Evaluation of the Antibacterial and Antifungal Activity of Isolated Bacteria from Appenzeller® Swiss Cheese

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November, 2020

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Special graduation project presented as partial requirement to obtain a Bachelor of Science degree in Food Science and Technology

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Abstract. Pathogenic bacteria are etiological agents that cause diseases related to food consumption, and spoilage microorganisms convert foods in unacceptable for consumers. In dairy products, there exist groups of microorganisms like bacteria, yeast, and molds. These bacteria can be isolated from dairy products like Appenzeller® Swiss cheese. The bacteria can inhibit other types of pathogenic and spoilage microorganisms that can increase the shelf-life of the products. The research study assessed the antimicrobial activity of isolated bacteria from Appenzeller® Swiss Cheese and identified the strains. Phase 1: Isolation of the bacteria where 27 morphological different strains were found. Phase 2: Characterization of the strains, discovering that *Bacillus* like the principal genus and *Staphylococcus.* Phase 3: Evaluation of the antimicrobial activity presented by six different strains, four only with inhibition activity against *Aspergillus fumigatus*, one with inhibition against *Bacillus amyloliquefaciens* and *Aspergillus fumigatus*, and the last one with inhibition of *Listeria innocua,* and the other two microorganisms evaluated. The antagonistic effect could be attributed to chitinase, protease, and volatile organic compounds production as butan-1-ol and 3-methylbutan-1-ol. The further applications for the isolated bacteria can be tested on *Aspergillus* and *Penicillium* that affect the cheese industry, *B. stearothermophilus,* and *B. coagulans* that produce the flat sour flavors, and *Aspergillus* genera with potential mycotoxin production. Further studies need to evaluate metabolite characterization and extend the spectrum of microorganisms evaluated.

Keywords: Antagonism, *Bacillus*, inhibition halo, metabolites.

Resumen. Las bacterias patógenas son agentes etiológicos que causan enfermedades relacionadas con el consumo de alimentos, y los microorganismos deterioradores vuelven los alimentos inaceptables para el consumidor. Dentro de los productos lácteos existen grupos de microorganismos como bacterias, levaduras y hongos. Estas bacterias pueden ser aisladas de los productos lácteos como el queso suizo Appenzeller®. Las bacterias pueden inhibir otros tipos de microorganismos patógenos y deterioradores, aumentando así la vida de anaquel de los productos. El estudio evaluó la actividad antimicrobiana de bacterias aisladas a partir de queso suizo Appenzeller® y la identificación de las cepas. Fase 1: Aislamiento de las bacterias donde fueron encontradas 27 cepas morfológicamente diferentes. Fase 2: Caracterización de las cepas, donde se encontró *Bacillus* como principal género y también *Staphylococcus.* Fase 3: Evaluación de la actividad antimicrobiana que presentaron seis cepas diferentes, solo cuatro con inhibición en contra de *Aspergillus fumigatus*, una con inhibición sobre *Bacillus amyloliquefaciens* y *A. fumigatus*, y la última con inhibición sobre *Listeria innocua,* y los otros dos microorganismos evaluados anteriormente. El efecto antagónico puede atribuirse a la producción de quitinasa, proteasa y compuestos orgánicos volátiles como butan-1-ol y 3-metilbutan-1-ol. Las futuras aplicaciones para las bacterias aisladas pueden ser evaluar contra *Aspergillus* y *Penicillium* que afectan la industria de quesos, *B. stearothermophilus,* y *B. coagulans* que producen el flat sour y en contra del género *Aspergillus,* que produce micotoxinas. Estudios futuros se necesitan para evaluar la caracterización de metabolitos y extender la gama de microorganismos evaluados.

Palabras clave: Antagonismo, *Bacillus*, halo de inhibición, metabolitos.

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1. INTRODUCTION

Foodborne diseases are the illness result for eating food contaminated at any stage of the food chain (WHO 2020). Exist over 200 diseases that are a public health problem that affects one in 10 people worldwide each year (WHO 2020). However, the food has not only the presence of pathogenic bacteria, but there is also another important change called spoilage food, these impacts in the matrix of the food making it unacceptable for human consumption. The pathogenic bacteria are etiological agents that cause diseases mainly related to the consumption of the huge spectrum of foods consuming daily, this way represent the greatest health problem both in developed and in third world countries (Haghi *et al.* 2015). These countries have poor control of the food manufacturing process and nevertheless an increase of outbreaks related to food than developed countries. On the other side, the spoilage of food includes insect damage, physical injury, the activity of indigenous enzymes, and chemical changes that can be developed by bacteria, yeast, and molds (Bourdichon and Rouzeau 2012). The characterization of the groups of bacteria present in fermented dairy products has a potential control in food safety and food quality because these groups have a specific benefit characteristic in the control of foodborne and spoilage. Through physic-chemical properties of some different strains, can reach the inhibition of microorganisms that cause damage to the microbiome of the digestive system (Garcia-Cano *et al.* 2019).

During the dairy product elaboration, a variety of microorganisms present in the place of manufacturing and starter cultures that are added to produce specific characteristics are involved. Both aggregates and those particular of the plant participate significantly in the final product. Lactic acid bacteria (LAB) are a common group of microorganisms present in dairy fermented and non-fermented products. However, other non-lactic acid bacteria can be part of the surface as *Arthrobacter nicotine,* brevibacteria, corynebacterial, micrococci, and *Staphylococcus* spp. (Brennan *et al.* 2002; Rademaker *et al.* 2005). This variety of microbes can change depending on the product and specific conditions as ripened or non-ripened, storage time, and environmental conditions.

Cheese is a group of fermented milk-based food products that have a great range of flavors and forms around the world (Fox 1994). In Switzerland, a widely known cheese, named Appenzeller® cheese, it is a semi-hard cheese known for its particular spicy flavor generated by the brine of herbs added during ripening (Guggisberg *et al.* 2017). Like other swiss cheeses, they are exposed to a series of complex microbiological, biochemical, and physicochemical changes during ripening (O'Mahony *et al.* 2005). At the beginning of preparation, a starter culture is added to promote fermentation, where the amount of LAB is controlled to be predominant. (Lee *et al.* 2015). However, this population is also varied by other important bacteria present in raw milk where it is assumed that they also affect the final characteristics of the product (Guggisberg *et al.* 2017). This information matches with that reported by Wolfe *et al.* (2014) who found in a study of 137 different cheeses made in 10 different countries between the United States and Europe that at least 60% of the bacteria and 25% of the fungi present in the cheeses are not part of starter cultures and these originate from the environment of the production place.

The pathogenic bacteria commonly encountered and transmitted through milk are *Listeria monocytogenes*, *Salmonella spp.*, *Staphylococcus aureus,* and *Escherichia coli*. However, there are

mechanisms to destroy these microorganisms such as pasteurization. Despite this, the treatment does not protect against contamination after pasteurization treatment. Raw milk is a perfect culture medium for the development of bacteria due to its concentration of nutrients (Arqués *et al.* 2015). This effect is intensified in dairy products such as cheese where the presence of fat and protein is increased.

The study of secondary metabolites of bacteria can positively impact the safety and quality of dairy products, in addition to helping to keep a balance in the microbiota inside of the digestive system of humans (Fernández *et al.* 2015). The impact of the study is of international magnitude because according to reports from the World Dairy Situation in 2016 document, the average per capita at the global level of consumption of dairy products, in general, is 111.3 kg with a growth of 0.6% compared to 2015 (Sumner 2018). The trend continues to rise and with it the probability of enriching the dairy products marketed with this type of bacteria with the purposes to get the benefits.

This document reports a chemical (catalase test and Gram staining), morphological and molecular analysis of different strains of bacteria found in the surface of Appenzeller® cheese with a potential application on the food industry and food safety. These strains produce important compounds that inhibit the growth and multiplication of *Escherichia coli*, *Staphylococcus epidermidis*, *Bacillus amyloliquefaciens*, *Candida albicans*, *Listeria innocua,* and *Aspergillus fumigatus* that can affect the commercialization chain of the dairy product and beyond the huge food industry around the world.

The objectives of this study were:

- Isolate bacteria present in Appenzeller® cheese.

- Evaluate the antimicrobial activity of bacteria isolated from Appenzeller® cheese.

- Identify the bacteria with antimicrobial activity.

2. MATERIALS AND METHODS

Location

The research was conducted entirely in J.T. "Stubby" Parker Dairy Foods laboratory, located in the Department of Food Science and Technology of The Ohio State University located in Columbus, Ohio, United States. The experiment was carried out between February and March 2020, and the written and presentation of the document was developed in two sites, The Ohio State University and Panamerican Agricultural School in Honduras, Central America.

Isolation of the bacteria

Obtaining and storing the sample. The sample used was the surface of Appenzeller® cheese from Switzerland. This sample was collected by Dr. Rafael Jiménez Flores, from a group of cheeses matured in 2019 in which the absence of microbial growth was observed on their surface, in contrast to other cheeses. The sample was placed in a sterile container and stored at -20 °C for later analysis.

Homogenization of the sample. Twenty grams sample were weighed into a special sterile bag for Stomacher, then 180 mL of saline solution (0.85% NaCl and pH 7) were added, next the sample was placed inside the Stomacher®80 Biomaster. After closing the system, the time and operating speed options were selected, these being 60 seconds and normal respectively. Subsequently, the homogenized solution was divided into 3 equal parts, in three sterile tubes with a capacity of 50 mL.

Sample dilution. Dilutions were made from 10^{-2} to 10^{-8}, these dilutions were made in sterile 15 mL tubes where 9 mL of saline solution were previously placed. From the solutions contained in the tubes with a capacity of 50 mL, 1 mL was taken and it was added to the 10^{-2} tube and from this, after homogenization, 1 mL was added to the subsequent tubes until the dilution of 10^{-8} was reached.

Plate inoculation and incubation. Man Rogosa Shape (MRS) culture medium with 1% w/v agar, Plate Count Agar (PCA), and Potato Dextrose Agar (PDA) were used to inoculate the dilutions; subsequently, they were incubated for 48 h at 37 °C searching LAB and aerobic plate count (APC). The PDA plates were incubated at room temperature (approx. 25 °C) for 3 - 5 days for the determination of molds and yeasts.

Colony isolation. With previously sterilized wooden toothpicks, the colonies identified morphologically different were minced, according to color, shape, margin, and size and then placed in two test tubes with 3 mL of culture medium, one with MRS broth and the other with nutrient roth, depending on the medium from which they come from isolation. They were covered and incubated at 37 °C between 24 and 48 h.

Bacteria isolation. The cultures of 24 h were streaked to isolate colonies on MRS plates with 1% w/v agar and on previously solidified PCA plates. Subsequently, they were incubated for 24 h at

37 °C. Bacteria isolated in PCA were minced with a bacteriological inoculation loop, later, they were inoculated in the MRS plate with 1% w/v agar and to check if they were already isolated in that culture medium. If they presented morphology different from the rest of the bacteria isolated from MRS, they were isolated again. The same procedure was repeated, in this way the purity of each of the cultures. Finally, the culture was carried out in 4 mL of MRS broth, incubating for 24 h at 37 °C. From the pure isolation culture, the 4 mL was placed in a 5 mL centrifuge tube to be centrifuged at 25,000 × g for 15 min. In the end, the supernatant was removed followed by adding 3 mL of MRS + glycerol (4:1 v/v), and 1 mL of this mixture was distributed for each cryogenic tube to later be stored at -80 °C.

Characterization of the strains

An amount of 3 mL of MRS was placed in a test tube and 10 µL of the isolated bacteria culture was added. It was homogenized and incubated overnight at 37 °C. The following day, each of the cultures was used for both the catalase test and the Gram staining.

Catalase test. An aliquot of the previous day's culture was placed on a slide, then a drop of 3% hydrogen peroxide (H_2O_2) was added to it. If immediately after placing the hydrogen peroxide, effervescence (bubbling) was visualized, this means a positive reaction to the test and otherwise a negative reaction (Figure 1) (Reiner 2010).

Figure 1. Positive (left) and negative (right) reaction to the catalase test.

Gram staining. An aliquot of the culture carried out the day before was taken and placed in the center of a slide, spread on the slide with the help of the handle, and gently passed over the burner three times to fix the sample. Next, the fixed sample was covered with crystal violet for 30 seconds, subsequently, the crystal violet was removed with distilled water. The same procedure was performed, but this time with Gram's iodine for 1 min. Then with the slide inclined, 95% ethanol was added for 20 sec, the remains were immediately removed with distilled water. Finally, the sample was dried and evaluated under the microscope with a 100x objective using immersion oil. Bacteria stained pink correspond to the Gram-negative group and Gram-positive bacteria to the purple color (Figure 2) (Moyes *et al.* 2009).

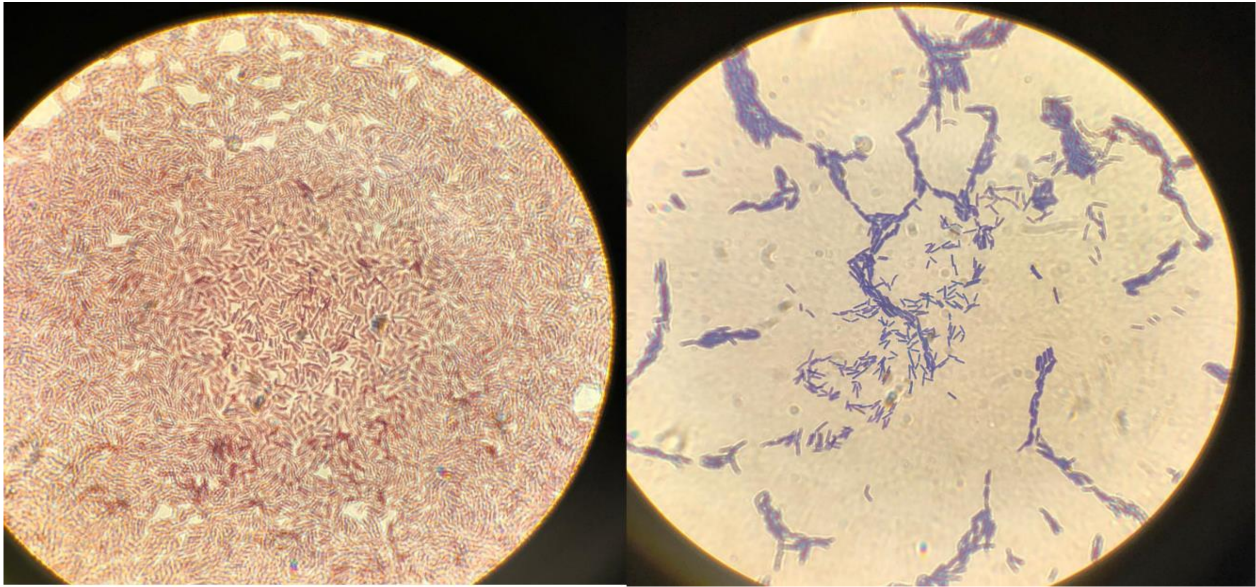

Figure 2. Gram-negative and Gram-positive (left to right).

Genetic characterization. The strains that presented any type of antimicrobial activity needed to be prepared for 16s rRNA analysis through DNA extraction with the principal aim to determine the species that produces the results. The DNA extraction was performed using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the protocol for Gram-Positive Bacteria with specific modification in add 70 µL of lytic enzymes, first incubation during 30 minutes, the first centrifuge for 3 minutes, add 7 µL of RNase solution and incubate it during 30 minutes, centrifuge during protein precipitation for 5 minutes. And finally, during the DNA precipitation and rehydration: transfer the supernatant to 800 µL of isopropanol, the first and the second centrifuge for 10 minutes.

The DNA extraction results were necessary to generate the amplification on a thermal cycler. After all, was necessary to clean-up each sample with the protocol called Wizard® SV Gel and PCR Clean-Up System for DNA Purification by Centrifugation (Promega, Madison, WI, USA). With the clean-up, resultant samples were made the agarose gel to be sure of the quality of the samples. The final product by each bacteria was tested for species identity using the 16s rDNA sequencing method by Macrogen (South Korea). With the chromatograms were selected the portions of the sequence with high peaks and eliminate the rest (purification). And then each sequence of nitrogen bases was analyzed by the sequence alignment analysis called Basic Local Alignment Search Tool (BLAST) from National Center for Biotechnology Information (NCBI) with specific selection in nucleotide BLAST, standard databases (nr, etc.), nucleotide collection (nr/nt) and highly similar sequences (megablast) those are pre-selected on the database. Analysis of the results was developed by each strain, the species from the output chart of the analysis by each sequence of nitrogen bases (forward and reverse) was selected by the smallest E value, the highest percent identity, the highest percent of query cover, and the highest total score to define the result of the identification.

Evaluation of the antibacterial and antifungal activity

Preparation of bacteria cultures. An amount of 10 microliters from each of the isolated bacterial strains in cryogenic tubes were placed in 1 mL of liquid MRS contained in centrifuge tubes. Subsequently, they were incubated at 37 °C overnight. The next day, each of the tubes was centrifuged at 1,520 × g, thus dividing into pellets and supernatants. The pellet was washed with 1 mL of saline solution, centrifuged again and the same pellet was resuspended in 1 mL of saline solution. On the other hand, the pH of the supernatant was regulated to neutral (7.0 ± 0.5), using drops of NaOH (0.05 M). Then pass the supernatant through a 0.45 micrometers syringe filter to get the culture filtrates of supernatant (CF). The division between pellet and CF was made to determine if, when antibacterial compounds existed, they were present in the cell wall of bacteria or extracellular compounds. Finally, the tubes containing the pellet and those containing the CF were stored at -20 °C for later use.

Preparation of microorganisms (bacteria and yeast) of interest. An amount of 10 microliters of *Escherichia coli*, *Staphylococcus epidermidis*, *Bacillus amyloliquefaciens*, *Candida albicans,* and *Listeria innocua* (strains from the J.T. "Stubby" Parker Dairy Foods lab) were placed in 5 mL of tryptic soy broth (TSB) and incubated at 37 °C overnight.

Plates preparation for evaluation of the antibacterial activity. With 20 µl aliquots of the microorganisms of interest prepared the day before were inoculated 100 mL of tryptic soy agar (TSA) previously sterilized and tempered at 45 °C ± 2 with constant agitation. For each of the microorganisms, 20 mL/plate were dispensed and allowed to solidify. Next, 10 µl of the re-suspended pellet and its respective CF were deposited on each of the plates. The plates were divided into eight spaces (Figure 3), where four bacteria (pellet and supernatant) were deposited for each plate. Finally, after drying, the Petri dishes were inverted and placed to incubate for 24 h at 37 °C.

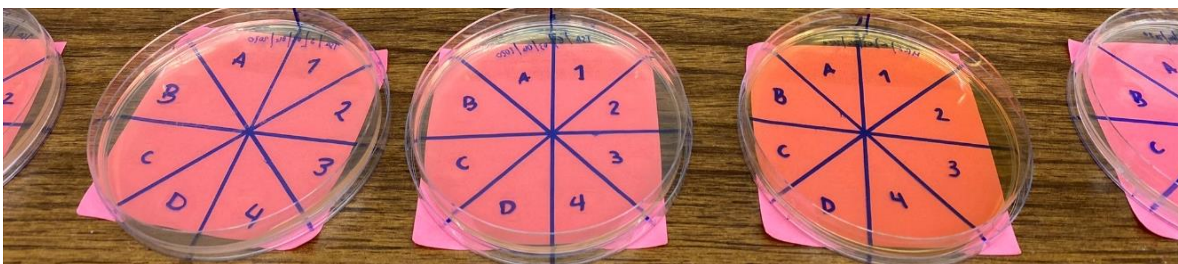

Figure 3. Eight sections into which each Petri dish was divided.

Plates preparation for antifungal activity evaluation. The medium of MRS with agar 1% w/v was sterilized, after sterilization, it was served in each of the Petri dishes and it was waited for them to dry. After being completely dry, 10 µl of the re-suspended pellet and of the CF were inoculated on them. Then, each Petri dish was divided into eight spaces, to evaluate four LAB isolates per plate. After drying the inoculum, the Petri dishes were inverted and incubated at 37 °C overnight. At the end of the 24 h incubation, the second layer of culture medium was added, but this time it was soft PDA (0.8% agar). This medium was previously inoculated with 1 mL of *Aspergillus fumigatus*, which was obtained by washing the surface of a Petri dish contaminated with *A.*

fumigatus with saline solution. And they were placed to incubate at room temperature 24 °C for at least 2 days to wait for the fungus development.

Reading results of antibacterial activity

At the end of the 24 h incubation, the Petri dishes were taken, and it was observed if there was a development of inhibition halos against each of the microorganisms of interest in the medium. This was the way of detecting the presence or absence of antibacterial compounds produced from the bacteria. The mentioned antibacterial compounds can come from the cell wall of bacteria or metabolites in the extracellular fluid of the cell. The above was verified through whether there was activity in a higher proportion in the re-suspended pellet or the CF. Finally, the measurement in millimeters (mm) of the inhibition halo of the bacteria that presented it was carried out to quantify its activity.

Reading results of antifungal activity

At the end of the incubation time of approximately 3 days, the plates were observed to detect whether the metabolites produced by the bacteria inhibited the growth of *A. fumigatus*. Otherwise, there was a uniform development along the entire PDA surface of the *A. fumigatus*. The last step was to measure in millimeters (mm) the inhibition halo of the bacteria on the fungus, to quantify its antifungal activity.

Statistical analysis

The data were collected at laboratory level. Measurements of the inhibition halo were made in millimeters (mm). The data were analyzed through a Completely Randomized Design (CRD). A factorial arrangement was used with 18 treatments using six isolated bacteria with antimicrobial effect as the first factor and *L. innocua, B. amyloliquefaciens,* and *A. fumigatus* for the second factor. Three repetitions by each treatment were used and the data were analyzed with the Statistical Analysis System software 9.4 (SAS 2016), across an analysis of variance (ANOVA), with a lineal general model (GLM), and mean adjust (DUNCAN) to analyze the main factors. The significant level used was 95%.

3. RESULTS AND DISCUSSION

Table 1 shows the amount and log of CFU/g present in the sample from the surface of Appenzeller® cheese. Where it can be seen that the detected amount of both bacteria on MRS and total aerobics is near to five log, which translates into 100,000 CFU/g. However, the presence of molds and yeasts is below 500 CFU/g. The results dictate that the total population of bacteria in the sample can be grown under MRS and PCA conditions.

Table 1. Count of microorganisms present in Appenzeller® cheese sample.

Indicator group	**Log CFU/g** **Mean ± Std. Dev.**
Bacteria on MRS	5.02 ± 0.06[a]
Bacteria on PCA	4.98 ± 0.02[a]
Total yeast and molds on PDA	1.56 ± 0.79[b]

[a-b]Means followed by a different letter are statistically different ($P < 0.05$). CV (%)= Coefficient of variation (%). The results represent the average ± standard deviation of three repetitions.

Through a Ducan mean adjust separation, it was determined that the number of bacteria present in MRS and PCA was the same. MRS has ammonium citrate which inhibits Gram-negative growth (Man *et al.* 1960). It could be possible only the presence of Gram-positive bacteria on the sample that did not have a difference with population growth on PCA. The results are shown in Figure 4, where it was proved that PCA bacterial isolation on MRS and these strains were the same. PDA is a medium to cultivate yeast and molds and it was incubated at room temperature, for this reason, it was not able to develop bacteria and only showed yeast and molds.

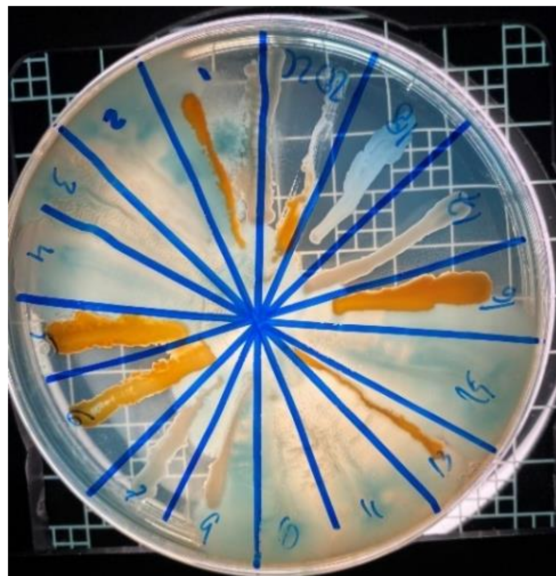

Figure 4. Isolated bacteria on PCA proved on MRS.

Table 2 shows a summary of the isolated bacteria. A total of 27 different morphological strains on the MRS medium Were isolated. It can be noted that 18.5% of the isolated bacteria showed antifungal activity against *Aspergillus fumigatus*. However, the percentage of isolated bacteria were decreasing while showing activity against other microorganisms 3.7% against *Listeria innocua,* 7.4% against *Bacillus amyloliquefaciens.* Finally, no bacteria presented inhibition against *Candida albicans, Escherichia coli,* and *Staphylococcus epidermidis.*

Table 2. Summary of isolated bacteria and strains with antimicrobial activity.

Category	Number of strains
Isolated strains	27
Antimicrobial activity against:	
Candida albicans	0
Escherichia coli	0
Staphylococcus epidermidis	0
Listeria innocua	1
Bacillus amyloliquefaciens	2
Aspergillus fumigatus	6

The total of isolated strains could be influenced by the possibility of the replication of bacteria, in other words, for the time of storage de sample the quantity of bacteria falls. The inhibition process was caused presumably by metabolites production specifically per bacteria, for that reason not all bacteria present the same inhibition amount because the species change. *Aspergillus fumigatus* showed more susceptibility during the evaluation. To understand the reasons for inhibition and quantity of the halo is necessary to know the compound of inhibition.

In Table 3 the results of 16s rRNA gene analysis for strains that demonstrated the antibacterial and/or antifungal activity are presented. The result of the Q+ strain sequence cannot be possible to be present because the DNA extraction did not pass the quality requirements in the laboratory analysis. On the other hand, four of the six bacteria with antimicrobial effects are part of the *Bacillus* group. Is necessary to mention that unknown strain, morphological, and catalase and Gram staining tests, fill the requirements as a *Bacillus* strain. Finally, only one strain belongs to the *Staphylococcus* group.

Table 3. Species of isolated strains with antimicrobial activity by 16s rRNA gene analysis.

Strain	Genera and species	Percent identity (%)
18	*Bacillus sp.*	100.00
-	*Bacillus altitudinis*	100.00
B	*Bacillus licheniformis*	100.00
E	*Bacillus thuringiensis*	99.89
Q-4	*Staphylococcus lentus*	99.15
Q+	Unknown	-

Table 4 shows the morphology of each of the isolated bacteria that presented antimicrobial activity. It is possible to see that they all have a circular shape. However, the margin varied between Undulate/Entire. The diameter of each was completely different from 0.9 to 12 millimeters for *B. altitudinis* and *Bacillus* sp. respectively. The color of the strains changes widely by each species.

Table 4. Morphological characteristics of strains with antimicrobial activity.

Strain	**Morphology**			
	Color	**Shape**	**Margin**	**Diameter (mm)**
Bacillus thuringiensis	Light pink	Circular	Undulate	8.00
Unknown	Aqua	Circular	Entire	1.59
Bacillus sp.	Aqua with blue center	Circular	Undulate	12.07
Bacillus licheniformis	Bluish white	Circular	Undulate	3.33
Bacillus altitudinis	Light blue	Circular	Entire	0.97
Staphylococcus lentus	Yellowish white	Circular	Entire	1.25

In Table 5 the results of the catalase test and Gram staining are displayed, which were obtained from the cells that showed antimicrobial activity. The six bacteria showed a positive reaction to Gram staining, 83.3% of bacteria with antimicrobial properties had a rod shape under 100x in the microscope. 17.7% (one strain) had cocci shape under the same conditions. During the catalase test, 83.3% of microorganisms presented a negative reaction, only one strain 17.7% presented a positive reaction to the catalase test. It is important to mention that unknown bacteria, by morphology and for the presence of other bacteria in the sample, the genus of it correspond to the *Bacillus* group like was mention in the Table 3 description. However, is recommendable to get a better quality of DNA to can be able to analyze by 16s sequence the strain.

Table 5. Gram staining and catalase test applied to the strains that showed activity.

Strain	**Shape**	**Gram staining**	**Catalase test**
Bacillus thuringiensis	Rods	Positive	Negative
Bacillus licheniformis	Rods	Positive	Positive
Bacillus altitudinis	Rods	Positive	Negative
Staphylococcus lentus	Cocci	Positive	Negative
Bacillus sp.	Rods	Positive	Negative
Unknown	Rods	Positive	Negative

Table 6, Figure 5, and Figure 6 present a summary of bacteria with antimicrobial activity (antifungal and antibacterial) against *Listeria innocua, Bacillus amyloliquefaciens,* and *Aspergillus fumigatus.* With the application of an evaluation of Duncan mean adjust, 0.982 R^2, general 22.009% of the coefficient of variation. The variation complies with the acceptability $\leq$ 30% despite be development of bacteria and with the presence and absence of halos of inhibition. *B.*

altitudinis inhibited *B. amyloliquefaciens* and *A. fumigatus;* unknown strain inhibited *L. innocua, B. amyloliquefaciens,* and *A. fumigatus.* Nevertheless, the six strains presented inhibition against *A. fumigatus.*

Table 6. Inhibition halos (mm) from isolated bacteria against *Listeria innocua, Bacillus amyloliquefaciens,* and *Aspergillus fumigatus*.

Strain	**Mean ± Std. Dev.**		
	Bacteria		**Mold**
	L. innocua	***B. amyloliquefaciens***	***A. fumigatus***
Bacillus licheniformis	0.00 ± 0.00[b]	0.00 ± 0.00[b]	85.33 ± 7.34[a]
Staphylococcus. lentus	0.00 ± 0.00[b]	0.00 ± 0.00[b]	84.57 ± 3.40[a]
Bacillus altitudinis	0.00 ± 0.00[b]	1.63 ± 0.24[a]	74.83 ± 3.35[ab]
Bacillus thuringiensis	0.00 ± 0.00[b]	0.00 ± 0.00[b]	68.00 ± 7.48[bc]
Bacillus sp	0.00 ± 0.00[b]	0.00 ± 0.00[b]	54.93 ± 9.64[c]
Unknown	1.37 ± 0.17[a]	1.57 ± 0.17[a]	18.17 ± 7.34[d]
CV (%)	37.31	32.55	12.85

[a-d]Mean followed by a different letter by each column represent a statistical difference among treatments ($P < 0.05$). CV (%) = Coefficient of variation by percentage. The results represent the average ± standard deviation of three repetitions.

Moshafi *et al.* (2011) showed antimicrobial activity of *Bacillus sp.* strains, during the assessment the supernatant produce as much as antibacterial and antifungal activity. Along with the evaluation the highest inhibition activity was caused against *Aspergillus niger* (Moshafi *et al.* 2011). On the other hand, *B. thuringiensis* and *B. licheniformis* presented antifungal activity against *Aspergillus fumigatus* it is believed that the cause of the inhibition was by different compounds produced by the bacteria. This hypothesis matches Gomaa (2012) who found that chitinase production from the two strains mentioned above caused antifungal biocontrol affecting the cell wall of the fungal. Besides was performed and evaluation on Loquat fruit from China to determinate the existence of antifungal compounds from *B. thuringiensis* and the results of gas chromatography showed the production of volatile organic compounds (VOCs) specifically Butan-1-ol, 3-methylbutan-1-ol,butane-2,3-dione, 3-hydroxybutan-2-one, 2-methylpropane, 2-methylnaphthalene, benzaldehyde, acetic acid among others, these caused inhibition in petri-dish of *Fusarium oxysporum, Botryosphaeria* sp., *Trichoderma atroviride, Colletotrichum gloeosporioides,* and *Penicillium expansum* (He *et al.* 2020). It is important to mention that iturin lipopeptide from the genus *Bacillus* including *B. altitudinis* has been classified as cyclic lipopeptide who exhibit promising activity against molds and bacteria (Goswami and Deka 2019). Finally, exopolysaccharides (EPSs) characterized as a heteropolysaccharide containing mannourinic acid, glucose, and sulfate where isolated from a *B. altitudinis* strain where presented antimicrobial activity against *B. subtilis, Staphylococcus aureus, Escherichia coli, Penicillium aeruginosa, Saccharomyces cerevisiae, Candida albicans, Aspergillus niger,* and *Fusarium oxysporum* (Goswami and Deka 2019).

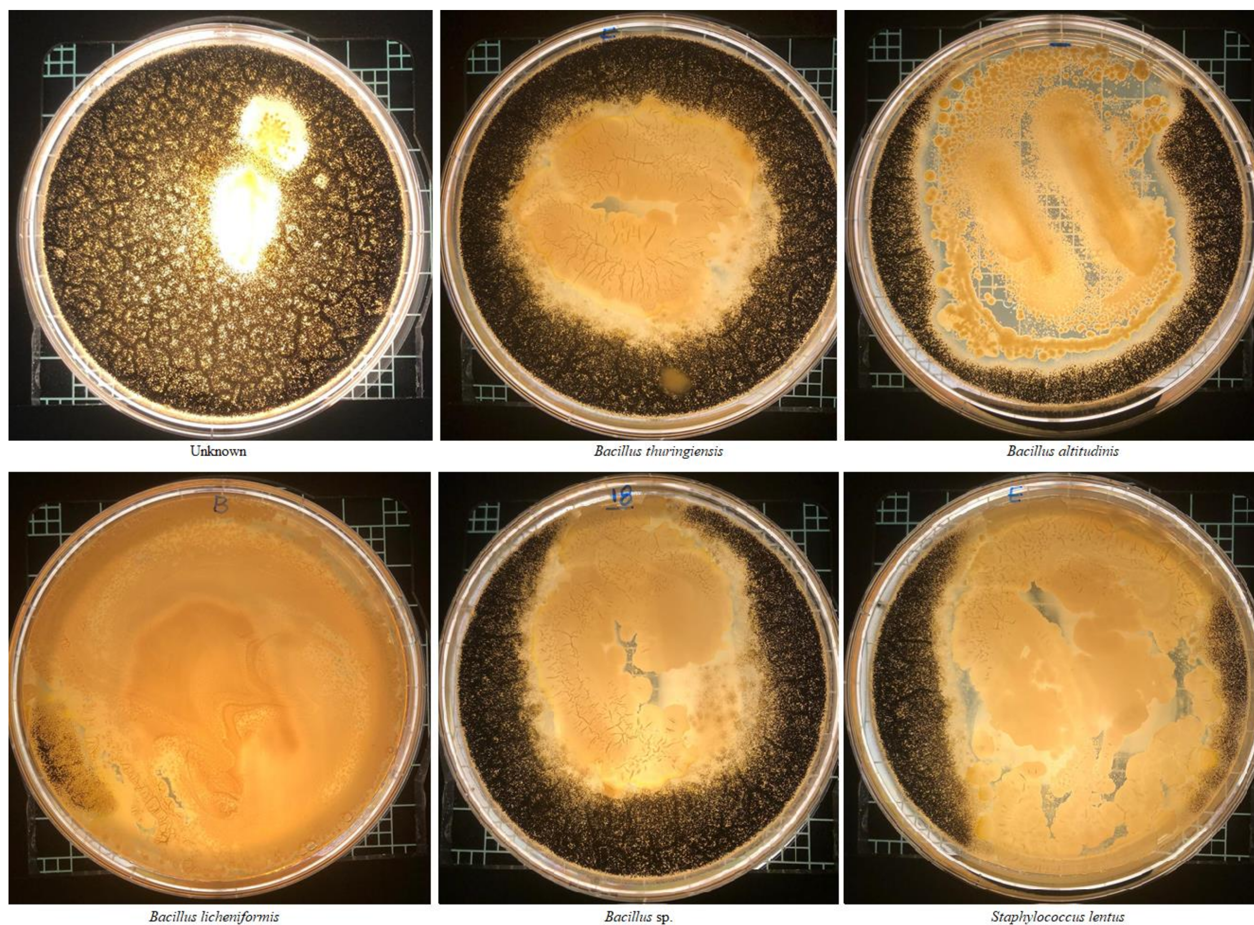

Figure 5. Six isolated bacteria inhibition against *Aspergillus fumigatus*.

Staphylococcus lentus present antifungal activity against *A. fumigatus* in another study it was evaluated for the antimicrobial activity against *Vibrio harveyi* however, not present inhibition zones in cultures of cell-free supernatant (Hamza *et al.* 2018). Like with the genus *Bacillus,* during an evaluation, *S. scuri* MarR44 presented the production of VOCs, and they produce inhibition of mycelial growth of *Colletotrichum nymphaeae.* Nevertheless, the MarR44 produced other compounds as protease, chitinase, hydrogen cyanide (HCN), siderophore, IAA, gibberellin, and biofilm, some of the fungal activity could be influenced by any or many of these compounds (Alijani *et al.* 2019). The VOCs that cause the antifungal activity mentioned above were determinate by gas chromatography-mass spectroscopy (GC-MS) and they were mesityl oxide, acetic acid, 2-methylpropyl ester, 4-methyldecane, 4-penten-2-one,4-methyl-, toluene, and xylene (Alijani *et al.* 2019). During an investigation, Barbieri *et al*. (2005) found that *S. pasteuri* produce a series of VOCs with antifungal characteristics as γ- patchoulene, 3-methoxy-2-cyclopentenone who has a similar structure to a new synthetic analog of strobilurin A (it is used to inhibit fungal conidia and mycelial. Lactic acid bacteria are typically found in cheese samples, however, it was not the results during this experiment because the moisture surface is the best predictor of rind community composition where the sample of this study had not the correct moisture to preserve the total population of bacteria (Wolfe *et al.* 2014). Maldonado *et al.* (2009) found that a metabolite

produced by *Bacillus sp.* that inhibits fungal growth is peptide, ester, or ketone links and saturated CH links of long-chain fatty acids.

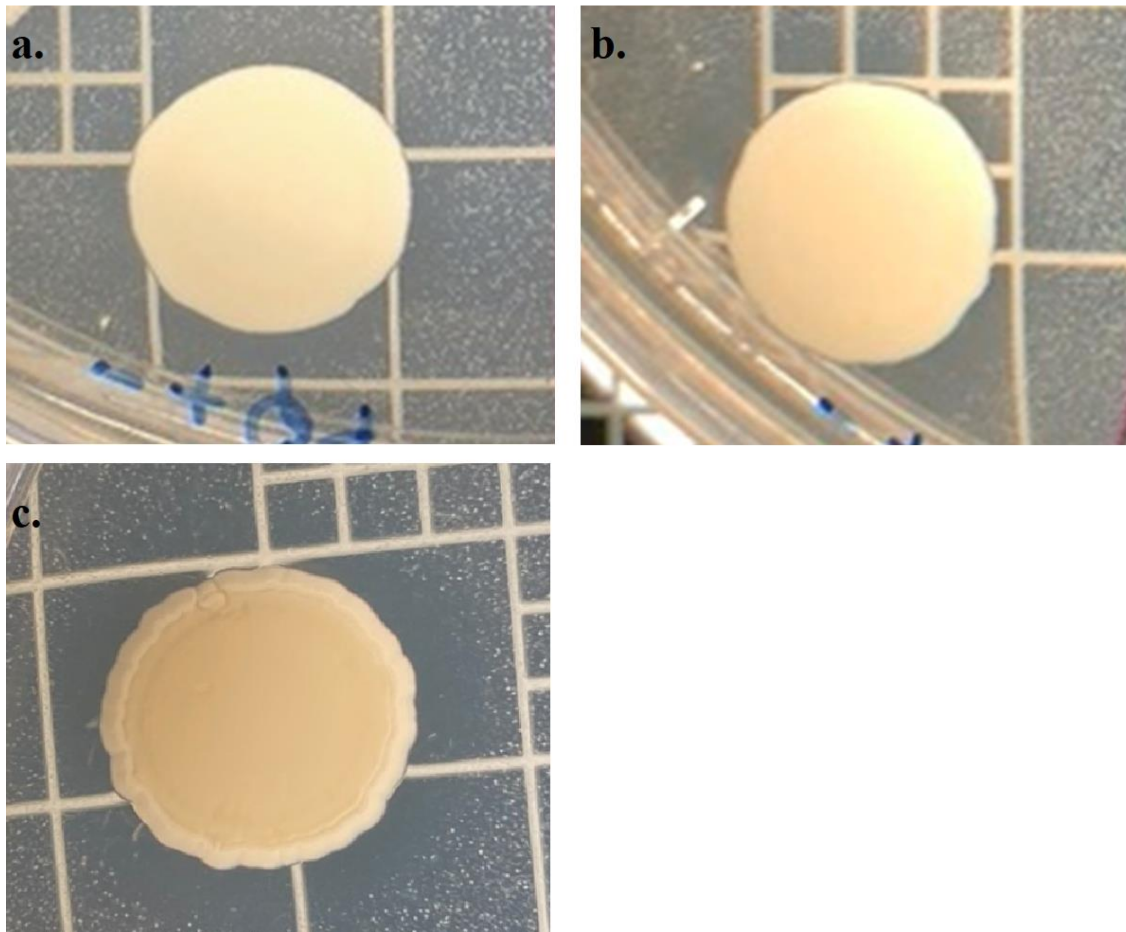

Figure 6. Antibacterial inhibition. Pictures a and b have shown inhibition of unknown bacteria against *Listeria innocua* and *Bacillus amyloliquefaciens*, respectively. Picture c show *B. amyloliquefaciens* inhibition caused by *B. altitudinis*.

Wolfe *et al.* (2014) studied 137 different pieces of cheese from 10 different countries of the United States of America and Europe where they found 24 widely distributed and culturable genera of bacteria and fungi. The presence of communities of bacteria and molds in a piece of cheese depends highly on the place of origin, milk animal precedence, milk treatment (raw or pasteurized), pH, moisture, and salt content (Wolfe *et al.* 2014). The presence of genera *Bacillus* and *Staphylococcus* among the bacteria isolation in high presence making the comparison with lactic acid bacteria was depending on the moisture and time of storage. However, the properties of these genera were not affected during the time.

Further applications of isolated bacteria with antimicrobial characteristics can be mentioned:

- Evaluate all *Bacillus* species found and *S. lentus* against the *Penicillium* and *Aspergillus* genera that are the most important in the cheese industry. The species that participate in the contamination of cheese may produce mycotoxins like ochratoxin A, cyclopiazonic acid, and sterigmatocystin that are stable under normal processing conditions (Kure and Skaar 2019). For that reason, is necessary to implement the use of metabolites of bacteria that control mold growth, to avoid mycotoxin production in cheese manufacturing.

- Prove the antibacterial effect of *B. altitudinis* against *B. stearothermophilus* and *B. coagulans* that are the principal bacteria along the year that causes losses of canned products. Montanari *et al.* (2018) mentioned that flat sour is the progressive acidification of carbohydrates, decreasing pH, and getting degraded product features as abnormal aroma and taste. The control of *B. altitudinis* on microorganism in the same genus increase the possibility to reach the control on *B. stearothermophilus* and *B. coagulans*.

- Assess the antifungal activity of *B. licheniformis* and *S. lentus* on other species of the genus *Aspergillus* that produce mycotoxins. *Aspergillus* genera produce important mycotoxins, *A. flavus* produces aflatoxin and *A. ochraceus* produce ochratoxin A these species among others are widely affecting the commodities like peanuts, maize, cotton, coffee cocoa, and more. The uncertainty created by the presence of mycotoxins carries out losses in agriculture production and agro-industrial manufacturing. The proposal is the focus on evaluating the affection of bacteria *B. licheniformis* and *S. lentus* on *Aspergillus* species as inhibition of growth and production of mycotoxins (Taniwaki *et al.* 2018).

4. CONCLUSIONS

- Twenty-seven strains of bacteria were isolated from Appenzeller® cheese.
- Six isolated bacteria showed antimicrobial activity against *L. innocua*, *B. amyloliquefaciens*, and *A. fumigatus*. The most sensitive to isolated strains was *A. fumigatus*.
- *Bacillus* was the most predominant genus among isolated bacteria with antimicrobial activity.

5. RECOMMENDATIONS

- Study the inhibition type of each strain of isolated bacteria to determine if their inhibition was the cause of secondary metabolites.

- Characterize the metabolite compounds of the isolated bacteria with antimicrobial activity through gas chromatography-mass spectroscopy.

- Evaluate the antimicrobial activity against other genera of molds and bacteria.

- Regulate the storage conditions (temperature, moisture, and amount) of the sample in future studies to detect the presence of lactic acid bacteria.

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7. APPENDIX

Appendix 1. Output table from NCBI web page for bacteria with antimicrobial activity.

Strain	Specie	Max score	Total score	Query cover	E value	Percent identity
Q4	*Staphylococcus lentus*	1496.5	5327.5	98.50%	0	99.15%
18	*Bacillus sp.*	1375.0	19150.0	100.00%	0	100.00%
B	*Bacillus licheniformis*	1598.0	12757.0	100.00%	0	100.00%
E	*Bacillus thuringiensis*	1676.0	25140.0	100.00%	0	99.89%
-	*Bacillus altitudinis*	1609.0	7281.5	100.00%	0	100.00%