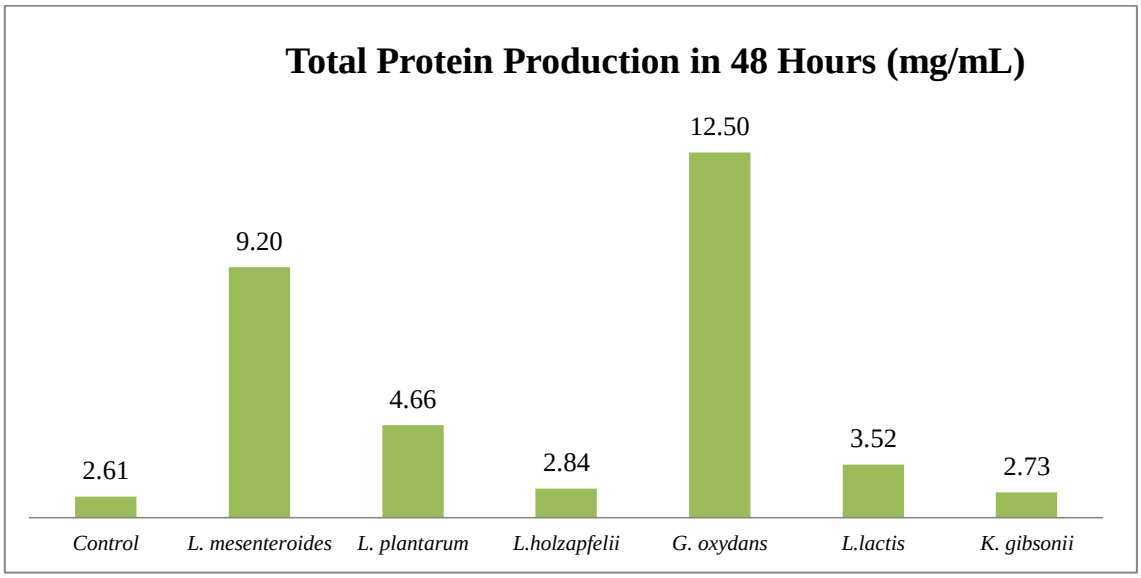


Figure 6. Total protein production measurements per bacterium through Bradford test. The measurements are expressed in mg/mL.

The highest protein concentration was in fermentations with *G. oxydans* (12.50 mg/mL) and *L. mesenteroides* (9.20 mg/mL). *L. plantarum* (4.66 mg/mL) and *L. lactis* (3.52 mg/mL) were quite good protein producers contrary to *L. holzapfelii* (2.84 mg/mL) and *K. gibsonii* (2.73 mg/mL) which produce low protein quantity. Notice that all bacteria increase protein concentration with respect to the control (2.61 mg/mL).

4.2.2. Lactic Acid Analysis

The analysis of lactic acid production to seek the bacteria's industrial potential for this metabolite was developed with a colorimetric test to detect ferric lactate ($(C_3H_5O_3)_3Fe$). For this, it was required a calibration curve performed with lactic acid 88%. To assemble the curve (Figure 6), the acid was diluted once at 1:10 and then in 1:2 serial dilutions. To figure out lactate on the fermentation products, these were diluted once at 4:23 (Figure 7).

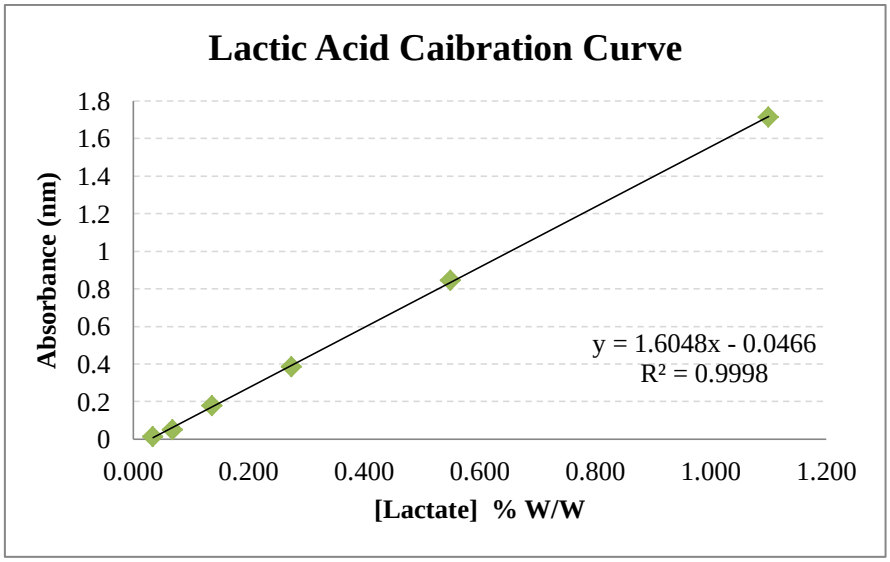


Figure 7. Lactic Acid Calibration Curve at 390nm. The figure also has the equation of the calibration curve where y, is absorbance at 390nm; x, is lactic acid concentration; and R^2 ,is the linear correlation coefficient.

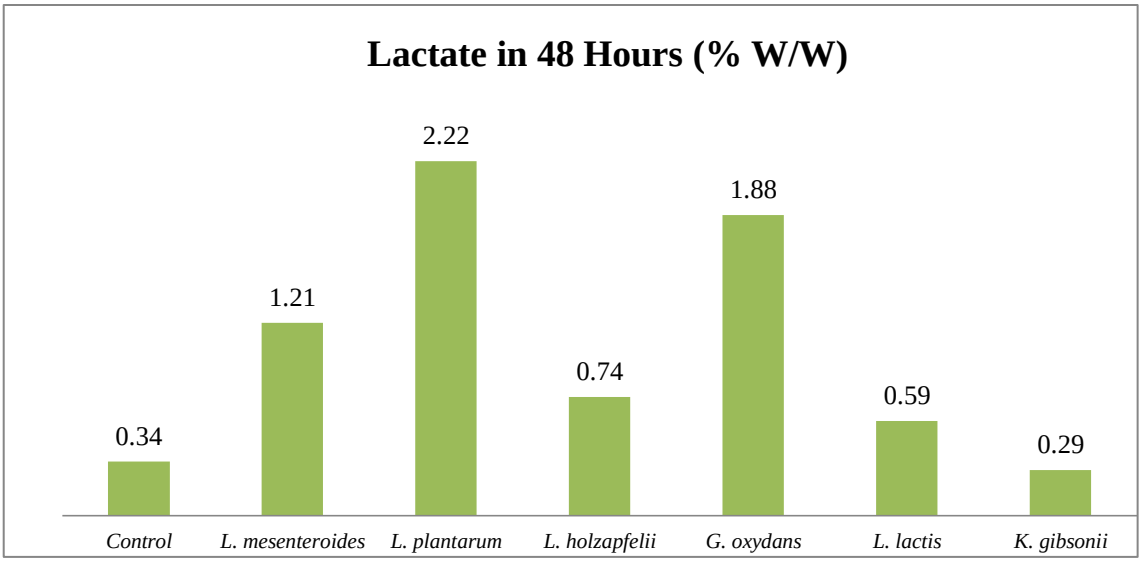


Figure 8. Lactate concentration measurements per bacterium through $FeCl_3$. The measurements are expressed in % W/W.

L.plantarum (2.22% W/W) and *G. oxydans* (1.88% W/W) were the bacteria with the highest lactate concentration. *L.mesenteroides* (1.21% W/W), *L. holzapfelii* (0.74% W/W), and *L. lactis* (0.59% W/W) also have lactate but in less quantity. On the other hand, *K. gibsonii* (0.29% W/W) lactate concentration was less than the present on the control (0.34% W/W).

5. DISCUSSION

Many different bacteria were isolated from the sugar production process and six bacterial species were identified. These were *Leuconostoc mesenteroides, Lactiplantibacillus plantarus, Leuconostoc holzapfelii, Gluconobacter oxydans, Lactococcus lactis,*and *Kurthia gibsonii.*

5.1. Sampling

The fact to have isolated *L. mesenteroides* from the marmaja, and *L. holzapfelii* from the primary juice, was not a surprise considering that despite *Leuconostoc* is a soil-born bacterium, it can grow on sugarcane tissues and juices (Misra *et al.*, 2020). In the same way, available information suggests that these bacteria grow in conditions where the pH ranges from 2.0 to 7.5 and at a temperature between 20 – 30°C. Nevertheless, a perfectly congenial environment are cane juices with a pH ranging from 5.0 – 5.5 (De Vos *et al.*, 2009; Misra *et al.*, 2020). Effectively in this study, *L. mesenteroides* and *L. holzapfelii* were isolated from sampling points where the pH was 5.63 and 5.31 and at temperatures of 26.5°C and 27.7°C, respectively (Table 2). Notice that pH 5.31 belongs to the primary juice, the most comfortable substrate.

Lactiplantibacillus plantarum is the recent reclassification for *Lactobacillus plantarum* (Zheng *et al.*, 2020), bacteria that can be isolated from fermented food, plant material, and human gastrointestinal tract (Sturme *et al.*, 2007; Plessas *et al.*, 2020). Fermented raw material derived from plants is the most frequent isolation source (Landete *et al.*, 2010). The ecological versatility and flexibility of this species remain on its largest genome among LAB. This special quality encodes for the biosynthesis of amino acids and lets bacterial grow on diverse sugar sources (Kleerebezem *et al.*, 2003; De Vos *et al.*, 2009). Therefore, it was possible to find *L. plantarum* on the squeezed

cane scraps of the trammel mill. Also, the literature suggests that *Lactobacillus* grows at acidic conditions with pH less or equal to 5 until 6.2 and through a wide temperature range from 2 – 53°C, however, the optimal is reduced to 30 – 40°C (De Vos *et al.*, 2009). The sampling point of this isolate was at a pH of 5.35 and 26.8°C (Table 2). These are not the optimum conditions but are not too far away from the range where *L. plantarum* has been reported.

The second bacterium obtained from the primary juice was *G. oxydans* which is reported to grow in garden soils but mainly at sugary niches because its natural habits are flowers and fruits (Gupta *et al.*, 2001; Prust *et al.*, 2005; Amaresen *et al.*, 2020). Literature show growth conditions at low pH on an interval between 5.5 and 6.0 and at temperatures ranging from 25 – 30°C (Gupta *et al.*, 2001). To this isolation, *G. oxydans* were obtained from a medium where the pH was 5.31 and at 27.7°C (Table 2).

A bacteria species isolated from the cachaza filter mud was *L. lactis* which commonly inhabits the dairy industry, plants-related products, and soil only after enrich it with plant material. This bacteria grows at neutral and at a wide temperature range from 10 to 40°C (De Vos *et al.*, 2009). However, *L. lactis* was isolated from a source with a pH of 6.65 and 23.8°C (Table 2).

Finally, the literature suggests that *K. gibsonii* is commonly isolated from decomposing organic material, principally from meat and meat products or animals' dung (chicken and pigs), yet, recent research reports this microorganism on spinach leaves (Dworkin *et al.*, 2006; Mukhopadhyay *et al.*, 2019). Nevertheless, in this study, *K. gibsonii* was isolated from a mud sample of the cachaza filter that contains: fibers, coagulated colloids, albuminoidal substances, calcium phosphate, and soil particles at a pH of 6.65 and 23.8°C (Table 2) (Procaña, no date). These values fit the proposed ranges for *Kurthia´*s growth and survival that go from pH 5.0 to 8.5 and at temperature between 10 – 45° (Dworkin *et al.*, 2006).

5.2. Characterization

To start, Tables 3 and 5 present characteristics of *L .mesenteroides* and *L. holzapfelii*, respectively. Both isolates belong to *Leuconostoc* genera that give them some standard features like positive gram staining, yet each one has its own traits. *L. mesenteroides* shows spherical cells that produce lumpy, irregular, white, and opaque

colonies, whereas *L. holzapfelii* presents ellipsoidal pairs that form well-defined mucous convex colonies (appearance related to exopolysaccharide production). These presented attributes coincided with reported ones (De Vos *et al.*, 2009).

Table 4 shows general features of *L. plantarum.* This table starts with the morphology, suggesting a simple description as rods, yet it is technically described as coryneform coccobacilli (De Vos *et al.*, 2009). As well as in the reported information, this bacterium showed characteristics of positive gram staining and white, opaque, and convex colonies with continuous margins. Nevertheless, in older cultures, colonies were prone to turn yellowish because of the pigment triterpenoid carotenoid 4,4´-diaponneuroponeurosporene (De Vos *et al.*, 2009).

Next, Table 6 collects the features of *G. oxydans* which is a ketogenic bacterium with negative gram staining and morphology of paired spheres. The literature supports these results because they mention the bacterial cells as ellipses that can occur in pairs (Gupta *et al.*, 2001; Amaresen *et al.*, 2020). Additionally, the colonies of this bacterium are mucus-rich because these microorganisms produce polysaccharides from sucrose (Amaresen *et al.*, 2020).

L. lactis features are shown in Table 7. There are reported characteristics that coincide with the obtained ones. Among these are positive gram staining, cells with a spherical shape, and small, circular, and white colonies. Yet, differ in the significant production of exopolysaccharides (De Vos *et al.*, 2009).

Finally, features of *K. gibsonni* are in Table 8, where are reported gram-positive unbranched elongated rods with different lengths. This size variation is normal and occurs according to the growth stage (De Vos *et al.*, 2009). Talking about macroscopic characteristics, the yellowish colonies' color and their irregular shape appear as a product of incubation at 35°C. Mentioned morphology is better described as rhizoid colonies that look like a bird´s feather (Shaw and Keddie, 1983; De Vos *et al.*, 2009).

According to the literature, *Leuconostoc* and *Lactococcus* have been reported as high exopolysaccharides producers (Mccleskey, Faville and Barnett, 1947; De Vos *et al.*, 2009). However, in this study, despite that the culture medium contained 10% of sucrose; *L. lactis* did not produce visible slime. However, it is possible to state that its milky appearance is due to exopolysaccharides production (Table 2 and 7). Likewise, *L.*

mesenteroides did not produce slimy exopolysaccharides but produced colonies with lumpy appearances that can be related to the exopolysaccharide production. An explanation for this low or even null exopolysaccharide production could be associated to the relatively low incubation temperature (35 °C) (Mccleskey, Faville and Barnett, 1947).

Above all, once each bacterium was characterized according to their phenotypical features and molecular identification; it was noticeable two main bacterial groups, Lactic Acid Bacteria (LAB) and Acetic Acid Bacteria (AAB). Most of the obtained microbial (*L. mesenteroides, L. holzapfelii, L. plantarum,* and *L.lactis*) belong to the first group, *G. oxydans* fits in the second group (Gupta *et al.*, 2001), and *K. gibsonii* does not pertain to any of these groups (Table 2). These results suggest an idea of the huge microbial diversity associated with sugarcane juices.

5.3. Preliminary Bacterial Industrial Potential

Bacteria consume sucrose as their source of energy and growth. From this biological activity, bacteria produce metabolites from which it is possible to obtain economic profits. To enhance and valorize this fact, waste or by-products rich in sucrose content were used (Kleerebezem *et al.*, 2003; Cuervo Mulet *et al.*, 2010; Misra *et al.*, 2020).

To test the industrial potential of the isolated bacteria in terms of protein and lactate production, the concentrations of these metabolites on a substrate that resulted from 48 hours of molasses fermentation were measured.

5.4. Fermentation

Isolated bacteria, mainly LAB, are considered as suitable microorganisms for industrial fermentation due to their non-pathogenicity, relatively rapid growth, short fermentation process (on this study results are visible from the beginning, see annex 9.7), ability to ferment cheap substrates (molasses), and protein secretion (De Vuyst and Vandamme, 1994). Similarly, *Gluconobacter spp*. have been reported for its ability to rapidly oxidize organic compounds to the corresponding acids and ketones (Silberbach *et al.*, 2003).

Even though fermentations aiming to produce lactic acid are reported to be performing at a pH ranging from 6.0 to 6.6 (Plessas *et al.*, 2020), this preliminary study was developed at a pH of 6.71. The obtained data suggest that fermentation under this condition can be also carried out because there is evidencing proteins and lactate production (Figure 6 and 8).

5.4.1. Protein Content

Bradford test allows estimating the total protein concentration through a colorimetric assay assessed with a spectrophotometer at 595 nm (Torres, Hernández and Peréz, 2013). To use this procedure, it is needed a calibration curve (Figure 5) that shows the relation between absorbance and total protein concentration. The correlation coefficient was 0.9981, a value close to 1 that suggests a linear correlation between the studied variables.

Data presented in Figure 6 was obtained by solving the protein concentration from the equation in Figure 5 and suggested that molasses inoculated with *G. oxydans* presented the highest protein concentration in 48 hours because the obtained value was 12.50 mg/mL. As well as that, *L. mesenteroides* and *L. plantarum* also present great concentration values 9.20 mg/mL and 4.66 mg/mL, respectively. Contrary to these, the lower values were obtained from media fermented with *L. lactis* (3.52 mg/mL), *L. holzapfelii* (2.84 mg/mL), and *K. gibsonii* (2.73 mg/mL). The last two values were not far apart from the control 2.61 mg/mL. At this point, it is important to consider that the biological activity of a strain is not defined by a certain amount of protein production instead by the protein biological potential (Torres, Hernández and Peréz, 2013).

5.4.2. Lactic Acid Analysis

Same as the previous method, a colorimetric assay was developed and it required a calibration curve. The curve served to measure lactate concentration and was assembled at 390nm (Figure 7). The curve shows a correlation coefficient of 0.9998 that suggests a linear correlation between absorbance and lactate concentration. That is to say, that as absorbance increases so does lactate concentration.

Data presented in Figure 8 was obtained by solving the lactate concentration from the equation on the calibration curve (Figure 7) and taking into account the dilution factor

(4:23). With this, interesting results were observed. To start, the control presented 0.34% W/W as lactate concentration and the lowest value was reported in *K. gibsonii* 0.29% W/W. On the other hand, media with the highest lactate concentration was *L. plantarum* with 2.22% W/W followed by *G. oxydans* with 1.88% W/W, *L.mesenteroides* with 1.21% W/W. Quite far was *L. holzapfelii* with 0.74% W/W and *L. lactis* with 0.59 % W/W.

The highest lactate concentration obtained by *L. plantarum* is related to its obligate saccharolytic fermentative metabolism that implies an efficient sugar breakdown (De Vos *et al.*, 2009). Contrary to this, the lowest value obtained by *K. gibsonii* could be explained because it produces acid from D, L-lactate (Dworkin *et al.*, 2006; De Vos *et al.*, 2009), so the bacteria consumed the available lactate instead of producing it.

Undoubtedly, the lactate available in the substrate of the molasses fermentation residues exists because the metabolic pathways of the used bacteria can ferment the sucrose present in molasses and transform it into lactate (Cuervo Mulet *et al.*, 2010). In the same way, it is important to consider that during lactic fermentation other metabolites also are produced *in situ,* these are hydrogen peroxide (H_2O_2) and bacteriocin-like compounds (De Vuyst and Vandamme, 1994; Ali *et al.*, 2020). These metabolites can be taken into account to consider them as profitable products.

6. CONCLUSIONS

In this work, the inspection of the sugar mill to discover bacteria growing in the sugar production pipelines allowed finding some species that belong to the LAB and AAB group that can produce both proteins and lactic acid. Whereas the bacterium that do not fit in any of these groups, *K. gibsonii,* shows low total protein production and an apparently lactate consumption. Knowing this, it can be stated that even though sugar mills harbor microorganisms that represent a risk for the industry due to sucrose consumption, the correct management and control of it will allow obtaining metabolites that can be profitable.

Although bacteria are considered damaging for sugarcane production, they possibly have industrial potential. In this way, *G. oxydans* can be considered as the best protein producer and a good lactate producer. In the same way, *L. plantarum* can be recognized

as the best lactate producer and a protein producer. Finally, *L. mesenteroides* is a good protein producer and a lactate producer. These microorganisms, especially *L. plantarum* and *G. oxydans,* can be used to valorize residues or by-products generated by sugar production processes. In this way, it could be possible to obtain profits that do not recompense sugar losses but could be an additional input for the industry.

In brief, diverse bacteria were obtained from the storage, extraction, and clarification of sugarcane juices. Most of them agree with the ones reported in articles and are associated with plant material and sugarcane. With this research, a small part of bacterial diversity associated with Ingenio Azucarero del Norte was known. Nevertheless, this is convenient because with this the industry could control and manage identified bacteria to avoid sugar production losses and could valorizing or give biological value to the sugar production by-products. In this way, profits can be produced.

7. RECOMMENDATIONS

Due to the dynamism of the processes of storage, clarification, and extraction of sugar cane juices, it is important to consider that the obtained bacteria are only a small fraction of the huge diversity that thrives on the sugar mill. Microbial flora sampling varies according to the weather, raw material, factory cleaning, and even the production process by-itself. By changing sucrose concentration, sampling season, incubation temperature, or agar concentration in the medium, new bacteria can be obtained.

Molasses fermentation study can be better developed by starting the fermentation with the same amount of bacteria in order to make a truly yield comparison. This is to obtain bacterial growth kinetics. I also recommend performing more essays to carry out statistical analysis that allows concluding more accurately.

To get profitable molasses fermentation, it could be a good idea to use them without adding compounds to enrich the substrate. In addition to saving costs for the enriching compounds this could shows which bacteria are better adapted to the natural conditions of the molasses. With this, molasses fermentation residues valorization would increase.

Another proposal can be studying molasses fermentation using bacterial consortia instead of a pure bacterium species. This can enhance protein and/or lactic acid production. In addition, this fermentation could be investigated under different temperature and °Brix conditions.

Develop a deeper analysis of the substrate obtained after 48 hours of fermentation. To carry this, it could be better to purify the substrate and assess the production through more specialized techniques. For example, for lactic acid concentration High-Performance Liquid Chromatography (HPLC) can be used, whereas to determine protein structure Hydroxyl Radical Protein Footprinting (HRPF) can be performed.

The isolation and characterization of microbes can also be focused on yeasts and molds at the same sampling points. For further studies, other sampling points can be considered, even for bacteria, and these could be cane stalk transport, mill yard, harvest machinery, and cane loaders stages.

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9. ANNEXES

9.1. *Leuconostoc mesenteroides* alignment.

Leuconostoc mesenteroides **consensus sequence**	**LENGTH:** 1337 bp

GCATTACAAACTCCCATGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGCGGCGTGCTGATCCGCGAT
TACTAGCGATTCCGACTTCATGTAGTCGAGTTGCAGACTACAATCCGAACTGAGACGTACTTTAAGAGATTAGCTCACCCT
CGCGGGCTGGCAACTCGTTGTATACGCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATCTGACGTCG
TCCCCGCCTTCCTCCGGTTTGTCACCGGCAGTCTCGCTAGAGTGCCCATCTGAATGCTGGCAACTAACAATAAGGGTTGCG
CTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACGACCATGCACCACCTGTCACTTTGTCTCCGAAGA
GAACACTTCTATCTCTAAAAGCTTCAAAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGC
TCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAACACTTAATGCG
TTAGCTTCGGCACTAAGAGGCGGAAACCTCCTAACACCTAGTGTTCATCGTTTACGGTGTGGACTACCAGGGTATCTAATC
CTGTTTGCTACCCACACTTTCGAGCCTCAACGTCAGTTGCAGTCCAGTAAGCCGCCTTCGCCACTGGTGTTCTTCCATATAT
CTACGCATTCCACCGCTACACATGGAGTTCCACTTACCTCTACTGCACTCAAGTTAACCAGTTTCCAATGCCATTCCGGAG
TTGAGCTCCGGGCTTTCACATCAGACTTAATAAACCGTCTGCGCTCGCTTTACGCCCAATAAATCCGGATAACGCTCGGGA
CATACGTATTACCGCGGCTGCTGGCACGTATTTAGCCGTCCCTTTCTGGTATGGTACCGTCAAACTAAAATCATTTCCTATT
CTAGCTGTTCTTCCCATACAACAGTGCTTTACGACCCGAAAGCCTTCATCACACACGCGGCGTTGCTCCATCAGGCTTTCG
CCCATTGTGGAAGATTCCCTACTGCAGCCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAATGTGGCCGATCAGTCTCT
CAACTCGGCTATGCATCATTGTCTTGGTAGGCCTTTACCCCACCAACTAACTAATGCACCGCGGATCCATCTCTAGGTGAC
GCCGAAGCGCCTTTTAACTTTGTGTCATGCGACACTAAGTTTTATTCGGTATTAGCATCTGTTTCCAAATGTTATCCCCAGC
CTTGAGGTAGGTTGTCCACGTGTTACTCACCCGTTCGCCAC

9.2. *Lactiplantibacillus plantarum* alignment.

Lactiplantibacillus plantarum **consensus sequence**	**LENGTH:** 1361 bp

GTGTTACAAACTCTCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCATGCTGATCCGCGAT
TACTAGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGCTTTAAGAGATTAGCTTACTCT
CGCGAGTTCGCAACTCGTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCAT
CCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCACCAGAGTGCCCAACTTAATGCTGGCAACTGATAATAAGGGTTGCGC
TCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTATCCATGTCCCCGAAGGG
AACGTCTAATCTCTTAGATTTGCATAGTATGTCAAGACCTGGTAAGGTTCTTCGCGTAGCTTCGAATTAAACCACATGCTC
CACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGCGGAATGCTTAATGCGTT
AGCTGCAGCACTGAAGGGCGGAAACCCTCCAACACTTAGCATTCATCGTTTACGGTATGGACTACCAGGGTATCTAATCC
TGTTTGCTACCCATACTTTCGAGCCTCAGCGTCAGTTACAGACCAGACAGCCGCCTTCGCCACTGGTGTTCTTCCATATATC
TACGCATTTCACCGCTACACATGGAGTTCCACTGTCCTCTTCTGCACTCAAGTTTCCCAGTTTCCGATGCACTTCTTCGGTT
GAGCCGAAGGCTTTCACATCAGACTTAAAAAACCGCCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTTGCCAC
CTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAAATACCGTCAATACCTGAACAGTTACTCTC
AGATATGTTCTTCTTTAACAACAGAGTTTTACGAGCCGAAACCCTTCTTCACTCACGCGGCGTTGCTCCATCAGACTTTCGT
CCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAATGTGGCCGATTACCCTCTC
AGGTCGGCTACGTATCATTGCCATGGTGAGCCGTTACCCCACCATCTAGCTAATACGCCGCGGGACCATCCAAAAGTGAT
AGCCGAAGCCATCTTTCAAGCTCGGACCTGCGGTCCAAGTTGTTATGCGGTATTAGCATCTGTTTCCAGGTGTTATCCCCC
GCTTCTGGGCAGGTTTCCCACGTGTTACTCACCAGTTCGCCACTCACTCAAATGTAAATCATGATGC

9.3. *Leuconostoc holzapfelii* alignment.

Leuconostoc holzapfelii **consensus sequence**	**LENGTH:** 1346 bp

ATTACAAACTCCCATGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGTATTCACCGCGGCGTGCTGATCCGCGATTA
CTAGCGATTCCGACTTCGTGCAGTCGAGTTGCAGACTGCAGTCCGAACTGAGACGTACTTTAAGAGATTAGCTCACCTTCG
CAGGTTGGCAACTCGTTGTATACGCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATCTGACGTCGTC
CCCGCCTTCCTCCGGTTTGTCACCGGCAGTCTCGCTAGAGTGCCCAACTGAATGCTGGCAACTAACAATAAGGGTTGCGCT
CGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACGACCATGCACCACCTGTCACTTTGTCTCCGAAGAGA
ACACTTCTATCTCTAAAAGCTTCAAAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTC
CACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAATACTTAATGCGTT
AGCTTCGGCACTAAGAGGCGGAAACCTCCTAACACCTAGTATTCATCGTTTACGGTGTGGACTACCAGGGTATCTAATCCT
GTTTGCTACCCACACTTTCGAGCCTCAACGTCAGTTGTTGTCCAGTAAGCCGCCTTCGCCACTGGTGTTCTTCCATATATCT
ACGCATTCCACCGCTACACATGGAGTTCCACTTACCTCTACAACACTCAAGTTAACCAGTTTCCAATGCCATTCCGGAGTT
GAGCTCCGGGCTTTCACATCAGACTTAATCAACCGTCTGCGCTCGCTTTACGCCCAATAAATCCGGATAACGCTCGGGACA
TACGTATTACCGCGGCTGCTGGCACGTATTTAGCCGTCCCTTTCTGGTATGGTACCGTCAAACTAAAATCATTCCCTATTTT
AGCATTTCTTCCCATACAACAGTGCTTTACGACCCGAAAGCCTTCATCACACACGCGGCGTTGCTCCATCAGGCTTGCGCC
CATTGTGGAAGATTCCCTACTGCAGCCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAATGTGGCCGATCAGTCTCTCA
ACTCGGCTATGCATCATCGTCTTGGTAAGCCTTTACCCCACCAACTAACTAATGCACCGCGGATCCATCTCTAGGTGACGC
CGTAGCGCCTTTTAACTTTGTATCATGCGATACTAAGTTTTATTCGGTATTAGCATCTGTTTCCAAATGTTATCCCCAGCCT
TGAGGCAGGTTATCCACGTGTTACTCACCCGTTCGCCACTCGCTTGAAAG

9.4. *Gluconobacter oxydans* alignment.

Gluconobacter oxydans **consensus sequence**	**LENGTH:** 1267 bp

AACGCGTAGGGATCTATCCACGGGTGGGGGACAACTTCGGGAAACTGGAGCTAATACCGCATGATACCTGAGGGTCAAA
GGCGCAAGTCGCCTGTGGAGGAACCTGCGTTCGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCGATAG
CTGGTTTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT
GGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTCGACGGGG
ACGATGATGACGGTACCCGTAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGT
TGCTCGGAATGACTGGGCGTAAAGGGCGCGTAGGCGGTTGTTACAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAAC
TGCATTTGATACGTGACGACTAGAGTTCGAGAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGG
AAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCGATACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCTGTAAACGATGTGTGCTGGATGTTGGGAAACTTAGTTTCTCAGTGTCGAAGCTAACGCGCT
AAGCACACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGCATGGGGAGGACCGGTTCAGAGATGGACCTTTCTTCG
GACCTCCCGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTGTCTTTAGTTGCCAGCACTTTCAGGTGGGCACTCTAGAGAGACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGAC
GTCAAGTCCTCATGGCCCTTATGTCCTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGAAGCTACATGGTGACAT
GGTGCTGATCTCTAAAAGCCGTCTCAGTTCGGATTGTACTCTGCAACTCGAGTACATGAAGGTGGAATCGCTAGTAATCGC
GGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTT

9.5. *Lactococcus lactis* alignment.

Lactococcus lactis **consensus sequence**	**LENGTH:** 1334 bp

TCCCAACTCCCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCGTGCTGATCCGCGATTACT
AGCGATTCCGACTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAATGGTTTTAAGAGATTAGCTAAACATCAC
TGTCTCGCGACTCGTTGTACCATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCC
CACCTTCCTCCGGTTTATCACCGGCAGTCTCGTTAGAGTGCCCAACTTAATGATGGCAACTAACAATAGGGGTTGCGCTCG
TTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTATCCCGTGTCCCGAAGGAACT
TCCTATCTCTAGGAATAGCACGAGTATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCAC
CGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAGGCGGAGTGCTTATTGCGTTAGCT
GCGATACAGAGAACTTATAGCTCCCTACATCTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTG
CTCCCCACGCTTTCGAGCCTCAGTGTCAGTTACAGGCCAGAGAGCCGCTTTCGCCACCGGTGTTCCTCCATATATCTACGC
ATTTCACCGCTACACATGGAATTCCACTCTCCTCTCCTGCACTCAAGTCTACCAGTTTCCAATGCATACAATGGTTGAGCC
ACTGCCTTTTACACCAGACTTAATAAACCACCTGCGCTCGCTTTACGCCCAATAAATCCGGACAACGCTCGGGACCTACGT
ATTACCGCGGCTGCTGGCACGTAGTTAGCCGTCCCTTTCTGGGTAGTTACCGTCACTTGATGAGCTTTCCACTCTCACCAA
CGTTCTTCTCTACCAACAGAGTTTTACGATCCGAAAACCTTCTTCACTCACGCGGCGTTGCTCGGTCAGACTTTCGTCCATT
GCCGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTTTGGGCCGTGTCTCAGTCCCAATGTGGCCGATCACCCTCTCAGGTC
GGCTATGTATCATCGCCTTGGTGAGCCTTTACCTCACCAACTAGCTAATACAACGCGGGATCATCTTTGAGTGATGCAATT
GCATCTTTCAAACTTAAAACTTGTGTTTAAAGTTTTTATGCGGTATTAGCATTCGTTTCCAAATGTTGTCCCCCGCTCAAAG
GCAGATTCCCCACGCGTTACTCACCCGTTCGCTGCTCA

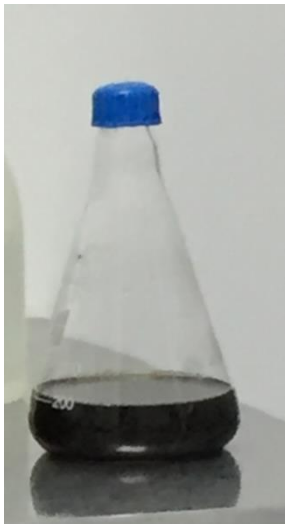
9.6. *Kurthia gibsonii* alignment.

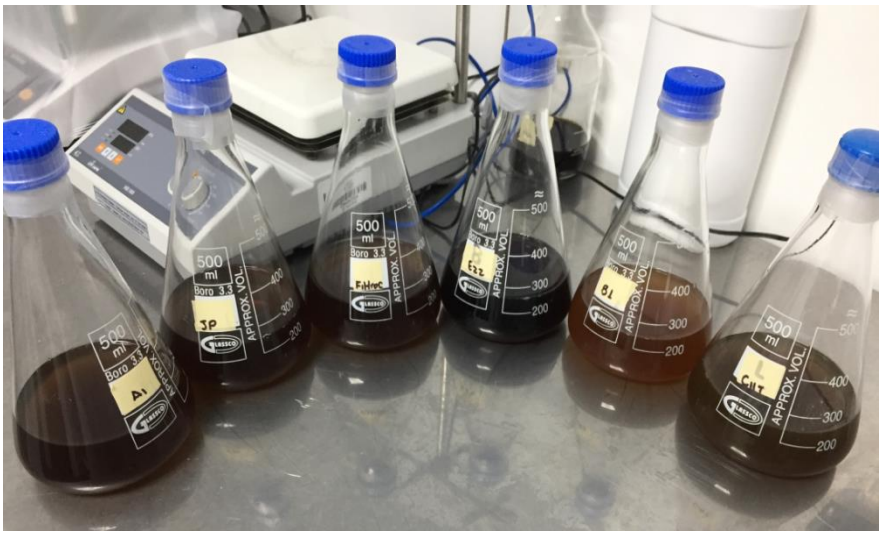
Kurthia gibsonii **consensus sequence**	**LENGTH:** 1386 bp

TCCTTACGGTTACCCCACCGACTTCGGGTGTTACAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGT
ATTCACCGCGGCATGCTGATCCGCGATTACTAGCGATTCCGGCTTCATGCAGGCGAGTTGCAGCCTACAATCCGAACTGA
GAACGATTTTATGAGATTAGCTTACCCTCGCGAGTTTGCGACTCTTTGTATCGTCCATTGTAGCACGTGTGTAGCCCAGGT
CATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCCTTAGAGTGCCCAACTAAA
TGATGGCAACTAAGAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATG
CACCACCTGTCACCAATGTCCCCGAAGGGAAAATCCTATCTCTAGAACGGTCATTGGGATGTCAAGACCTGGTAAGGTTC
TTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTC
GTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTT
TACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGTGTCAGTTACAGACCAGACAGT
CGCCTTCGCCACTGGTGTTCCTCCAAATCTCTACGCATTTCACCGCTACACTTGGAATTCCACTATCCTCTTCTGCACTCAA
GTTCCCCAGTTTCCAATGACCCTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGAAACCACCTGCGCGCGCTTTA
CGCCCAATAAATCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTAATAA
GGTACCGTCAAGGTACGTTCATTTCCTAACGTACTTGTTCTTCCCTTACAACAGAGTTTTACGATCCGAAAACCTTCATCAC
TCACGCGGCGTTGCTCCATCAGACTTTCGTCCATTGTGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTTTGGGCCGTGT
CTCAGTCCCAATGTGGCCGATCACCCTCTCAGGTCGGCTATGCATCGTTGCCTTGGTAGGCCGTTACCCCACCAACTAGCT
AATGCACCGCGGGCCCATCCTACAGTGACGCCGAAGCGCCTTTCAACTTCAAAACATGTGATTCGAAGGATTATCCGGTA
TTAGCCCAGGTTTCCCTGAGTTATCCCGATCTGTAGGGCAGGTTGCCCACGTGTTACTCACCCGTCCGCCGCTAAATCAGA
GAAGCAAGCTTC

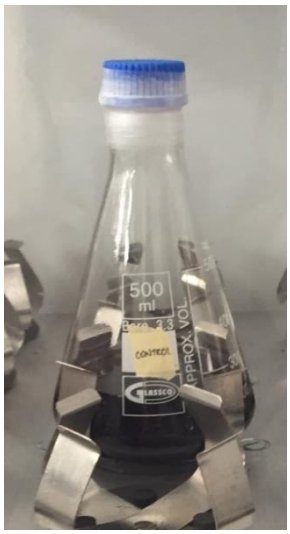
9.7. Molasses Fermentation

9.7.1. **Molasses Fermentation at time 0.** Control and Isolated Bacteria.





9.7.2.**Molasses Fermentation at 24 hours.** Control and Isolated Bacteria.



9.7.3. Molasses Fermentation at 48 hours. Control and Isolated Bacteria.