Universidad de Oviedo
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Master Thesis

Antagonistic relationships between lactic acid bacteria and main bacterial and fungal food contaminants:

Finding ways to improve food safety in developing countries

MASTER IN BIOTECNOLOGY OF ENVIRONMENT AND HEALTH

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The research entitled "Antagonistic relationships between lactic acid bacteria and main bacterial and fungal food contaminants" has been made under my supervision by Mr./Ms. **Sara Jáimez Velázquez**, graduated from the Degree of Biology by the University of Oviedo, in the Dairy Starters and Biopreservation Group (DairySafe) of research of the Institute of Dairy Products of Asturias (IPLA-CSIC).

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In Oviedo, 15 July of 2019

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Resumen

La contaminación de comida es origen de importantes pérdidas económicas y de millones de casos de enfermedad en el mundo cada año. Se produce principalmente por bacterias y hongos que, en algunos casos, además de estropear la comida, producen toxinas dañinas para los consumidores y esporas que pueden provocar la aparición de alergias. Esta es especialmente preocupante en países en vías en desarrollo, en los que la falta de recursos, la falta de controles de seguridad en la comida y la malnutrición, hacen a sus ciudadanos especialmente vulnerables.

El uso de microorganismos o de los compuestos que estos producen en alimentación, conocido como "bio-preservación", está recibiendo creciente atención como posible solución a este problema. Las bacterias ácido-lácticas (BAL) son de gran interés en este campo, ya que la mayoría son consideradas seguras para el consumo humano y su actividad frente a distintos patógenos ha sido descrita en numerosos trabajos científicos. En este Trabajo de Fin de Máster se propone su uso en papilla nutricional para niños con malnutrición en países en vías de desarrollo. Por este motivo, se estudió el potencial inhibidor de *Lactobacillus plantarum* CECT 8962 frente a *Penicillium expansum* CECT 2278 en dicho producto, observándose que se trata de un medio complejo en el que especies como las del género *Bacillus* también son capaces de crecer, siendo responsables de la inhibición del hongo y viéndose fuertemente inhibidas por la actividad del lactobacilo.

Debido a estas observaciones, se decidió evaluar el efecto de cepas BAL sobre el crecimiento de los *Bacillus* contaminantes, y el efecto de estos sobre importantes hongos patógenos. En el segundo caso, se vio una clara inhibición de los hongos tanto en cocultivo como usando los sobrenadantes de los *Bacillus*. En cuanto a las BAL, en cambio, solo se vio actividad en algunos de los cocultivos.

Abstract

Food contamination is the origin of important economic losses and of illness in millions of people every year. It is mainly caused by fungi and bacteria which, in some cases, apart from spoiling food, produce toxins that harm consumers, and spores that can elicit allergic reactions. It is especially concerning in developing countries, where the lack of resources and food safety controls, together with malnutrition, make individuals particularly vulnerable.

To solve this problem, the application of microorganisms or of the compounds they produce to food, which is known as "biopreservation", is receiving increasing attention. Lactic acid bacteria (LAB) are of very much interest in this field, as most of the species are considered to be safe for human consumption and have been previously reported to present inhibitory activities against important pathogens in many scientific papers. In this Master Thesis, we propose their use for preservation of nutritional formula for malnourished kids in developing countries. For this reason, firstly, the inhibitory potential of *Lactobacillus plantarum* CECT 8962 against *Penicillium expansum* CECT 2278 was studied in this product. It was observed that nutritional formula was a complex environment where some *Bacillus* spp. could also grow, that these species were responsible for the inhibition of the fungus, and that *L. plantarum* CECT 8962 had a strong antagonistic activity over them.

Due to these observations, it was decided to also assess the effect of an array of LAB strains on the growth of different *Bacillus* that are frequent food contaminants, and the effect of those *Bacillus* over important fungal pathogens. Clear inhibition by *Bacillus* was observed over fungi, both in co-culture and when using *Bacillus* supernatants, in most of the cases. Regarding LAB and *Bacillus*, however, inhibitory activities where only demonstrated in the case of some co-cultures.

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Master Thesis

Study of the antagonistic relationships between lactic acid bacteria (LAB) and some of the main bacterial and fungal food contaminants:

Finding ways to improve food safety in developing countries

1) Introduction

1.1. Background

1.1.1. The problem of food contamination:

Food contamination, defined as the presence of microorganisms or toxic compounds in food that makes it unsuitable for consumption, is considered by the World Health Organization (WHO) a global challenge (Hussain, 2016). It is estimated that around 600 million cases of food-borne illnesses occur every year (WHO, 2019), and the number of people that die per year because of this reason was estimated to be of 420,000 people in 2016 (WHO, 2016). Food contamination is also the origin of important economic losses, mainly caused by food spoilage and by the acquisition of undesirable odors and flavors that reduce food products' market value. It can take place during all stages of the food chain.

The main causative agents are fungi and bacteria, which can inflict important negative effects on consumer's health (Australian Institute of Food Safety, 2019). Regarding fungi, the main issue related to their presence in food is the production of spores and mycotoxins, as they can be allergenic, immunotoxic, teratogenic, neurotoxic, nephrotoxic, hepatotoxic and, in some cases, even carcinogenic (Varsha, 2015). Some of the most important contaminant genus are: *Fusarium, Penicillium, Aspergillus, Cladosporium, Rhizopus,* and *Gibberella* (Gajbhiye and Kapadnis, 2016). This work will be focused, more precisely, on the pathogens *Penicillium expansum* CECT 2278,

Gibberella moniliformis CECT 2987, and *Aspergillus parasiticus* CECT 2681. *P. expansum* CECT 2278, which causes the "blue mold disease", is ubiquitous on nature, being a frequent in agricultural products (Russo *et al.,* 2017). It shows the best growth at 25ºC and pH of 5.6 (Tannous *et al.,* 2017). It is one of the main toxin producers in food, being patulin its main product, which has potent teratogenic, cytotoxic, genotoxic and immunotoxic effects, even at low concentrations (Russo *et al.,* 2017), apart from causing gastrointestinal disorders and disruption of renal function. It is resistant to most food-processing methods. It also produces other mycotoxins such as citrinin, with a high nephrotoxicity, roquefortine C, which is neurotoxic, or chaetoglobosins A and C, which inhibit cell division and cause mammalian cells death (Tannous *et al.,* 2017). *Gibberella moniliformis* (*Fusarium verticillioides*) CECT 2987 is another important plant pathogen with a worldwide distribution (de la Torre-Hernández *et al.,* 2014). It contaminates both food and feed animals (Jurgenson, Zeller and Leslie, 2002), and it produces important amounts of toxins, including fusaric acid, naphthoquinones, moniliformin, and fumonisins, being the latter the most abundant ones (de la Torre-Hernández *et al.,* 2014). Some are known to have carcinogenic effects on humans (Jurgenson, Zeller and Leslie, 2002). In regard to *Aspergillus parasiticus* CECT 2681, it is a frequent contaminant of cereal grains and peanuts (Yuan, Liu and Chen, 1995). It produces different aflatoxins which are, in general, important carcinogens, having also hepatotoxic and mutagenic effects. The aflatoxin AFB_1 is especially concerning, as it is the third main cause of liver cancer, above all in developing countries (Fouad *et al.,* 2019).

In the case of bacterial contaminants, *Salmonella* spp., *Listeria* spp., *Escherichia coli*, *Clostridium* spp., *Campylobacter* spp., *Shigella* spp., *Staphylococcus* spp. and *Bacillus* spp. are some of the most frequent ones (Hussain, 2016). In infant nutritional formula, which is the food product that this Master Thesis is going to be focused on, and the ingredients that it is composed of, such as powdered milk and flours, *Bacillus* species are the main hazard, especially the ones belonging to the *Bacillus cereus* group (*B. cereus, B. thuringiensis, B. cytotoxicus, B. weihenstephanensis, B. mycoides, B. pseudomycoides, B. toyonensis*, *B. anthracis* (Xin *et al.,* 2014) and *B. wiedmannii*) (Miller *et al.,* 2016).

Species of the *B. subtilis* group (such as *B. subtilis, B. amyloliquefaciens, B. licheniformis* or *B. pumilus*, among others), which are known antagonists of plant pathogens (Alina, Constantinscu and Petruta, 2015) and generally considered not hazardous for humans, can also be found in this kind of foods and can act as opportunistic pathogens (Schleifer, 2009). Members of these groups are Gram-positive, usually motile, spore-forming microorganisms. Their cells are of irregular and variable shapes, found singly or forming pairs or chains. They can support a wide range of temperatures, with an optimal growth at 20-50ºC, depending on the species, and grow well at slightly acidic and neutral pH (Schleifer, 2009). They are extensively distributed along the world, probably due to their capability to grow under very different conditions and to the production of spores, which allows them to survive in especially harsh environments (MesselhäuBer and Ehling-Schulz, 2018). Being known producers of toxins such as the hemolysin BL or the non-hemolytic enterotoxin, among many others (IVAMI), they are mostly related to diarrheal episodes and mild symptoms of gastrointestinal disease (abdominal cramps, nausea, vomiting, weakness…). Their infections can pose severe complications in some cases, though, causing gangrene, kidney failure, brain or nerve damage, or even death (Tajkarimi, 2007).

Apart from the previously mentioned risks that contaminated food may pose for human health, food contamination presents an additional problem, which is that contaminants may be resistant to antibiotics, and these resistances can be transmitted to other microorganisms used in food production or living in our microbiome. This is a very concerning problem nowadays, as it decreases the possibilities to fight against infections (Scientific Committee of the Food Safety Authority of Ireland, 2015).

1.1.2. Food contamination in developing countries:

Access to safe food is a basic human right. However, in developing countries it is not always ensured, with more than 2 million deaths estimated to take place every year due to food-borne diseases. This affects especially old people, children and

immunocompromised individuals, due to their reduced immunological function (Odeyemi, 2016). Several factors contribute to the high incidence of food-borne illnesses (table 1) (Odeyemi, 2016; Käferstein, 2003):

Table 1. Factors that contribute to the high incidence of food-borne diseases in developing countries.

Factors causing the high incidence of food-borne illnesses
- Growing, ageing and malnourished population → Increased susceptibility to diseases
- Lack of a clean water supply for cooking and lack of personnel's hygiene
- Unavailability of efficient food-processing technologies due to lack of economic resources
- Poor knowledge about safety along the food chain → Improper processing, cooking and handling
- Lengthening of the food chain → More possibilities of contamination
- High environmental temperatures and relative humidity → Good growth of contaminants
- Lack of strict food quality and safety controls

1.1.3. Possible solutions:

Until recently, most efforts for prevention of food-borne diseases in developing countries was focused on water and sanitation, not being enough to solve the problem (Käferstein, 2003). An alternative is the implementation of appropriate physic-chemical food-processing technologies, such as ultrasounds, thermal treatments or the use of chemical additives (Das and Rajyalakshmi, 2015), but apart from causing changes in products' organoleptic properties, developing countries do not usually have the economic resources and infrastructures needed to carry out those processes (Käferstein, 2003). Additionally, some contaminant species, mainly the ones producing spores, are resistant to those techniques (Andersson, Rönner and Granum, 1995). Due to these facts, additional measures are needed to fight food contamination, such as the combination of different preservative methods (Käferstein, 2003). Moreover, nowadays there is a demand for more "natural" foods in which security and a long shelf-life are ensured meanwhile avoiding the use of chemical additives, that can have negative

effects on human health and alter organoleptic properties. For all those reasons, biopreservation is now receiving an increasing interest (García *et al.,* 2010).

1.1.4. A more consumer-friendly option - Lactic acid bacteria (LAB):

Biopreservation is the use of microorganisms, or of the compounds they produce, in order to keep food products in a safe state for consumption. They can be present in food naturally or can be intentionally added by us (Singh, 2018). Lactic acid bacteria (LAB) seem to be very appropriate for this use, as they are part of our microbiota and most species have acquired the "Qualified Presumption of Safety" (QPS) status given by the European Food Safety Authority (EFSA). They are also "Generally Recognized As Safe (GRAS)" microorganisms, according to the United States Food and Drug Administration (FDA). They have, in fact, been used for decades to produce foods such as dairy products and many studies have been published in relation to their capability of inhibiting the growth of important bacterial and fungal pathogens. *Lactobacillus* is one of the main genera of this group, formed by Gram-positive, non-spore forming, generally non-motile bacteria. They provide desirable organoleptic properties of texture, flavor or aroma to foods, and they can improve their nutritional value by the production of vitamins. They are also known to be probiotic, as some strains can exert beneficial effects on human health, including the evasion of colonization of the gastrointestinal tract by pathogenic microorganisms, the enhancement of the immunological function or antimutagenic activity, among many others (Florou-Paneri, Christaki and Bonos, 2013). It is known that LAB have inhibitory activity against many bacterial and fungal species, but their mechanisms of inhibition are not very well known yet. The main hypothesis is the production of organic acids (Russo *et al.,* 2017) which diffuse through the target's cellular membrane and, once inside, cause a decrease in the intracellular pH, interfering with enzymatic processes (Sudhanshu, Ramesh and Nevijo, 2018), ultimately leading to death. Competition for substrates where to grow or for nutrients can be another way of reducing the development of pathogens by LAB (Russo *et al.,* 2017). Additionally, recent

studies have suggested that another possible mechanism is the ability of these strains to bind to some mycotoxins, such as the aflatoxin B1, reducing their bioavailability (Varsha, 2015). And, at last, another way to inhibit the growth of other species is the production of compounds with anti-bacterial and/or anti-fungal activity, such as cyclic dipeptides, phenylacetic and fatty acids (Yépez *et al.,* 2017) or bacteriocins.

1.1.5. The use of LAB bacteriocins as bio-preservatives:

Currently, the use of bacteriocins in food preservation is being extensively studied. These are ribosomally synthesized antimicrobial peptides produced by bacteria that have the potential to inhibit generally closely-related species. They can have bacteriostatic or bactericidal activities, with or without the lysis of the target cell. Suitable for their use in foods as they are regarded as safe, considered non-toxic to eukaryotes; they are rapidly inactivated by proteases in the gastrointestinal tract, not affecting our microbiota; and they extend food's shelf-life and prevent food-borne pathogens' transmission. Bacteriocins' effectiveness depends on many factors, including the availability of nutrients for the producer on the food matrix, the load of producer strain or of the compound added, or the susceptibility of the target species, among others. They may not be enough to ensure food safety when present on their own, but they can reduce the use of chemicals and other preservation techniques, improving their efficiency and being a more "natural" way to produce safe foods while keeping their organoleptic properties and nutritional values (Gálvez *et al.,* 2007).

1.2. The project - *CYTED-Proinfant*

This Master's Thesis is part of the *CYTED-Proinfant Project: Vegetal foods with probiotic functions for malnourished infant populations*, which is being carried out by some Spanish researchers in collaboration with researchers from several South American countries. The main goal of this project is to establish ways of elaborating "functional

foods" for malnourished children that can exert beneficial effects on their health and that are ensured to be safe, reducing their probability of suffering infectious diseases. These products will be produced using Andean vegetal ingredients, as the objective is to establish food production processes that developing countries will be able to implement, focusing on the use of LAB as probiotics and biopreservatives (Aznar and Ruas-Madiedo, 2018).

1.3. Motivation of the work

All the reasons previously presented lead us to think that food contamination is a problem of big concern, especially in developing countries, which this work focuses on. Because of them, we propose the use of species of the *Lactobacillus* genus as biopreservatives for nutritional formula for kids, with the main goal of applying this method in developing countries so as to ensure food safety and help minimizing children malnutrition. In order to do so, we consider that pilot experiments are needed to prove that, in fact, *Lactobacillus* spp. possess antagonistic activities against the most frequent fungal and bacterial contaminants of milk powder and flours. The selection of the strains chosen to carry out these experiments was based on another experiment that had been previously performed in the IPLA, which consisted on the contamination of infant nutritional formula using different LAB and fungal species in order to determine which LAB had the highest inhibitory activities against each fungus and which fungi showed better growth in coculture with those bacteria (Álvarez, 2018).

2) Goal and objectives

The main goal of this Master Thesis was to find ways to improve the safety of nutritional formula for malnourished children in developing countries by the use of microorganisms as an alternative or complement to conventional chemical and physical food treatments. In order to achieve this, three objectives were established:

1 - To determine the antagonistic activity of *Lactobacillus plantarum* CECT 8962 against *Penicillium expansum* CECT 2278, an important spoilage and toxigenic fungus, in nutritional formula.

2 - To seek for anti-*Bacillus* activities amongst different strains of LAB, both by *in vitro* antagonistic assays and by the *in silico* prediction of genes related to the production of bacteriocins.

3 - To search for inhibitory activities against fungal pathogens amongst *Bacillus* strains.

3) Materials and methods

3.1. *In vitro* assessment of the antagonistic activity of *Lactobacillus plantarum* CECT 8962 against fungal and bacterial contaminants of nutritional formula

3.1.1. Strains used and culture conditions:

L. plantarum CECT 8962 was grown in MRS (BD Biosciences) broth for 24 h at 30ºC; then, cell pellet was washed with buffered peptone water (BPW) in order to remove broth and was used to inoculate infant nutritional formula at 2% (10^7 CFU/ml). A suspension of *P. expansum* CECT 2278 spores (25 CFU/ml) in BPW was used to contaminate the formula.

3.1.2. Preparation of infant nutritional formula:

200 ml of formula were prepared using ingredient from South American countries and following the instructions given by the Food and Agriculture Organization of the United Nations (FAO). It was composed of 19.5 g of maize flour, 4.5 g of soy flour, 6 g of powdered milk, and 170 ml of sterile distilled water. Once all the ingredients were mixed, formula was agitated for 5 minutes to homogenize it and then heated in a water bath at 95ºC for 10 min, with constant manual shaking (Russo *et al.,* 2017).

3.1.3. Contamination assay:

Nutritional formula was first contaminated with 25 CFU/ml of *P. expansum* CECT 2278 and dispensed into two bottles. One of the bottles was inoculated with 10^7 CFU/ml of *L. plantarum* CECT 8962. The other one was used as a control to study fungal growth in the absence of *Lactobacillus*, so the same volume of BPW was added instead of the bacterial culture. Lastly, formula was distributed in 5-ml portions (samples) which were kept at 25ºC until the next day. A summary of the protocol is shown in figure 1. The assay was carried out in two replicates of 200 ml of nutritional formula.

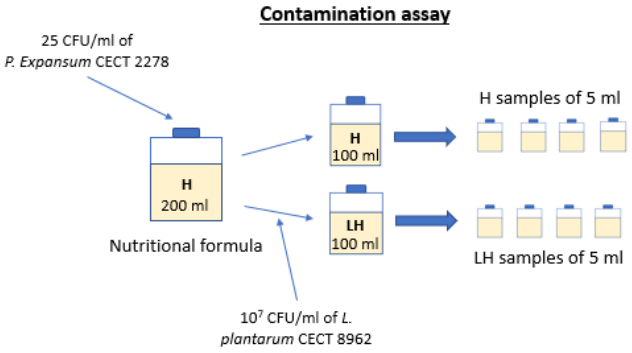

Figure 1. Schematic representation of the procedure followed in order to contaminate the nutritional formula. "H" refers to the bottle with formula that was only contaminated with the fungus, while "LH" refers to the one that was contaminated by the fungus and inoculated with *L. plantarum* CECT 2278.

3.1.4. Sample processing:

At T0 (right after inoculation), the first sample processing was carried out. It consisted on taking a sample from the formula inoculated with the *Lactobacillus* and contaminated by the fungus, and another from the formula that was only contaminated. Both were weighed and their pH was measured. They were then homogenized in 20 ml of sterile BPW with vigorous agitation on a shaker prior to preparing serial decimal dilutions in the same diluent. Appropriate dilutions were plated on MRS (BD Biosciences) 2% agar plates for counting *Lactobacillus*, on Yeast Extract Glucose Chloramphenicol Agar (YGC) (Merck) 2% agar plates for the fungus, and on *Bacillus cereus* Selective Agar

(BCA) (Scharlau Microbiology) 2% plates for *Bacillus* species. MRS and BCA plates were incubated at 30ºC for 48 hours, while YGC ones were kept at 25ºC for around 4 days. The next day, T1, half of the 5-ml samples were introduced in an incubator at 25ºC and kept there until their processing day, in order to follow populations' evolution during fermentation; the other half were stored at 6ºC. Posterior sample processing were carried out following the same protocol used in T0, which is summed up in figure 2, on days T1, T2, T5 and T9 of the experiment for samples at 25ºC, and on days T1, T7, T15 and T21 for samples at 6ºC.

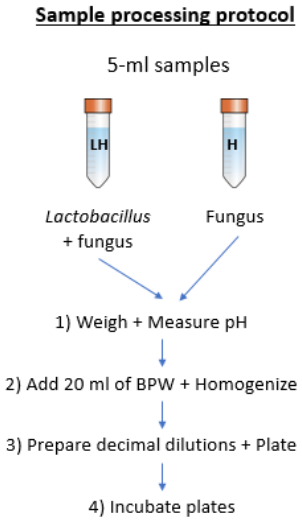

Figure 2. Protocol followed in order to process samples along the experiment. Every processing day, two samples were used: one with the *Lactobacillus a*nd fungus (LH), and another one with only the fungus (H), to compare counts of bacterial and fungal populations in both cases.

3.1.5. Identification of bacterial species by 16S-DNA sequencing:

In order to identify bacterial species, their DNA had to be extracted by resuspending one bacterial colony in 20 µl of a mixture of SDS 0.25% and NaOH 50 mM used to destabilize cellular membranes. Samples were then boiled for 5 minutes at 95ºC, and 180 µl of distilled water were added to each of them. After vigorous agitation, they were centrifuged and their supernatants were transferred to clean tubes. Polymerase chain reaction (PCR) was then used to amplify the gene that encodes the ribosomal 16S DNA

with primers 1492r and 27f-YM (Frank *et al.,* 2008). Reaction mixtures were prepared containing: 1 µl of the extracted DNA, 25 µl of the Taq DNA Polymerase 2x Master Mix RED (1.5 mM $MgCl_2$ final concentration) (Ampliqon III PCR Enzymes & Reagents), which is composed of 0,4 mM of each dNTP, Tris-HCl pH 8.5, 3 mM $MgCl_2$, 0.2% Tween 20, the ampliqon Taq DNA polymerase, and a red dye; 1 µl of each of the primers 10 µM, and 22 µl of distilled water, following manufacturer's instructions (Ampliqon A/S, 2017). Thermocycler was programmed as follows: 1) initial denaturalization by heating to 95ºC for 5 min; 2) 34 cycles consisting on denaturalization at 95ºC for 20 s, primers' hybridization and initial DNA replication at 50ºC for 25 s, and DNA elongation for 1 min and 30 s at 72ºC; 3) final elongation step at 72ºC for 5 min. Once PCR had finished, electrophoresis was carried out for 40 min at a constant voltage of 130 V, and the fluorescent EZ-Vision loading buffer (VWR, Part of Avantor) was used in order to visualize the pattern of bands and see if DNA was correctly amplified or not. Then, DNA was purified using the *Illustra GFX PCR DNA and gel band purification kit* (GE Healthcare), which is based on the use of different buffers in order to achieve DNA binding to a silica column, the washing of unbound substances present in the sample, and the elution of the DNA (GE Healthcare, 2019). Lastly, purified DNA samples were sent to Macrogen Inc., together with the primers used in the PCR, for their sequencing.

3.2. Search for anti-*Bacillus* activities amongst different LAB strains

3.2.1. Strains used and culture conditions:

88 LAB strains coming from different sources were used as possible antagonists to *Bacillus* indicators. 9 of those strains had been isolated from Andean fermented products: *Lactobacillus fermentum* CECT 9269 from tocosh, *L. plantarum* strains CECT 8962, 8965, 8963, 8964 and 8966 from chicha, *L. plantarum* CECT 8493 and 8492 from atole, and *L. sakei* CECT 9267 from tocosh; 16 strains were obtained from bread doughs (from now on named as P1-P16), and 63 strains from organic milk were also utilized (named as L1-L64, because L22 was not available). They were all grown in a microtiter

plate with MRS broth by inoculating each well with a colony of the correspondent strain and incubating the plate at 30ºC for 24 h. Four LAB strains were used as positive controls, as they are known producers of bacteriocins: *Lactococcus lactis* IPLA 517, producer of Nisin Z, isolated from Afuega'l pitu cheese; *Lactobacillus plantarum* LL441, obtained from Cabrales cheese, being producer of Plantaricin C; and *Lactobacillus paraplantarum* C23 and *Lactococcus lactis* subsp. *lactis* 270, producers of Coagulin C23 and Lacticin 481, respectively, both isolated from Casín cheese. *Lactobacillus sakei* CECT 906 was also used as a control because it is very sensitive to bacteriocins, but it does not produce any. Controls were also cultured in MRS broth at 30ºC for 24 h. As indicators of the antibacterial activity of the LAB strains, 7 strains of *Bacillus* were used: *B. safensis/B. pumilus* 2M, *B. subtilis* 3M, *B. wiedmannii* CS1, *B. licheniformis* C5R and *B. licheniformis* C6, all isolated from fermented nutritional formula; and *B. subtilis* BD630 and *B. cereus* CECT 131, both coming from strain collections. They were all grown in TSB broth supplemented with 0.25% glucose, with agitation at 250 rpm at 37ºC for 20 h.

3.2.2. Antagonistic assays:

3.2.2.1. Direct antagonistic assay

In a 200-µl microtiter plate, each well was filled with 150 µl of MRS broth and 5 µl of the correspondent LAB cultures, which were extracted from the microtiter plate previously prepared; the new plate was then incubated for 24 h at 30ºC. 128 x 128 mm Petri dishes were filled with 60 ml of MRS 2% agar and let solidify prior to inoculation with the LAB strains and controls from the microtiter plate, using a multichannel micropipette to stab the agar, and were then let dry for 30 min. Afterwards, plates were covered with 20 ml of TSB 0.7% agar (0.25% glucose) previously inoculated with 200 µl of the correspondent overnight *Bacillus* culture, and finally incubated at 30ºC for 20 h. The next morning, plates were checked for inhibition halos and results were scored as follows: (-) when the indicator was resistant to the putative producer, (+) when a small and opaque halo was observed, and (+++) when a big and transparent halo was seen (only inhibition halos of

6 mm of diameter or more were taken into consideration). The matrix obtained with the results can be seen in Annex 1.

3.2.2.2. Indirect antagonistic assay

Cell-free supernatants of the LAB strain used in the direct screening were prepared by centrifugation of the overnight cultures (obtained after inoculation of MRS broth with one colony and incubation at 30ºC) at 14,000 rpm for 10 min, discarding cell pellets. Supernatants were neutralized to pH of 6-7 and filtered using Whatman filters of 0.45 µm of diameter and NORM-JECT syringes of 1 ml of volume. Finally, different tests were performed in order to establish the best experimental conditions for the visualization of inhibition halos caused by the presence of compounds with antibacterial activity.

3.2.3. Clustering of antagonistic profiles:

For classification of the antagonistic profiles of the LAB strains we applied clustering analysis, which groups data into homogeneous groups (Tryfos, 1997); more precisely, a hierarchical agglomerative clustering method in which all the input data are at first considered as individual clusters and then, in each clustering step, the two most similar groups are clustered together (Kassambara, 2018). Once clusters were obtained, the online tool *Heatmapper* (http://www.heatmapper.ca/) (Wishart Research Group, 2019) was used to elaborate a dendrogram in which data was represented as different branches of a tree according to the similarities between observations. Then, a matrix was created in order to transform the qualitative data (-, +, +++) obtained from the previous screenings into quantitative values: 1, *Bacillus* indicator resistant to the LAB strain; 2, indicator slightly sensitive, and 3, strongly sensitive. The matrix (Annex 2) was then uploaded to *Morpheus* website (https://software.broadinstitute.org/morpheus/) (Broad Institute, 2019) in order to create a heatmap that would made results clearer and easier to interpret. The following parameters were selected: 1) scale type: row; 2)

clustering method: average linking; 3) distance measurement method: Euclidean; 4) apply clustering to: rows, columns. The *Average Linking Clustering Method*, also known as *Unweighted Pair Group Mean Averaging* (UPGMA), defines distance between two different observations on the data as the average distance between observations from the first cluster and observations from the second one (Eberly College of Science, 2019). *Euclidean distance measurement* calculates distance as the length of the straight-line between those two different points, putting together points that are close to each other (GitHub Inc., 2019). A maximum distance of 1.5 between LAB strains was fixed as the point where to separate two strains into different groups, what means that strains located at less than 1.5 of distance in the scale will be in the same cluster.

3.2.4. Statistical analysis of the data:

To determine if the differences observed for the inhibitory potential of LAB strains depending on their source of origin were significative or not, a statistical analysis was carried out using the XLSTAT tool for Excel. More precisely, three t tests for two independent samples were performed, establishing the following hypotheses:

1) - H0 = differences on the average number of *Bacillus* strains inhibited by LAB depending on if LAB came from Andean fermented products or from organic milk were not significative.

- H1 = differences were significative.

2) - H0 = differences on the average number of *Bacillus* strains inhibited by LAB depending on if LAB came from Andean fermented products or from bread doughs were not significative.

- H1 = differences were significative.

3) - H0 = differences on the average number of *Bacillus* strains inhibited by LAB depending on if LAB came from bread doughs or from organic milk were not significative.

- H1 = differences were significative.

A significance level (α) of 0.05 was chosen in order to determine when to accept and when to refuse the null hypothesis (H0). When the p-value obtained in the t test was bigger than α, H0 was accepted, therefore meaning that differences observed were not significative. On the contrary, when the p-value was lower than α, the H0 was rejected and therefore the alternative hypothesis (H1) accepted, suggesting that the observed differences were significative.

3.2.5. *In silico* prediction of genes related with the production of bacteriocins:

Two webs were used to analyze the genetic sequences of some of the LAB strains previously used: BAGEL 4 (http://bagel4.molgenrug.nl/) (BAGEL 4, 2019) and antiSMASH database (https://antismash.secondarymetabolites.org/#!/start) (antiSMASH, 2019). The genomes of *L. plantarum* CECT 8962 (accession number on NCBI: OKQP00000000), CECT 8963 (OKQT00000000), CECT 8964 (OKQV00000000), CECT 8965 (OMOO00000000), CECT 8966 (OMOP00000000) and LL441 (ASM175402v1), *Lactobacillus fermentum* CECT 9269 (OKQY00000000) and *Lactobacillus sakei* CECT 906 (ASM237035v1) were analyzed using their whole genome sequences, which were available online at the National Center for Biotechnology Information (NCBI)'s Assembly database (https://www.ncbi.nlm.nih.gov/assembly/?term=) (NCBI, 2019) in FASTA format. Posteriorly, in order to extract more information about the bacteriocin-encoding genes, BACTIBASE (http://bactibase.hammamilab.org/main.php) (BACTIBASE, 2017), UniProt (https://www.uniprot.org/) (UniProt, 2019) and InterPro (https://www.ebi.ac.uk/interpro/) (InterPro, 2019) webpages were used.

3.3. Identification of inhibitory activity against fungal pathogens by different *Bacillus* strains

3.3.1. Strains used and culture conditions:

Three fungal pathogen species were used as indicators of *Bacillus* antifungal activity: *P. expansum* CECT 2278, *G. moniliformis* CECT 2987 and *A. parasiticus* CECT 2681. Decimal dilutions of the spore stocks (10^4 spores/ml) were plated on mal extract 2% agar Petri dishes which were then incubated at 25ºC for 5 days to determine CFU counts. Finally, spore suspensions of 10^4 spores/ml were prepared in BPW. As putative producers of antifungal compounds, five different *Bacillus* strains isolated from infant nutritional formula were used: *B. subtilis* 3M, *B. safensis/B. pumilus* 2M, *B. licheniformis* C5R and C6, and *B. wiedmannii* CS1. They were all grown by inoculating 10 ml of TSB broth supplemented with 0.25% glucose with single colonies, and incubating them at 37ºC, on a shaker at 250 rpm, for 14-16 h.

3.3.2. Determination of fungal growth inhibition by co-culture with *Bacillus*:

For each fungus, 25 ml of melted malt extract (ME) 1.2% agar was inoculated with 250 µl of the correspondent *Bacillus* overnight culture, mixed by inversion and poured on a plate; as controls, plates with ME 1.2% not inoculated with *Bacillus* were prepared. Then, 10 µl of the correspondent fungal spore suspension (10^4 spores/ml) were added in the center of its control and assay plates and let dry for 20 min; then, they were incubated at 25ºC for 10 days. Every day, measurements of the diameter of fungal colonies were made in order to compare fungal growth in both situations, and the percentages of growth inhibition along the experiment were determined (Fonseca *et al.,* 2016).

3.3.3. Detection of the production of antifungal compounds by *Bacillus:*

Bacillus overnight cultures were centrifuged at 14,000 rpm for 10 min and cell pellets were discarded. They were neutralized to pH of 6-7 and filtered using polyethersulfone sterile syringe filters of 0.2 µm of diameter (VWR International). Dilutions $^{1}/_{2}$, $^{1}/_{4}$, $^{1}/_{10}$, $^{1}/_{50}$, $^{1}/_{100}$ and $^{1}/_{200}$ of each supernatant were prepared in Ringer. Then, for each pair of fungal indicator/*Bacillus*, 4 ml of melted ME 1.2% agar were mixed with 2 ml of the correspondent dilution (or with 2 ml of TSBG broth for the controls) and plated on a portion of four-vented Petri dishes until they solidified. The next step was to inoculate the correspondent portions of the plates with 10 µl of the fungal suspensions, to let them dry for 20 min and incubate the plates at 25ºC for 5 days. Measurements of colony diameters were taken every day in order to calculate fungal growth rate in the presence and absence of *Bacillus* and the percentages of inhibition (Russo *et al.,* 2017). This protocol was used for the following fungus-*Bacillus* pairs: *P. expansum* CECT 2278/*B. licheniformis* C6, *P. expansum* CECT 2278/*B. licheniformis* C5R, *A. parasiticus* CECT 2681/*B. licheniformis* C6, *A. parasiticus* CECT 2681/*B. licheniformis* C5R, and *G. moniliformis* CECT 2987/*B. licheniformis* C6. A little modification of the protocol was made for the rest of the pairs of *Bacillus* and fungi. In these cases, 10-cm Petri dishes were prepared with mixtures of: A) 10 ml of TSB 0.25% glucose broth + 15 ml of melted ME 2% agar for controls; B) 10 ml of *Bacillus* supernatant + 15 ml of melted ME 2% agar; C) a $^{1}/_{10}$ dilution of the *Bacillus* supernatant prepared in TSBG broth + 15 ml of ME 2% for plates corresponding to diluted supernatants. 10 µl of the correspondent fungal spore suspension (10^4 spores/ml) were added to each plate and, finally, plates were incubated at 25ºC.

4) Results

4.1. *In vitro* assessment of the antagonistic activity of *Lactobacillus plantarum* CECT 8962 against fungal and bacterial contaminants of nutritional formula

4.1.1. Determination of the activity of *L. plantarum* CECT 8962 against *Penicillium expansum* CECT 2278 and species of the *Bacillus* genus:

The main goal of this experiment was to determine the presence or absence of antifungal activity by *L. plantarum* CECT 8962 in nutritional formula for malnourished kids, both in conditions of fermentation (at 25ºC) and in storage at cold temperatures (6ºC). This LAB strain was chosen according to a previous study realized in nutritional formula of the same composition where it was observed that, among the LAB strains screened, this was the one with the highest inhibitory potential. *P. expansum* CECT 2278 was also selected according to the results of that study, as it was the fungus with the best growth under the presence of LAB (Álvarez, 2018). Due to these observations, the evolution of both species when cocultured on formula was studied by colony counts on selective culture media. Along the experiment, pH measures, which are represented in figure 3, were taken each processing day in order to register any change that could affect results.

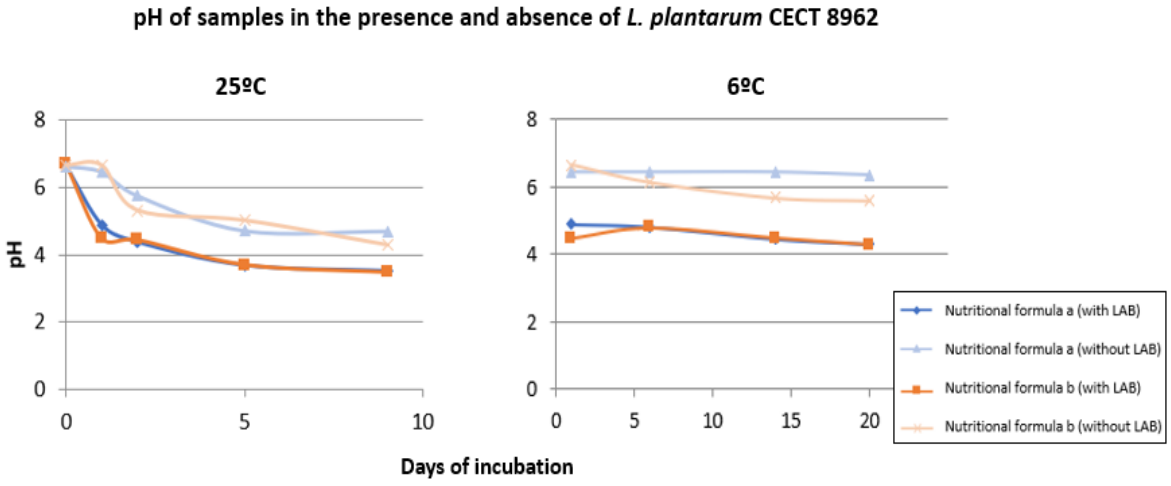

Figure 3. Representation of the pH changes in samples observed along the experiment, both at 25ºC (left) and at 6ºC (right), in the two replicates of nutritional formula.

4As the graphs show, both in samples at 25ºC and in those kept at 6ºC, after the first 24 h of fermentation pH was always lower in samples that had been inoculated with lactobacilli and pH decreased over time, though in samples stored at 6ºC the decrease was not that marked.

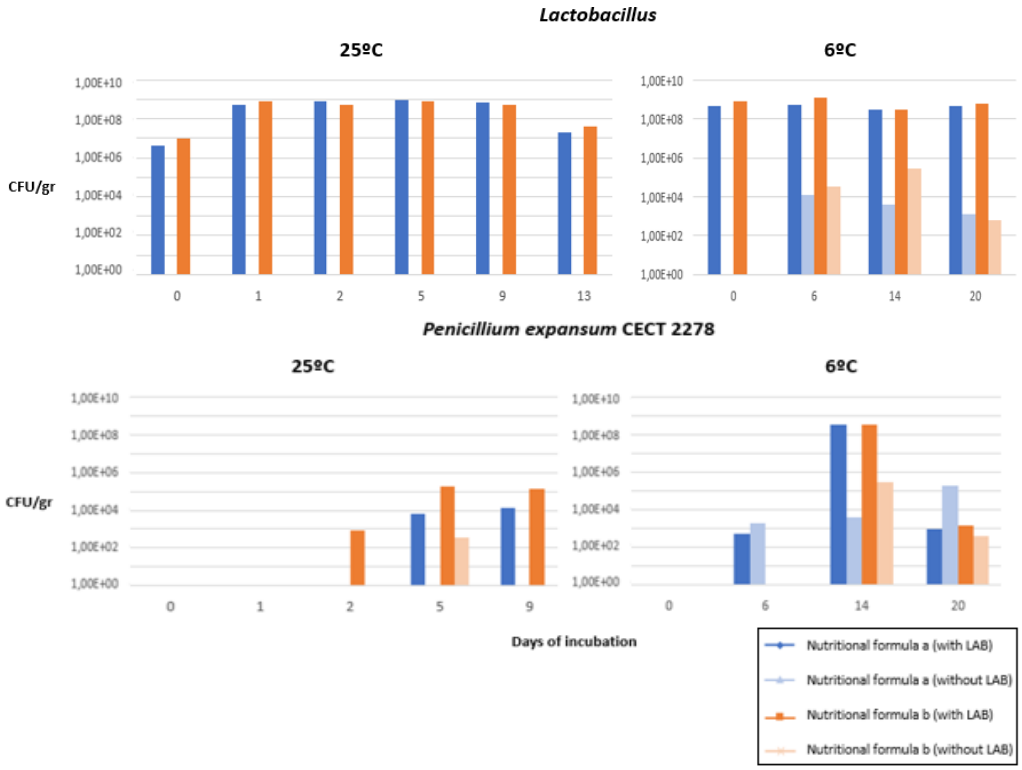

Figure 4. Average of *L. plantarum* CECT 8962 (upper graphs) and *P. expansum* CECT 2278 (bottom graphs) CFU counted per gram of sample along time, made on two nutritional formula duplicates. Results for duplicate a) are shown in blue, results for duplicate b) in orange, being samples with inoculated LAB shown in darker tones and samples without inoculated LAB in lighter colour. Graphs on the left represent samples incubated at 25ºC, which were studied for 13 days for *Lactobacillus* and 9 for the fungus; the right ones belong to samples stored at 6ºC after a 24-h incubation at 25ºC, studied for 21 days (for samples at 6ºC, T0 corresponds to T1 at 25ºC).

Figure 4 shows the results of colony counts of the bacillus and the fungus in fermentation and in storage at 6ºC. In samples incubated at 25ºC, where species were carrying out the fermentation of the nutritional formula, *L. plantarum* CECT 8962 reached a concentration of 10^9 CFU/ml, whereas *P. expansum* CECT 2278 highest concentration was of 10^5 CFU/ml, both after 5 days of incubation. When comparing

samples kept at 25ºC with the ones that were stored at 6ºC after 24 h of incubation at 25ºC, it was observed that colony counts decreased at a higher rate in the first ones for both species. In addition, when looking at the results obtained for *Lactobacillus,* higher colony counts were found in the samples that had been previously inoculated with the species, compared to the ones that were supposed to only contain the fungus.

4.1.2. Isolation and identification of bacterial species contaminating previously prepared infant nutritional formula:

While carrying out the previous experiment, two bacterial contaminants were observed on MRS and BCA agar plates. The latter culture medium is selective for the growth of *Bacillus* spp. and was used because it is known that these species are frequent contaminants of dehydrated foods. Colony counts in BCA were, therefore, performed at different times of the experiment, both for samples fermented at 25ºC and for the ones stored at 6ºC after a 24-h fermentation at 25ºC. Results are shown in figure 5 and, as it can be seen in the graph, contaminants reached a maximum concentration of 10^6 CFU/g of sample after 6 days of fermentation at 25ºC. In samples stored at 6ºC, bacteria seemed to stay viable, maintaining a concentration similar to the maximal reached at 25ºC, for almost two weeks; in the case of samples kept at 25ºC, colony counts decreased at a faster rate. It has also to be noted that *Bacillus* grew better in samples that had not been previously inoculated with *L. plantarum* CECT 8962, whereas in samples that had been inoculated they were not able to develop in most of the cases.

In order to identify the contaminants, they were isolated on selective culture media and their DNA was extracted, purified and then sent to Microgen for sequencing of their 16S rRNA genes. Sequences were then compared to the ones in the NCBI 16S-RNA database and one of the contaminants was found to be *Bacillus subtilis* (from now on will be called *B. subtilis* 3M), while the other was rather a member of *B. safensis* or *B. pumilis* species (from now on termed as *B. safensis/B. pumilus* 2M). The exact species of the latter could not be determined due to the high similarity between their 16S rRNA sequences.

Sequencing additional genes, such as the ones encoding the DNA gyrase (*gyrA* and *gyrB*), which have a high variability among strains, would have been useful in this case (Satomi, La Duc and Venkateswaran, 2006).

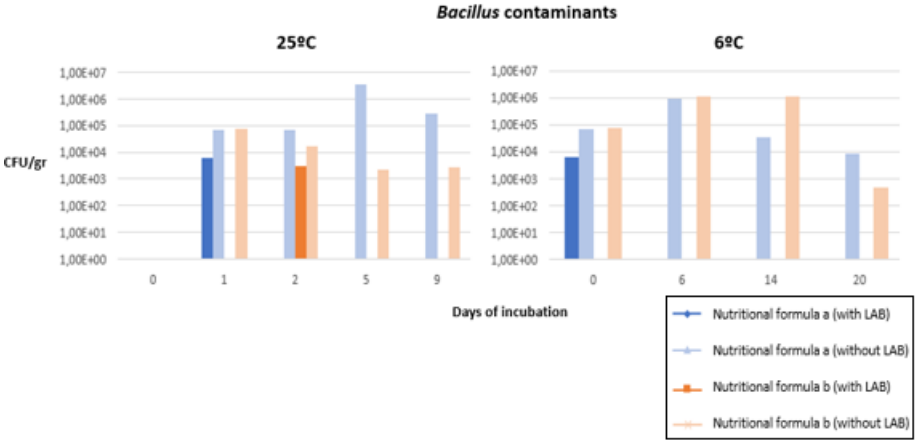

Figure 5. CFU of *Bacillus* present per gr of sample. Counts were carried out from two nutritional formula duplicates, being results for duplicates a) shown in blue and the ones for duplicate b) in orange. Samples with *L. plantarum* CECT 8962 inoculated are shown in darker tones compared to the ones that had not been inoculated with *Lactobacillus*. Graph on the left represents the results obtained for samples kept incubating at 25ºC, whereas the one on the right belongs to the samples stored at 6ºC after 24 h of incubation at 25ºC.

4.2. Search for anti-*Bacillus* activities amongst different LAB strains

In the first experiment it was observed that bacterial species of the *Bacillus* genus were able to grow in infant nutritional formula, but that they seemed to be strongly inhibited when *L. plantarum* CECT 8962 was also present. As a result of this observation, the second objective of this Master Thesis was established, which was to demonstrate the existence of inhibitory activities of this and other 88 LAB strains coming from different sources (organic milk, bread doughs and Andean fermented products) against 7 *Bacillus* spp. (including the two contaminants detected on BCA). These indicators were: *B. subtilis* 3M and *B. safensis/B. pumilus* 2M, *B. licheniformis* C5R, *B. licheniformis* C6 and *B. wiedmannii* CS1, from nutritional formula, and *B. subtilis* BD630 and *B. cereus* CECT 131, from strain collections.

4.2.1. Antagonistic assays:

Two kinds of antagonistic assays were carried out. The first one, the direct assay, consisted on a coculture of LAB strains with each of the indicators for 20 h at 30ºC, in order to see if LAB strains were active against them or not. The second one, the indirect assay, consisted on the addition of LAB supernatants to plates previously inoculated with the *Bacillus,* with the aim of showing the production of compounds with antibacterial activity. It has to be noted that, among the 88 LAB strains used in the first assay or screening, results for strains L30, L33, L34, L53 and L61 were finally not taken into account, as they seemed to be dead after a few days of storage at 4ºC. Also important is the fact that four of the LAB strains were known bacteriocin-producers, included as positive controls so as to determine if the methods employed were effective for the detection of inhibition halos or not. *Lactococcus lactis* IPLA 517, for example, produces Nisin Z, which has been shown to have inhibitory activity against some *Bacillus* strains, such as *B. subtilis* C1 (Matsusaki, Sonomoto and Ishizaki, 1998).

As it is represented in figure 6, among all the *Bacillus* used, *B. subtilis* 3M seems to be the most susceptible, inhibited by 63 LAB strains of a total of 88 (including 3 controls: *L. lactis* IPLA 517, *L. plantarum* LL441 and *L. paraplantarum* C23). *B. licheniformis* C5R was the second most sensitive, being susceptible of inhibition by 51 possible producers and by all the positive controls (*L. lactis* IPLA 517, *L. plantarum* LL441, *L. paraplantarum* C23 and *L. lactis* subsp. C270), followed closely by *B. safensis/B. pumilus* 2M, which showed resistance against 38 LAB strains, being sensitive to all the controls, too. *B. wiedmannii* CS1 presented an intermediate sensibility to LAB, inhibited by 26 strains, including controls *L. lactis* IPLA 517 and *L. lactis* subsp. *lactis* C270. *B. subtilis* BD630, *B. licheniformis* C6 and *B. cereus* CECT 1313 were, by far, the most resistant of all indicators. *B. subtilis* BD630 was sensitive to only 6 strains, including controls *L. lactis* IPLA 517 and *L. paraplantarum* C23; *B. licheniformis* C6 and *B. cereus* CECT 131 were both sensitive just to 4 strains, including the control *L. lactis* subsp. *lactis* C270 in the case of the first

one, and controls *L. lactis* IPLA 517 and *L. plantarum* LL441 in the case of the second one.

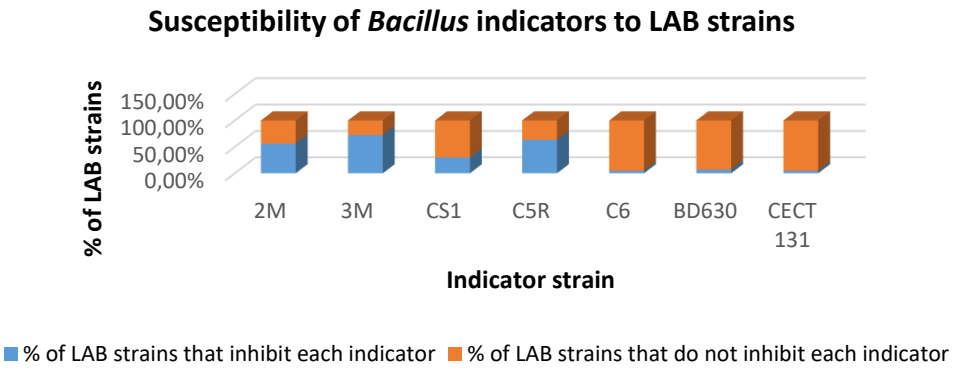

Figure 6. Level of susceptibility to different LAB strains of the 7 *Bacillus* indicators: *B. safensis/B. pumilus* 2M, *B. subtilis* 3M, *B. wiedmannii* CS1, *B. licheniformis* C5R, *B. licheniformis* C6, *B. subtilis* BD630 and *B. cereus* CECT 131. The graph shows the percentage of LAB strains that were able to inhibit each indicator (in blue), and the percentage of strains that were not able to produce that inhibition (in orange).

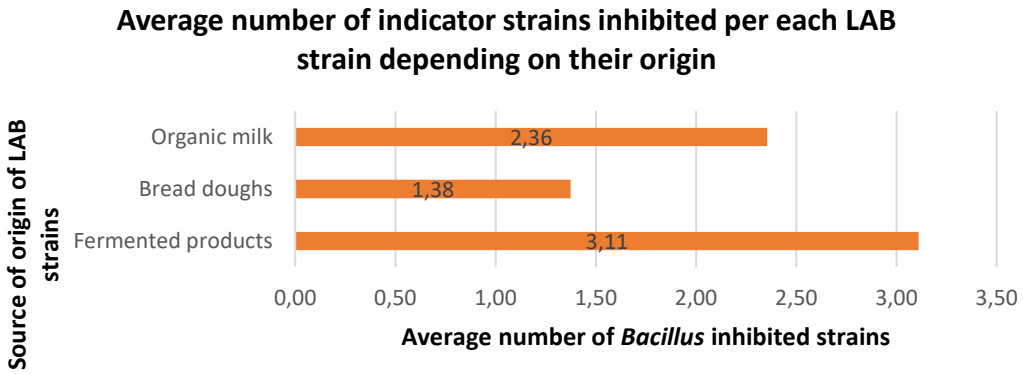

Figure 7. Graphical representation of the average number of *Bacillus* indicator strains inhibited by LAB strains depending on the source of origin of the latter: organic milk, bread doughs, or Andean fermented products.

When analyzing the results taking into account the origin of each LAB strain (figure 7), it was seen that *B. subtilis* 3M was sensitive mainly to strains coming from organic milk. In the case of *B. licheniformis* C5R, it was more susceptible to strains coming from organic milk rather than from bread doughs. For its part, *B. safensis/B. pumilus* 2M was sensitive

to LAB from all the different origins, but especially to the ones from Andean fermented products. For *B. wiedmannii* CS1, *B. licheniformis* C6, *B. subtilis* BD630 and *B. cereus* CECT 131 no preferences were observed. LAB from Andean fermented products were, in general, the ones with the highest inhibitory potential, meanwhile the ones coming from bread doughs were the least active.

Three t tests for two independent samples were carried out in XLSTAT (Excel) in order to see if these differences were significative or not. Results from the three tests are shown in figure 8.

1)

	Minimum	Maximum	Average	Standard deviation
Fermented products	3.00	4.00	3.25	0.46
Organic milk	0.00	5.00	2.39	1.36

Significance level (alpha)	0.05
p-value (bilateral)	0.081

2)

	Minimum	Maximum	Average	Standard deviation
Bread doughs	0.00	5.00	1.33	1.59
Fermented products	3.00	4.00	3.25	0.46

Significance level (alpha)	0.05
p-value (bilateral)	0.003

3)

	Minimum	Maximum	Average	Standard deviation
Organic milk	0.00	5.00	2.39	1.36
Bread doughs	3.00	4.00	3.25	0.46

Significance level (alpha)	0.05
p-value (bilateral)	0.081

Figure 8. Results of the t tests for two independent samples carried out on XLSTAT. 1) belongs to the hypothesis contrast regarding the differences on the average number of *Bacillus* indicators inhibited by LAB strains coming from Andean fermented products and organic milk; 2) compares the average number of indicators inhibited by LAB coming from bread doughs and fermented products; 3) compared LAB strains coming from organic milk to the ones coming from bread doughs. Cases where the p-value obtained in the contrast is lower than the significance level established (alpha = 0.05) lead to reject the null hypothesis (H0), meaning that the alternative hypothesis (H1) is true and that the differences observed were significative; on the other hand, when the p-value was higher than alpha, H0 was accepted, so differences observed were regarded as non-significative.

In the case of the first hypothesis contrast, where differences among LAB strains coming from Andean fermented products and from organic milk were analyzed, according to the criteria established in the section of Methodology, the null hypothesis (H0) had to be accepted, as the p-value obtained was higher than the significance level (α), so in this case differences could not be considered significative. For the second hypothesis

contrast, which compared the average number of *Bacillus* indicator strains inhibited by LAB strains coming from bread doughs to the ones coming from Andean fermented products, a p-value lower than α was obtained, so H0 was rejected, meaning that differences among strains isolated from those both sources were significative. Finally, in the case of LAB strains coming from organic milk and bread doughs, the differences among them were stated to not be significative. According to these results, it would be interesting to carry out further experiments in order to find out the causes of the differences among LAB strains coming from fermented products and bread doughs.

4.2.2. Clustering of antagonistic LAB isolates according to their antagonistic profile:

To assess the biodiversity of antagonistic activities within the 88 LAB strains assayed, a clustering analysis was carried out based on their overall inhibitory potential against the 7 *Bacillus* indicators. A heatmap, which is shown in figure 9, was created using the data obtained in order to make results easier to read. Looking at it, it can be seen that there is a region where the blue color, corresponding to non-sensitive indicators for a certain producer, is predominant. This region was where *B. licheniformis* C6, *B. subtilis* BD630 and *B. cereus* CECT 131 strains were located, showing their resistance to most LAB producers. *B. subtilis* 3M, on the contrary, seems to be the most sensitive indicator, mostly in red on the heatmap, followed by *B. safensis/B. pumilus* 2M and *B. licheniformis* C5R. It is also seen that, again, there is a clear difference on the inhibitory potential of the LAB strains depending on their source of origin: on white, in the left, strains from Andean fermented products were in general the most active, able to inhibit all the indicators except *B. safensis/B. pumilus* 2M; next to it, another group of LAB, in blue, composed of strains isolated from bread doughs, to which most indicators were resistant, showing their low inhibitory potential. A third cluster including LAB strains from organic milk exhibited a more heterogeneous pattern of inhibitory activity. Lastly, another clearly distinguishable area is the one corresponding to the positive controls

Lactococcus lactis IPLA 517, *Lactobacillus plantarum* LL441, *Lactobacillus paraplantarum* C23 and *Lactococcus lactis* subsp. *lactis* C270, which are well-known producers of bacteriocins and were capable of inhibiting the growth of most of the indicators.

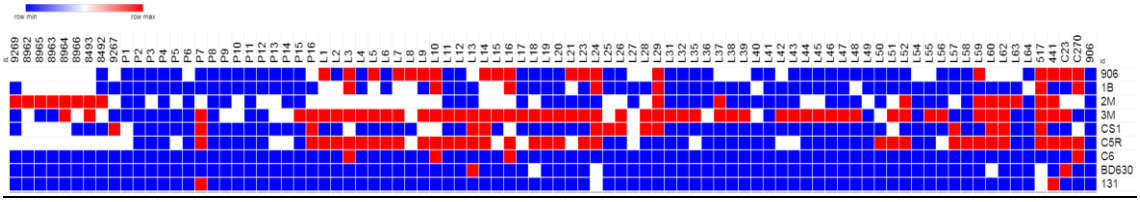

Figure 9. Heatmap obtained using the online tool *Morpheus*. Resistant strains to a certain producer are shown in blue, slightly sensitive strains in white, and very sensitive ones appear in red. LAB producers are shown in the horizontal axis and *Bacillus* indicators in the vertical one.

A dendrogram was also elaborated in order to cluster LAB strains into distinct groups regarding their inhibitory potentials over *Bacillus*. At the end, 27 different clusters composed of 37 different strains were obtained, as it can be seen in figure 10. This dendrogram was used in order to choose which of the strains used in the anti-*Bacillus* screening would be used in posterior experiments. For this, an addition of all the inhibition values (1, 2 or 3, included in the matrix that was used in order to carry out the clustering) of each LAB was carried out so as to choose the ones with the highest inhibitory activities (the ones with the highest overall scores). Among them, a putative producer from each source of origin (organic milk, bread doughs or Andean fermented products) was chosen from each cluster, in order to ensure representativity of the sample in further assays.

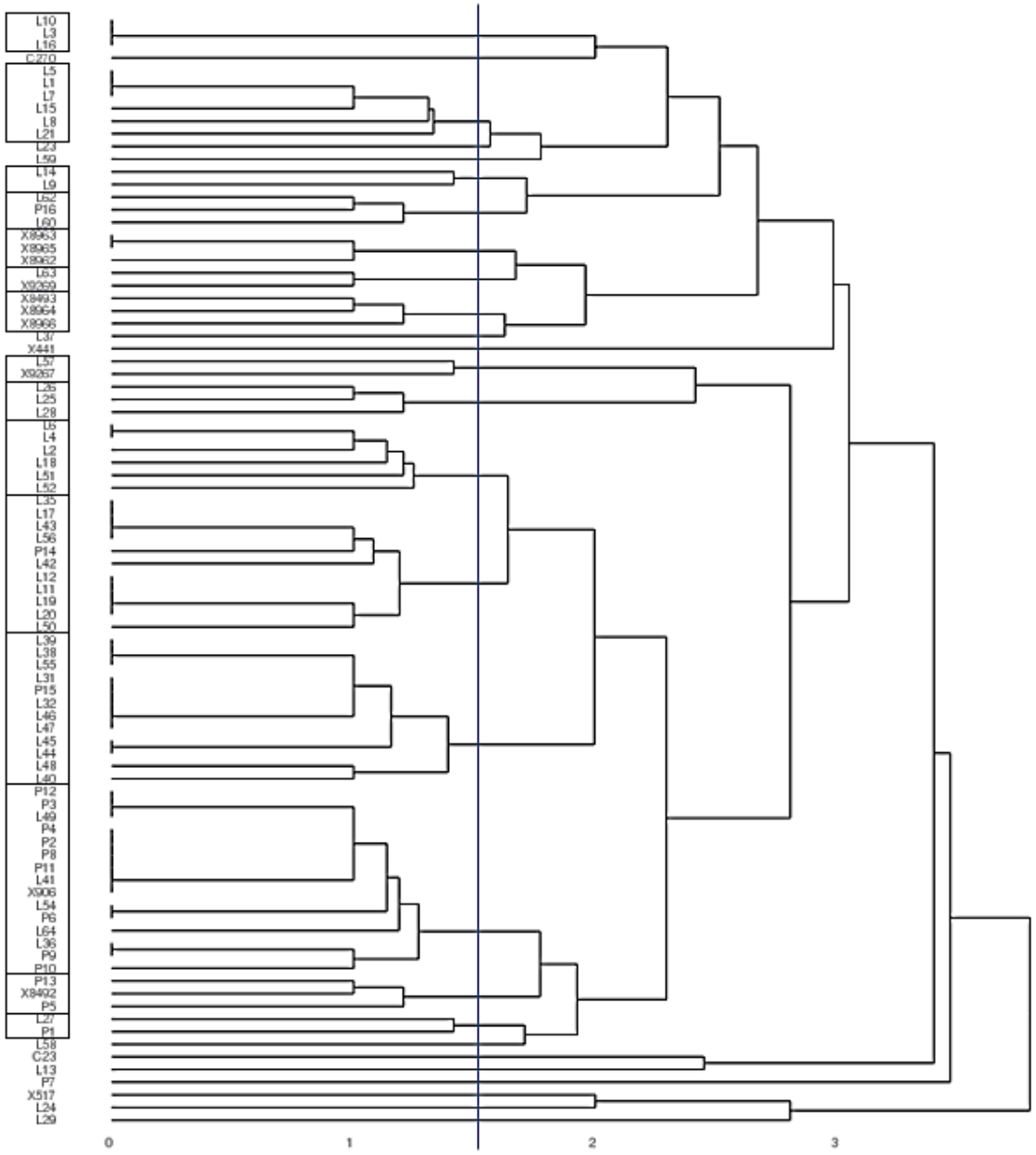

Figure 10. Representation of the clusters obtained after performing the clustering analysis, using the online tool *Heatmapper*, based on the different inhibitory activities observed in the 88 LAB strains against the 7 *Bacillus* indicators. LAB producer strains are indicated in the Y axis, while the X axis shows a scale of distance/dissimilarity between LAB strains ranging from 0 (strains that have the same attributes) to 4 (strains that are the most different compared to the others). The vertical blue line corresponds to a distance of 1.5, which was used to establish which strains belonged to the same cluster and which to different clusters. LAB strains on the left included inside the same square belong to the same group, while the strains that are not inside of any square form single-strain clusters on their own.

4.2.3. *In silico* prediction of genes related with the production of bacteriocins:

With the objective of identifying which compounds could be causing the appearance of anti-*Bacillus* activities among the LAB strains screened, an *in silico* approach was used for the detection of bacteriocin-related genes on LAB genomic sequences, using online tools BAGEL 4 and antiSMASH. Table 2 sums up the main results obtained in this search. In the case of *L. plantarum* CECT 8962 and *L. fermentum* CECT 9269, no genes related to the production of bacteriocins could be found. On the contrary, *L. plantarum* CECT 8963, *L. plantarum* CECT 8964, *L. plantarum* CECT 8965 and *L. plantarum* CECT 8966 had multiple bacteriocin-related genes on their genomes, being the same in all the strains. These were related to the production of Plantaricin A, sactipeptides, Plantaricin E/F, Plantaricin J/K, and Enterolysin X β chain. For *L. plantarum* LL441, genes for sactipeptides and Salivaricin A were observed. In regard to *L. sakei* CECT 906, its genome had genes coding for Carnobacteriocin B2.

Table 2. Table showing the results obtained for the *in silico* search for bacteriocins.

	Bacteriocins								
Strain	**Pln A**	**Sact.**	**Pln E/F**	**Pln J/K**	**Enteroc. X**	**Enterol. A**	**Nisin Z**	**Saliv. A**	**Carn. B2**
L. plantarum CECT 8962									
L. plantarum CECT 8963	x	x	x	x	x				
L. plantarum CECT 8964	x	x	x	x	x				
L. plantarum CECT 8965	x	x	x	x	x				
L. plantarum CECT 8966	x	x	x	x	x				
L. fermentum CECT 9269									
L. plantarum LL441		x						x	
L. sakei CECT 906									x

4.3. Detection of inhibitory activity against fungal pathogens by different *Bacillus* strains

4.3.1. Determination of fungal growth inhibition by co-culture with *Bacillus*:

As it was seen in the first experiment, in which the antifungal potential of *L. plantarum* CECT 8962 against *P. expansum* CECT 2278 was assessed, the growth of the fungus was reduced because of the presence of bacterial contaminants on the nutritional formula

prepared, then identified as members of *B. subtilis* and *B. safensis/B. pumilus* species. In the view of these result, the third objective of this Master Thesis was established, which consisted on performing two different antagonistic assays in order to demonstrate the existence of antifungal activities among different *Bacillus* strains (*B. licheniformis* C5R, *B. licheniformis* C6, *B. subtilis* 3M, *B. safensis/B. pumilus* 2M and *B. wiedmannii* CS1). The first one consisted on a coculture of the *Bacillus* strains and three important fungal pathogens: *P. expansum* CECT 2278, *A. parasiticus* CECT 2681 and *G. moniliformis* CECT 2987. Photographs of the plates obtained from this assay are shown in figure 11 and, as it can be seen by comparing the diameter of fungi in control plates with the diameter of fungi in plates that had been previously inoculated with *Bacillus,* all *Bacillus* exerted an inhibitory effect against the fungal indicators, shown by their reduction in size. *P. expansum* CECT 2278 was the fungus with the lower growth, showing a very high susceptibility to the presence of *Bacillus*, with *B. subtilis* 3M completely inhibiting its growth and *B. licheniformis* C5R and *B. safensis/B. pumilus* 2M having a highly inhibitory effect. Regarding *G. moniliformis* CECT 2987, this fungus showed the biggest colony among control plates and a very good growth in the presence of *B. licheniformis* C6; meanwhile, it was highly inhibited by *B. subtilis* 3M and showed a moderate susceptibility to *B. licheniformis* C5R and *B. safensis/B. pumilus* 2M. At last, *A. parasiticus* CECT 2681 was the most resistant fungus to *B. licheniformis* C5R, whereas it was extremely sensitive to *B. subtilis* 3M and *B. safensis/B. pumilus* 2M, being *B. safensis/B. pumilus* 2M capable of completely inhibiting it (data for *B. licheniformis* C6 not available). As it is shown in table 3, *B. subtilis* 3M was the *Bacillus* strain with the highest inhibitory potential, completely reducing the growth of *P. expansum* CECT 2278; this strain was also observed to reduce the growth of *G. moniliformis* CECT 2897 in a 61.29% and of *A. parasiticus* CECT 2681 in an 88.00%. In the case of *B. safensis/B. pumilus* 2M, reductions were of a 79.07%, a 56.45% and a 100%, respectively. *B. licheniformis* C5R showed an intermediate inhibitory activity, with reductions of a 60.47% versus *P. expansum* CECT 2278, a 43.55% against *G. moniliformis* CECT 2987 and a 30.00% when confronted with *A. parasiticus* 2681. Finally, *B. licheniformis* C6 was the

least active strain, reducing *P. expansum* CECT 2278 by only a 23.26% and *G. moniliformis* CECT 2987 in a 14.52% (results for *A. parasiticus* CECT 2681 are not available for this *Bacillus* strain due to contamination of the plates).

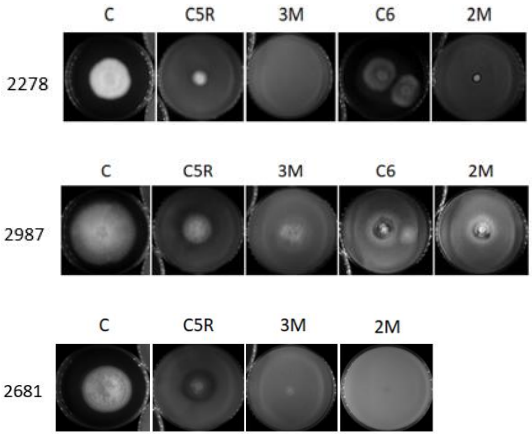

Figure 11. Photographs of the colonies of the three fungal indicators (*P. expansum* CECT 2278 (up), *G. moniliformis* CECT 2987 (in the middle) and *A. parasiticus* CECT 2681 (at the bottom)), in the absence (control) and presence of four *Bacillus* strains (*B. licheniformis* C5R, *B. subtilis* 3M, *B. licheniformis* C6, and *B. safensis/B. pumilus* 2M), after coculture and incubation at 25ºC for 7 days.

Table 3. % of reduction of the growth of the three fungal indicators (*P. expansum* CECT 2278, *G. moniliformis* CECT 2987 and *A. parasiticus* CECT 2681) when cocultured with four *Bacillus* strains: *B. licheniformis* C5R, B. *subtilis* 3M, *B. safensis/B. pumilus* 2M, and *B. licheniformis* C6. "N/A" indicates that data is not available.

Fungus	*B. lichen.* C5R	*B. subtilis* 3M	*B. saf./B. pum.* 2M	*B. lichen.* C6
CECT 2278	60.47%	100.00%	79.07%	23.26%
CECT 2987	43.55%	61.29%	56.45%	14.52%
CECT 2681	30.00%	88.00%	100.00%	N/A

4.3.2. Detection of the production of antifungal compounds by *Bacillus:*

To determine if fungal growth inhibition observed was due to the production of antifungal compounds or not, an indirect antagonistic assay was carried out using the supernatants obtained from *Bacillus* overnight cultures. Results from this experiment are summed up in table 4, which shows the % of inhibition of the growth all fungal indicators. Reductions in size were observed in almost all cases in the presence of

Bacillus supernatants when compared to control plates. In general, *B. licheniformis* C5R showed the highest inhibitory activity, together with *B. subtilis* 3M, and that *P. expansum* CECT 2278 and *A. parasiticus* CECT 2681 were the most sensitive indicators. When the same experiment was carried out using $^{1}/_{10}$ dilutions of the supernatants, *B. subtilis* 3M and *B. licheniformis* C5R were still the most active against fungal indicators, being the strongest inhibitory effect exerted by *B. licheniformis* C5R over *P. expansum* CECT 2278. In many cases, though, fungal growth seemed to be enhanced by the diluted supernatants when their size was compared with the colonies present in control plates.

Table 4. % of fungal growth inhibition of *P. expansum* CECT 2278, *G. moniliformis* CECT 2987 and *A. parasiticus* CECT 2681, in the presence and absence of the supernatants of *Bacillus* strains *B. wiedmannii* CS1, *B. safensis/B. pumilus* 2M, *B. subtilis* 3M, and *B. licheniformis* C6 and C5R. Cases in which no reduction on fungal development was observed are represented as "No inh."; "N/A" indicates that data is not available.

		B. wied. CS1	*B. saf./B. pum.* 2M	*B. subtilis* 3M	*B. lichen.* C6	*B. lichen.* C5R
Supernatant	CECT 2278	5.36%	5.13%	No inh.	5.13%	19.96%
	CECT 2987	3.80%	No inh.	1.23%	3.85%	5.45%
	CECT 2681	3.33%	2.17%	23.81%	9.80%	18.28%
S. 1/10 dilution	CECT 2278	No inh.	2.56%	No inh.	No inh.	14.87%
	CECT 2987	No inh.	No inh.	No inh.	1.92%	5.45%
	CECT 2681	No inh.	No inh.	9.52%	No inh.	N/A

5) Discussion

5.1. *In vitro* assessment of the antagonistic activity of *Lactobacillus plantarum* CECT 8962 against fungal and bacterial contaminants of nutritional formula

As it was previously described, the first experiment of this Master Thesis was carried out with the main aim of proving the antifungal activity of *L. plantarum* CECT 8962 against *P. expansum* CECT 2278. One of the main conclusions of that experiment was that,

contrary to what was firstly hypothesized, the fungus showed a much better growth in the presence of *Lactobacillus.* This makes sense when it is taken into account the fact that in the nutritional formula there were other bacterial species present, so the network of relationships established in that food was more complex than it had been previously thought. The most probable explanation for this is that the contaminant species probably had a higher inhibitory potential against the fungus than the one exerted by the *Lactobacillus*. This coincided with the fact that, in samples were *Lactobacillus w*as not inoculated, contaminants showed a good growth, inhibiting the fungus which could barely grow; in samples were *Lactobacillus* had been inoculated, on the contrary, *Bacillus* were inhibited and could not grow, therefore allowing the fungus to develop. This is consistent with scientific reports that had been published before, such as the one by Rossland *et al.* (2003), in which lower counts of *B. cereus* were observed in the presence of *Lactobacillus* when compared with cultures of *Bacillus* alone. Something similar had been observed by Suomalainen *et al.* (1999) when mixing *L. rhamnosus* and different *Bacillus* species in bakery products. Another possible reason could be the enhanced expression of genes related to fungal virulence under acidic pH, phenomenon that was reported by Prusky D. *et al.* in 2004 in a study about the changes in the size of the wounds caused by the fungus with changes in pH (Prusky *et al.,* 2004). Something similar could have happened in this case, as the minimum pH observed during this experiment was also of around 4. Maybe if extremely acidic pH was reached, fungal inhibition could have been achieved by *Lactobacillus*.

Regarding counts of *L. plantarum* CECT 8962 and *P. expansum* CECT 2278 under the two situations studied: (a) fermentation at 25ºC; b) storage at 6ºC after a 24-h incubation at 25ºC), it was observed that both populations decreased faster on samples incubated at 25ºC, showing that storage at 6ºC allowed both species to stay viable for a longer period of time; in samples at 25ºC individuals started to die sooner. Higher numbers of fungal colonies were observed in samples at 6ºC, maybe due to the fact that these samples were studied for a longer period of time. This is not contrary to the observations made

by Tannous J. e*t al.* in 2016 in a research about this fungus and its ability to produce patulin, where it was seen that at 6ºC a lag phase took place in the growth of the fungus but notable counts were still reached. This proved that low temperatures are not enough to prevent food spoilage by *P. expansum*, as it only delays its growth (Tannous *et al.,* 2016), what has also been shown in our results.

5.2. Search for anti-*Bacillus* activities amongst different LAB strains

As carrying the antifungal assay with *L. plantarum* CECT 8962 it was seen that this strain seemed to inhibit bacterial contaminants growing on nutritional formula, the second objective of this Master Thesis was proposed. This objective was to look for anti-*Bacillus* activities among a set of LAB strains, carrying out two antagonistic assays in the lab and an *in silico* approach. The fact that L. plantarum CECT 8962 inhibits *Bacillus* was proven on coculture by the clear observation of inhibition halos around *Bacillus* indicators *B. safensis/B. pumilus* 2M, *B. subtilis* 3M, *B. wiedmannii* CS1 and *B. licheniformis* C5R. After the antagonistic assays were performed, it was also seen that the inhibitory activities of LAB were strain-dependent, as the same LAB strain could show a very strong inhibition against one indicator and, at the same time, not be able to inhibit others. There were some LAB that showed very high inhibitory activities in general and some that were unable to inhibit any of the Bacillus, though.

Despite the large array of antagonistic LAB detected on the direct antagonist test, all the efforts invested on trying to detect inhibitory activities on LAB supernatants, so as to see if those activities were due to the secretion of antimicrobial metabolites or to other factors, were unsuccessful. A possible explanation is that the activities observed in the direct antagonistic assay could have been due to the production of lactic acid and the concomitant local reduction of pH, and not to the production of any metabolite. This would not be surprising, as it is known that *Bacillus* spp. normally grow under pH around 5-8 and are sensitive to acidic pH, which causes stress on their cells, having to develop

stress-response mechanisms towards it (Wilks *et al,* 2009). Another possible reason could be that, if there was production of an antimicrobial compound, its production or its release may only take place in the presence of the target bacteria in the culture medium, therefore only being manifested in coculture, but not when only using the LAB supernatants (Chanos and Mygind, 2016). It could also have been due to the fact that maybe the antibacterial compound was only active at acidic pH, and it lost its activity when supernatants were neutralized. And, lastly, another possible reason, which is very plausible when working strains such as *B. subtilis* 3M, that show an excessive growth, forming dense biofilms that cover the whole Petri dish in a short period of time, could be that that our methodology was not sensitive enough to observe the inhibition halos with the *Bacillus* indicators growing that much.

In spite of not having been able to detect antagonistic activities on supernatants, when analyzing the LAB genomes that were available online with BAGEL 4 and antiSMASH tools, some bacteriocin-related genes were detected, as it can be seen in table 2. Strains of *L. plantarum* CECT 8963/8964/8965/8966 were found to have the same genes, showing their genetic closeness to each other. All of them had structural genes for Plantaricins A, E/F, and J/K, which are bacteriocins typically produced by strains of *L. plantarum* (ScienceDirect, 2019), Sactipeptides, and the β chain of Enterocin X. **Plantaricin A** (*plnA*) is a post-translationally unmodified bacteriocin of less than 10 kDa that belongs to class II, according to the classification proposed by Álvarez-Sieiro P. e*t al.* (2016). As any other class II bacteriocin, it requires a peptidase and a transporter (Pal and Srivastava, 2015), and it is produced as a pre-peptide in order to prevent its biological activity inside of the producer, needing the cleavage of a N-terminal leader peptide so as to be active. The leader peptide is of double-glycine type, and it is associated with an ATP-binding cassette (ABC) transporter, what was proven by the presence of a gene coding for an **ABC transporter-peptidase C39** in the genome of these strains. The transporter removes the leader peptide from the pre-peptide and causes translocation of the mature bacteriocin across the cytoplasmic membrane (Todorov,

2009). Its production is regulated by a membrane-bound **histidine kinase** (HK), whose gene was also, that monitors the concentration of the bacteriocin in the environment and, in response, induces the phosphorylation of response regulators such as **PlnC**, an activator which was also found to be present in genomes (UniProt, 2019). According to Sand SL *et al.* (2012), Plantaricin A is a pheromone that induces the production of other bacteriocins, but it also acts as a membrane-permeabilizer, therefore having an antimicrobial effect. Structural genes for **Plantaricin E/F** and **Plantaricin J/K**, which belong to the class II b, were also found. They are composed of two peptides which need to be together in order to present full activity (Pal and Srivastava, 2015). The gene for the **PlnD response regulator**, transcriptional repressor contrary to PlnC was also observed, probably helping the activator to regulate the expression of Plantaricin-production genes in a "quorum sensing" way. In the same gene cluster ***HlyD*** gene was included, encoding a membrane fusion protein which acts as accessory factor for the ABC-transporter PlnH (UniProt, 2019). Finally, the **immunity protein PlnI** was also present, protecting producers from their own bacteriocins. A structural gene for one peptide of another two-peptide bacteriocin, **Enterocin X β chain**, was also found in their genomes, though it is normally produced by *Enterococcus faecium* (Hu e*t al.,* 2010). A gene for the correspondent immunity protein was also present in the same gene cluster. Structural genes were also discovered for **Sactipeptides or sactibiotics,** class I c type bacteriocins, which are post-translationally modified peptides that have uncommon amino acids and structures, a small size of less than 10 kDa, and are heat-stable. As it happens with class II bacteriocins, they also need a leader peptide so that enzymes can recognize and modify them (Pal and Srivastava, 2015).

In regard to *L. plantarum* LL441, structural genes for **Sactipeptides** together with genes coding for 2 **ABC-transporters** were found in the same gene cluster. Additionally, the structural gene for the lantibiotic **Salivaricin A** was also observed, bacteriocin that is normally produced by *Streptococcus* species and that acts by the formation of pores on target cell membrane (UniProt, 2019). This strain is also a known producer of Plantaricin

C, a pore-forming bacteriocin that inhibits target's cell wall synthesis, but genes related to its production were not found using BAGEL 4 and antiSMASH, as they are codified in a plasmid-derived contig which was not included in the assembled sequence analyzed in this work (Flórez and Mayo, 2018).

Lastly, in the case of *L. sakei* CECT 906, the only gene encoding for a bacteriocin present in its genome belonged to **Carnocin CP52 or carnobacteriocin B2**, which is a class II a or pediocin-like bacteriocin normally produced by *Carnobacterium piscicola*. It acts by binding itself to the receptors of the sugar transporter mannose phosphotransferase system in the membrane of the target bacteria, which allows it to insert on the membrane and form a pore (Pal and Srivastava, 2015). It has to be mentioned that, on the antagonistic assays carried out against *Bacillus* indicators, no antimicrobial activity was detected for this strain.

Taking all these data into account, we can presumably say that most of the strains studied are capable of producing, or at least have the machinery needed to produce, some antimicrobial compounds. In some cases, such as Salivaricin A in *L. plantarum* LL441 or Carnocin CP52 in *L. sakei* CECT 906, which are bacteriocins known to be produced by other species, the presence of genes related to their production is a probable indicator of horizontal gene transfer among the original producers and the species that received the genes.

5.3. Identification of inhibitory activity against fungal pathogens by different *Bacillus* strains

The last objective of this Master Thesis, also in accordance with the results obtained in the assay of the antifungal activity of *L. plantarum* CECT 8962, in which *Bacillus* contaminants present in nutritional formula seemed to inhibit *P. expansum* CECT 2278, was to prove the existence of inhibitory activity by *Bacillus* strains against some important fungal pathogens. This was done by: 1) coculture; 2) indirect antagonist assay using *Bacillus* supernatants.

As it can be seen in table 3, where % of inhibition of fungal growth in cocultures have been represented, it can be seen that all *Bacillus* strains showed inhibitory activities over fungi, though some strains had a stronger effect than others. *B. subtilis* 3M was the *Bacillus* strain with the highest overall inhibitory potential, completely reducing the growth of *P. expansum* CECT 2278. This coincides with the fact, in the first experiment included in this Master Thesis, this fungus was unable to grow in nutritional formula under the presence of contaminant *Bacillus*, among which *B. subtilis* 3M was included, proving our hypothesis that those contaminants were inhibiting fungal growth. When comparing those results with the ones obtained using *Bacillus* supernatants (table 4), it can be seen that inhibitory activities were maintained in most of the cases, what indicates that there must have been something present in the supernatants that was exerting those effects. In most of the cases, however, inhibition in the assay with supernatants was usually lower than in cocultures, what suggests that the effect observed in cocultures was probably due to an additive activity of the antimicrobial compounds produced by the *Bacillus* and other factors affecting fungal growth.

In both experiments, *B. subtilis* 3M, *B. safensis/B. pumilus* 2M and *B. licheniformis* C5R have been shown to be the most active strains against the fungal indicators used, though the compounds produced by *B. subtilis* 3M and *B. safensis/B. pumilus* 2M did not have such a strong effect when compared to the ones produced by *B. licheniformis* C5R. In the case of C5R, on the contrary, there is not a very big difference between its potential of inhibition when present in co-culture and when exposing the fungi to only the supernatants, from what we can deduce that, in this case, the inhibitory activity is mostly due to the production of antifungal substances. This production of compounds with antagonistic activity towards fungi by *Bacillus* species had already been reported in several scientific works published during the last years. In 2012, Islam *et al.* described the inhibition of various fungal plant pathogens by a strain of *Bacillus licheniformis*, which was suggested to present that activity thanks to the production of volatile organic compounds (Islam et al., 2012). In 2013, Zhiquiong *et al.* reported the inhibitory activity of a of *B. subtilis* strain against plant pathogens such as *Aspergillus niger* or *Fusarium oxysporum*, among others, caused by the production of a protein. Regarding *B. safensis*, in 2017, Mayer e*t al.* described an inhibitory effect over different pathogens, such as *Clostridium neoformans* or *Candida albicans*, affecting their capsule production, morphology, and biofilm formation. These are only a few examples, but it is well-known that *Bacillus* spp. are able to produce different kinds of antibiotics, toxins, lipopeptides and enzymes that affect the growth of fungal pathogens (Orberá, Serrat and Ortega, 2014). When focusing only on the results obtained with the supernatants it can be seen that, in many cases, the sizes of fungal colonies were bigger in the plates with *Bacillus* diluted supernatants than in control plates (without supernatants). This was possibly due to the fact that, when adding a supernatant to the medium, additional nutrients might also have been added, enhancing fungal growth. This phenomenon may have been masked in plates with non-diluted supernatants because, in those cases, the inhibitory activities were too strong, but with the diluted supernatants there could have been slight inhibitions that could have been overcome by the enhanced growth caused by the availability of more nutrients in respect to controls.

All these data proves that some *Bacillus s*trains may be good tools for food biopreservation, always when their consumption is ensured to be safe, as they have been demonstrated to have a clear antagonistic effect against some important fungal pathogens. For example, *B. subtilis* has been shown to have several probiotic effects including the regulation of pro-inflammatory and autoimmune processes, to help in food digestion, or the production of many important nutrients such as vitamins, apart from producing many different kinds of antibacterial and antifungal compounds. This species has been shown to inhibit the growth of important pathogens such as *Escherichia coli*, *Salmonella enterica, Listeria monocytogenes* or *Clostridium perfringens*, among some others, and has, in fact, been used as a starter culture in some oriental beverages. But a potential for biopreservation has also been observed in other *Bacillus* species, such as *B. licheniformis*, which was observed to inhibit the growth of *E. coli* and many Gram-positive strains due to the production of bacteriocins (Nath, Chowdhury and Dora, 2015). Apart from their potential for food preservation, some *Bacillus* strains seem to be interesting for their use for biocontrol in agriculture, as many strains have been reported as good antagonists of fungal phytopathogens. This is, for example, the case of *B. subtilis* or *B. licheniformis* against fungi like *Fusarium* spp. (Khan *et al.,* 2018), *Botrytis cinerea* or *Phytophtora infestans* (Nigris *et al.,* 2018).

However, it has to be taken into account that, due to the lack of time, these experiments were carried out only once for each pair of *Bacillus* and fungal indicator, so that replicates should be made in order to assess the reproducibility of results and to extract definitive conclusions.

6) Conclusions and future perspectives

1. *P. expansum* CECT 2278 grows better in the presence of *L. plantarum* CECT 8962 when cocultured in infant nutritional formula, contrary to what was firstly hypothesized. This is due to the presence on food of other bacterial species, such as *Bacillus*, which exert an inhibitory effect on the fungus when the *Lactobacillus* is not present. This shows that

fermented food products have a very complex network of biological interactions, so a previous case-by-case analysis of each product and of the species that grow in them should be performed before trying to establish the most adequate biopreservation method-

2. Many LAB strains show inhibitory activities against different *Bacillus* species. The mechanism that caused these activities could not be established, but bacteriocin-biosynthetic genes were detected in some of their genomes, suggesting that they could be due to the production of antimicrobial compounds. Additional experiments and an improvement on methodology is needed in order to establish which are those mechanisms.

3. *Bacillus* spp. are a good source of antifungal compounds, as it has been proven in the case of *B. licheniformis* C5R and *B. subtilis* 3M, which were shown to secrete compounds that were able to delay fungal growth. Further characterization is required to investigate their nature and mechanisms of action. In addition, other *Bacillus* strains were highly competitive when growing in coculture with the fungus. These facts make some strains good tools for food biopreservation or other applications, such as biocontrol of plant pathogens.

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Annexes

Annex 1. Matrix obtained after the screening for anti-Bacillus activities from different LAB:

	Indicators								
Producers	906	1B	2M	3M	CS1	C5R	C6	BD630	131
9269	+	-	+++	-	-	+	-	-	-
8962	+	+	+++	+	+	+	-	-	-
8965	+	+	+++	-	+	+	-	-	-
8963	+	+	+++	-	+	+	-	-	-
8964	+	+	+++	+++	+	+	-	-	-
8966	+	+	+++	+	-	+	-	-	-
8493	+	+	+++	+++	-	+	-	-	-
8492	-	-	+++	+	-	+	-	-	-
9267	+	-	+	-	+++	+	-	-	-
P1	-	-	-	-	+	+	-	-	-
P2	-	-	-	-	-	-	-	-	-
P3	-	-	+	-	-	-	-	-	-
P4	-	-	-	-	-	-	-	-	-
P5	-	-	+	-	-	+	-	-	-
P6	+	-	-	-	-	-	-	-	-
P7	-	-	+	+++	+++	+++	-	-	+++
P8	-	-	-	-	-	-	-	-	-
P9	-	-	-	-	-	-	-	-	-
P10	-	-	+	-	-	-	-	-	-
P11	-	-	-	-	-	-	-	-	-
P12	-	-	+	-	-	-	-	-	-
P13	-	-	+	+	-	+	-	-	-
P14	-	-	-	+	-	+	-	-	-
P15	-	-	-	+++	-	-	-	-	-
P16	+	-	+	+++	+++	+++	-	-	-
L1	+++	+	+	+++	-	+++	-	-	-
L2	-	+	+	+++	-	+++	-	-	-
L3	+++	+++	+	+++	+	+++	+	-	-
L4	-	-	+	+++	-	+++	-	-	-
L5	+++	+	+	+++	-	+++	-	-	-
L6	-	-	+	+++	-	+++	-	-	-
L7	+++	+	+	+++	-	+++	-	-	-
L8	+++	+	+	+	-	+	-	-	-
L9	+++	-	+	+++	+	+++	-	-	-
L10	+++	+++	+	+++	+	+++	+	-	-
L11	-	-	-	+++	-	+++	-	-	-
L12	-	-	-	+++	-	+++	-	-	-
L13	+	-	-	+++	+++	+++	-	+++	-
L14	+++	+	+	+++	+++	+++	-	-	-
L15	+++	+	+	+++	-	+	-	-	-
L16	+++	+++	+	+++	+	+++	+	-	-
L17	-	-	-	+++	-	+	-	-	-
L18	-	-	+	+++	-	+++	-	+	-

	Indicators								
Producers	906	1B	2M	3M	CS1	C5R	C6	BD630	131
L19	-	-	-	+++	-	+++	-	-	-
L20	-	-	-	+++	-	+++	-	-	-
L21	+++	+	-	+++	-	+	-	-	-
L23	+++	-	-	+++	-	+++	-	-	-
L24	+++	+++	-	+++	+++	+++	-	+	+
L25	-	-	+	+	+++	-	-	-	-
L26	-	-	+	+++	+++	-	-	-	-
L27	+	-	-	+	+	+	-	-	-
L28	+	-	+	+++	+++	-	-	-	-
L29	+++	+++	+++	+++	+++	-	-	-	-
L30	-	-	+++	-	-	-	-	-	-
L31	-	-	-	+++	-	-	-	-	-
L32	-	-	-	+++	-	-	-	-	-
L33	-	-	-	-	-	-	-	-	-
L34	-	-	-	-	-	-	-	-	-
L35	-	-	-	+++	-	+	-	-	-
L36	-	-	-	+	-	-	-	-	-
L37	+	-	+++	+++	-	-	-	-	-
L38	+	-	-	+++	-	-	-	-	-
L39	+	-	-	+++	-	-	-	-	-
L40	-	+	-	+	-	-	-	-	-
L41	-	-	-	-	-	-	-	-	-
L42	+	-	-	+++	-	+	-	-	-
L43	-	-	-	+++	-	+	-	-	-
L44	-	-	+	+++	-	-	-	-	-
L45	-	-	+	+++	-	-	-	-	-
L46	-	-	-	+++	-	-	-	-	-
L47	-	-	-	+++	-	-	-	-	-
L48	-	+	-	+++	-	-	-	-	-
L49	-	-	+	-	-	-	-	-	-
L50	-	-	-	+	-	+++	-	-	-
L51	-	-	+	+++	+	+++	-	-	-
L52	-	-	+++	+++	-	+++	-	-	-
L53	-	-	+	-	-	-	-	-	-
L54	+	-	-	-	-	-	-	-	-
L55	+	-	-	+++	-	-	-	-	-
L56	-	-	-	+++	-	+	-	-	-
L57	-	-	+	-	+++	+++	-	-	-
L58	-	-	-	-	-	+++	-	-	-
L59	+++	-	+++	+++	-	+++	-	-	-
L60	+	-	+++	+++	+++	+++	-	+	-
L61	-	+	+	-	-	-	-	-	-
L62	+	-	+++	+++	+++	+++	-	-	-

	Indicators								
Producers	906	1B	2M	3M	CS1	C5R	C6	BD630	131
L63	+	-	+++	-	-	-	-	-	-
L64	-	+	-	-	-	-	-	-	-
517	+++	+++	+++	+++	+++	+++	-	+	+
441	+++	-	+++	+++	-	+++	-	-	+++
C23	+++	-	+	+++	-	+++	-	+++	-
C270	+++	+++	+	-	+	+++	+	-	-
906	-	-	-	-	-	-	-	-	-
Control	-	-	-	-	-	-	-	-	-
Control	-	-	-	-	-	-	-	-	-
Control	-	-	-	-	-	-	-	-	-

Rows in red correspond to putative producer strains that did not show a good growth on the microtiter plate, so they were finally not taken into account nor included as part of the results.

Annex 2. Transformed matrix in order to use it for the creation the heatmaps and a dendrogram:

Matrix showing the different sensitivities/resistances observed during the screening for activities against Bacillus strains. Indicator strains are named in red, possible LAB producer strains in green, and positive (IPLA 517, LL441, C23, C270) and negative (CECT 906) controls in blue. For each pair of producer/indicator, a value of sensitivity was given: 1 = indicator resistant to the producer; 2 = indicator slightly sensitive to the producer; 3 = indicator very sensitive to the producer.

	Indicators								
Producers	906	1B	2M	3M	CS1	C5R	C6	BD630	131
CECT 9269	2	1	3	1	1	2	1	1	1
CECT 8962	2	2	3	2	2	2	1	1	1
CECT 8965	2	2	3	1	2	2	1	1	1
CECT 8963	2	2	3	1	2	2	1	1	1
CECT 8964	2	2	3	3	2	2	1	1	1
CECT 8966	2	2	3	2	1	2	1	1	1
CECT 8493	2	2	3	3	1	2	1	1	1
CECT 8492	1	1	3	2	1	2	1	1	1
CECT 9267	2	1	2	1	3	2	1	1	1
P1	1	1	1	1	2	2	1	1	1
P2	1	1	1	1	1	1	1	1	1
P3	1	1	2	1	1	1	1	1	1
P4	1	1	1	1	1	1	1	1	1
P5	1	1	2	1	1	2	1	1	1
P6	2	1	1	1	1	1	1	1	1
P7	1	1	2	3	3	3	1	1	3
P8	1	1	1	1	1	1	1	1	1
P9	1	1	1	2	1	1	1	1	1
P10	1	1	2	2	1	1	1	1	1
P11	1	1	1	1	1	1	1	1	1
P12	1	1	2	1	1	1	1	1	1
P13	1	1	2	2	1	2	1	1	1
P14	1	1	1	2	1	2	1	1	1
P15	1	1	1	3	1	1	1	1	1
P16	2	1	2	3	3	3	1	1	1
L1	3	2	2	3	1	3	1	1	1
L2	1	2	2	3	1	3	1	1	1
L3	3	3	2	3	2	3	2	1	1
L4	1	1	2	3	1	3	1	1	1
L5	3	2	2	3	1	3	1	1	1
L6	1	1	2	3	1	3	1	1	1
L7	3	2	2	3	1	3	1	1	1

Indicators

Producers	906	1B	2M	3M	CS1	C5R	C6	BD630	131
L8	3	2	2	2	1	2	1	1	1
L9	3	1	2	3	2	3	1	1	1
L10	3	3	2	3	2	3	2	1	1
L11	1	1	1	3	1	3	1	1	1
L12	1	1	1	3	1	3	1	1	1
L13	2	1	1	3	3	3	1	3	1
L14	3	2	2	3	3	3	1	1	1
L15	3	2	2	3	1	2	1	1	1
L16	3	3	2	3	2	3	2	1	1
L17	1	1	1	3	1	2	1	1	1
L18	1	1	2	3	1	3	1	2	1
L19	1	1	1	3	1	3	1	1	1
L20	1	1	1	3	1	3	1	1	1
L21	3	2	1	3	1	2	1	1	1
L23	3	1	1	3	1	3	1	1	1
L24	3	3	1	3	3	3	1	2	2
L25	1	1	2	2	3	1	1	1	1
L26	1	1	2	3	3	1	1	1	1
L27	2	1	1	2	2	2	1	1	1
L28	2	1	2	3	3	1	1	1	1
L29	3	3	3	3	3	1	1	1	1
L31	1	1	1	3	1	1	1	1	1
L32	1	1	1	3	1	1	1	1	1
L35	1	1	1	3	1	2	1	1	1
L36	1	1	1	2	1	1	1	1	1
L37	2	1	3	3	1	1	1	1	1
L38	2	1	1	3	1	1	1	1	1
L39	2	1	1	3	1	1	1	1	1
L40	1	2	1	2	1	1	1	1	1
L41	1	1	1	1	1	1	1	1	1
L42	2	1	1	3	1	2	1	1	1
L43	1	1	1	3	1	2	1	1	1
L44	1	1	2	3	1	1	1	1	1
L45	1	1	2	3	1	1	1	1	1
L46	1	1	1	3	1	1	1	1	1

Indicators

Producers	906	1B	2M	3M	CS1	C5R	C6	BD630	131
L47	1	1	1	3	1	1	1	1	1
L48	1	2	1	3	1	1	1	1	1
L49	1	1	2	1	1	1	1	1	1
L50	1	1	1	2	1	3	1	1	1
L51	1	1	2	3	2	3	1	1	1
L52	1	1	3	3	1	3	1	1	1
L54	2	1	1	1	1	1	1	1	1
L55	2	1	1	3	1	1	1	1	1
L56	1	1	1	3	1	2	1	1	1
L57	1	1	2	1	3	3	1	1	1
L58	1	1	1	1	1	3	1	1	1
L59	3	1	3	3	1	3	1	1	1
L60	2	1	3	3	3	3	1	2	1
L62	2	1	3	3	3	3	1	1	1
L63	2	1	3	1	1	1	1	1	1
L64	1	2	1	1	1	1	1	1	1
IPLA 517	3	3	3	3	3	3	1	2	2
LL441	3	1	3	3	1	3	1	1	3
C23	3	1	2	3	1	3	1	3	1
C270	3	3	2	1	2	3	2	1	1
CECT 906	1	1	1	1	1	1	1	1	1