



CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA

PORTO

EVALUATION OF MICROBIAL BEHAVIOR IN SELECTIVE CULTURE MEDIA FOR THE
MICROBIOLOGICAL ANALYSIS OF FRUIT PREPARATIONS

by

Beatriz Gonçalves

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MICROBIOLOGICAL ANALYSIS OF FRUIT PREPARATIONS

Training Placement Report presented to *Escola Superior de Biotecnologia* of the
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Food Engineering

by

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Resumo

Na indústria Alimentar, quando uma análise microbiológica é realizada, esta geralmente baseia-se na detecção de microrganismos por métodos imunológicos, visuais ou bioquímicos. Atualmente é possível cultivar microrganismos e induzir a proliferação de células através da adição de quantidades suficientes de nutrientes aos meios de cultura.

O estudo desenvolvido teve como principal objetivo desenvolver um meio de cultura ótimo para o crescimento de leveduras, bactérias e bolores. Estes meios de cultura pretendem mimetizar os preparados de fruta e garantir que se as amostras estiverem contaminadas, o microrganismo crescerá no meio, de forma mais rápida e formando colónias maiores para que a sua identificação seja fácil.

Para isso, primeiro foi feita uma visão geral, através da adição de diferentes componentes a meios de cultura mais simples, como NA e PDA, para perceber como cada componente afetaria o crescimento dos diferentes microrganismos, sendo que os microrganismos utilizados ao longo do estudo foram *Bacillus cereus*, *Escherichia coli*, *Leuconostoc mesenteroides*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Pichia fermentans*, *Candida intermedia* e *Neosartorya fischeri*. Posteriormente, foi efetuada uma confirmação, para verificar se a nível estatístico e qualitativo os componentes adicionados afetaram negativamente ou positivamente o crescimento dos microrganismos. Por fim, os componentes com os melhores resultados foram misturados nos meios.

Os meios de cultura que melhor potenciaram o crescimento bacteriano foram o 201 e o 203, onde em ambos foi adicionado sacarose e lisina, no 201 magnésio e no 203 magnésio e ferro, uma vez que para as três bactérias em estudo foram obtidos resultados positivos nos 3 inóculos para ambos os meios de cultura. Para as leveduras, dos seis meios testados, apenas um se destacou, sendo ele o 601, onde foi incorporado no meio frutose, lisina, biotina, tiamina, magnésio e ferro. Neste meio foram obtidos resultados positivos para duas leveduras, *S. cerevisiae* e *P. fermentans*. Neste meio, não foi melhorado nem comprometido o crescimento da levedura *C. intermedia*. O único bolor testado foi *N. fischeri* e foi possível perceber que este cresce melhor em PDA do que nos outros meios testados, logo é possível supor que para este bolor meios de cultura mais simples, como o PDA, são melhores para o seu crescimento.

Foi então possível concluir que a combinação de certos componentes, quando adicionados a meios simples, fizeram com que as colónias de bactérias e de leveduras crescessem maiores. Sendo que o oposto foi aferido para o caso do bolor, em que as suas colónias cresciam maiores em meios simples.

Palavras Chave: Meios de Cultura, microrganismos, leveduras, bactérias e bolores.

Abstract

In the food industry, when a microbiological analysis is performed, it is generally based on the detection of microorganisms in immunological, visual, or biochemical ways. Nowadays, it is possible to cultivate microorganisms and induce the proliferation of cells by adding sufficient nutrients to the culture media.

The study developed had the main objective of developing an optimum culture media for the growth of yeasts, bacteria and moulds. These culture media are intended to mimic the fruit preparations and guarantee that if the samples are contaminated, the microorganism will grow in the media and grow faster and more significantly to identify the microorganism quickly.

To achieve this first was made an overview by adding different components to simpler culture media, like NA and PDA, to understand how each component affected the growth of the different microorganisms, being the microorganisms used throughout the study *Bacillus cereus*, *Escherichia coli*, *Leuconostoc mesenteroides*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Pichia fermentans*, *Candida intermedia* e *Neosartorya fischeri*. Later, a confirmation was performed to see if, at a statistical and qualitative level, the components negatively or positively affected the growth of the microorganisms. Finally, the components with better results were mixed in the media.

The culture media that better enhanced the bacteria growth were 201 and 203, where both sucrose and lysine were added, 201 magnesium and 203 magnesium and iron, once three different bacteria had positive outcomes in their three inoculums for both culture media. For yeasts, of the six media tested, only one stood out, being 601, where fructose, lysine, biotin thiamine, magnesium and iron were incorporated into the medium. This media had a positive result for two yeasts, *S. cerevisiae* and *P. fermentans*, and the yeast *C. intermedia*. The media may not have had a positive outcome but did not have a negative result, which means that it does not negatively compromise the growth of the yeast. The only mould tested was *N. fischeri*, and it was possible to perceive that it grow better in PDA than in the tested media, so it is possible to assume that simpler culture media, like PDA, are better for growth for this mould.

Then was possible to conclude that the combination of certain components, when added to simpler media, made the bacteria and yeasts colonies to grow larger. The opposite was observed in the case of the mould, in which its colonies grew larger in simpler media.

Key Words: Culture media, microorganisms, yeasts, bacteria and moulds.

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Contents

Resumo	V
Abstract	VII
Acknowledgements	IX
List of Figures	XV
List of tables	XVII
List of abbreviations	XXI
Introduction	1
1. Company Presentation	1
2. Food Safety	2
2.1. Microbial Foodborne Diseases.....	2
2.2. Food Pathogen Detection.....	3
2.3. Food Pathogen Detection: Microbiological Analysis.....	3
2.4. Traditional Culture Methods.....	3
3. Culture Media	4
3.1. Sugars as a Carbon Source.....	4
3.2. Alcohols as a Carbon Source.....	6
3.3. Sodium Salts.....	7
3.4. Amino Acids.....	7
3.5. Vitamins.....	8
3.6. Mineral Salts.....	9
3.7. Culture Media pH.....	9
4. Microorganisms	9
4.1. Bacteria.....	10
4.1.1. <i>Bacillus cereus</i>	10
4.1.2. <i>Escherichia coli</i>	10
4.1.3. <i>Leuconostoc mesenteroides</i>	10
4.1.4. <i>Pseudomonas aeruginosa</i>	11
4.2. Yeasts.....	11
4.2.1. <i>Candida intermedia</i>	11
4.2.2. <i>Pichia fermentans</i>	11
4.2.3. <i>Saccharomyces cerevisiae</i>	11
4.3. Molds.....	12
4.3.1. <i>Neosartorya fischeri</i>	12

Work Objectives	12
Material and Methods	14
1. Preparation of Culture Media	14
1.1. Preparation of Culture Media for Yeasts and Molds.....	14
1.1.1. Preparation of the Control Culture Media	14
1.1.2. Preparation of the Culture Media With the addition of Sugars	14
1.1.3. Preparation of the Culture Media With the addition of Alcohols.....	14
1.1.4. Preparation of the Culture Media With the addition of Vitamins.....	15
1.1.5. Preparation of the Culture Media With the addition of Proteins	15
1.1.6. Preparation of the Culture Media With the addition of Mineral Salts.....	15
1.2. Preparation of Culture Media for Bacteria	16
1.2.1. <i>Preparation of the Control Culture Media</i>	16
1.2.2. Preparation of the Culture Media With the addition of Sugars	16
1.2.3. Preparation of the Culture Media With the addition of Proteins	16
1.2.4. Preparation of the Culture Media With the addition of Mineral Salts.....	17
2. Preparation of Culture Media: Synergism	17
2.1. Preparation of Culture Media for Yeasts	17
2.1.1. Preparation of the Control Culture Media	17
2.1.2. Preparation of the Culture Media.....	17
2.2. Preparation of Culture Media for Molds.....	18
2.2.1. Preparation of the Control Culture Media	18
2.2.2. Preparation of the Culture Media.....	18
2.3. Preparation of Culture Media for Bacteria	18
2.3.1. Preparation of the Control Culture Media	18
2.3.2. Preparation of the Culture Media.....	19
3. Microorganisms Plating	19
3.1. Microbial Cultures	19
3.2. Inoculum Preparation	19
3.3. Yeasts, Moulds, and <i>Pseudomonas aeruginosa</i> Plating.....	20
3.4. Bacteria Plating	20
4. Qualitative Analysis	21
5. Statistical Analysis	22
Results and Discussion	23

1. Phase 1: Overview	23
2. Phase 2: Confirmation	28
2.1. Bacteria.....	28
2.2. Yeasts	31
2.3. Mould	32
3. Phase 3: Synergism Between Nutrients	34
3.1. Bacteria.....	34
3.2. Yeasts	37
3.3. Mould	39
Conclusions	42
Future Work	43
References	44
Appendix	50
A. Results obtained for Phase 2	50
A.1. Results obtained in Phase 2 for Bacteria	50
A.2. Results obtained in Phase 2 for Yeasts	55
A.3. Results obtained in Phase 2 for the Mold	63
B. Results obtained for Phase 3	66
B.1. Results obtained in Phase 3 for the Bacteria	66
B.2. Results obtained in Phase 3 for the Yeasts	70
B.3. Results obtained in Phase 3 for the Mold	74
B.4. Results obtained in Phase 3 for the pH.....	75

List of Figures

Figure 1 - ATP synthesis through glycolysis and TCA cycle (<i>in Fernie, Carrari & Heiden, 2011</i>).....	5
Figure 2 - Representation of the two pathways of the degradation of glycerol in yeasts. i - glycerol degradation via glycerol-3-phosphate; ii - glycerol degradation via dihydroxyacetone. (<i>in Klein et al., 2017</i>).....	7
Figure 3 - Secretion of amino acids by cells. (<i>in Krämer, 1994</i>).....	8
Figure 4 - An example of a positive impact on the growth of <i>E. coli</i> when grown in NA_SAC_4, compared to NA.	21
Figure 5 - An example of a negative impact on the growth of <i>S. cerevisiae</i> in PDA_FRU compared to PDA.	21
Figure 6 - An example of no impact on the growth of <i>C. intermedia</i> , when grown in PDA_GLU_6 compared to PDA.	22
Figure 7 - Comparison of the bacterial growth between Nutrient Agar and Nutrient Agar with 40 g/L of Sucrose. BC_NA: <i>B. cereus</i> on Nutrient Agar; BC_NA_SAC_4: <i>B. cereus</i> on Nutrient Agar with 40 g/L of sucrose; EC_NA: <i>E. coli</i> on Nutrient Agar; EC_NA_SAC_4: <i>E. coli</i> on Nutrient Agar with 40 g/L of sucrose; LM_NA: <i>L. mesenteroides</i> on Nutrient Agar; LM_NA_SAC_4: <i>L. mesenteroides</i> on Nutrient Agar with 40 g/L of sucrose; PA_NA: <i>P. aeruginosa</i> on Nutrient Agar; PA_NA_SAC_4: <i>P. aeruginosa</i> on Nutrient Agar with 40 g/L of sucrose.	24
Figure 8 - Comparison of the bacterial growth between Nutrient Agar and Nutrient Agar with 40 g/L of Sucrose. BC_NA: <i>B. cereus</i> on Nutrient Agar; BC_NA_SAC_4: <i>B. cereus</i> on Nutrient Agar with 40 g/L of sucrose; EC_NA: <i>E. coli</i> on Nutrient Agar; EC_NA_SAC_4: <i>E. coli</i> on Nutrient Agar with 40 g/L of sucrose; LM_NA: <i>L. mesenteroides</i> on Nutrient Agar; LM_NA_SAC_4: <i>L. mesenteroides</i> on Nutrient Agar with 40 g/L of sucrose; PA_NA: <i>P. aeruginosa</i> on Nutrient Agar; PA_NA_SAC_4: <i>P. aeruginosa</i> on Nutrient Agar with 40 g/L of sucrose.	24
Figure 9 - Comparison of the yeast growth between PDA and PDA with thiamine. SC_PDA: <i>S. cerevisiae</i> on PDA; SC_PDA_THI: <i>S. cerevisiae</i> on PDA with thiamine; CI_PDA: <i>C. intermedia</i> on PDA; CI_PDA_THI: <i>C. intermedia</i> on PDA with thiamine; PF_PDA: <i>P. fermentans</i> on PDA; PF_PDA_THI: <i>P. fermentans</i> on PDA with thiamine.	26
Figure 10 - Results obtained in Phase 2 for the different inoculums of <i>L. mesenteroides</i> in the culture media with 40 g/L of sucrose (NA_SAC_4).	30
Figure 11 - Results obtained in Phase 3 for the different inoculums of <i>E. coli</i> in all the different culture media tested.	36
Figure 12 - Results obtained for the culture media 601 for the growth of the 3 inoculums of <i>S. cerevisiae</i> and <i>P. fermentans</i>	39
Figure 13 - Results obtained for the culture media 701, 802 and 901 for the 3 inoculums of <i>N. fischeri</i>	41

List of tables

Table 1 - Preparation of the Culture Media for yeasts and molds with the addition of Sugars.	14
Table 2 - Preparation of the Culture Media for yeasts and molds with the addition of Alcohol.	14
Table 3 - Preparation of the Culture Media for yeasts and molds with the addition of Mineral Salts.....	15
Table 4 - Preparation of the Culture Media for bacteria with the addition of Sugars.....	16
Table 5 - Preparation of the Culture Media for bacteria with the addition of Mineral Salts.....	17
Table 6 - Preparation of the Culture Media for yeasts with the addition of sugars, protein, vitamins and mineral salts to test the synergism between them.....	17
Table 7 - Preparation of the Culture Media for molds with the addition of sugars, protein, vitamins and mineral salts to test the synergism between them.....	18
Table 8 - Preparation of the Culture Media for bacteria with the addition of sugars, protein and mineral salts to test the synergism between them.....	19
Table 9 - Results from microorganisms' growth in NA (for bacteria) and PDA (for yeast and moulds) with the addition of different sugars.....	23
Table 10 - Results from yeast growth in PDA with the addition of different glycerol concentrations.....	25
Table 11 - Results from yeast and mold growth in PDA with the addition of biotin and thiamine.....	25
Table 12 – Qualitative results from microorganisms growth on NA, for bacteria, and PDA, for yeast and moulds, with the addition of different concentrations of lysine.....	26
Table 13 - Results from microorganisms growth in NA, for bacteria, and PDA, for yeast and moulds, with the addition of different minerals.....	27
Table 14 - Results obtained for bacteria in Phase 2, in which there was a statistically significant difference between the tested culture media and NA.....	28
Table 15 - Qualitative results of bacteria from Phase 2 obtained by comparing the morphology of the colonies with their growth in NA and with their growth in NA with the addition of one nutrient.....	30
Table 16 - Statistical Data Analysis from Phase 2 of the yeasts.....	31
Table 17 - Qualitative results of yeasts from Phase 2 obtained by comparing the morphology of the colonies with their growth in NA and with their growth in NA with the addition of one nutrient.....	32
Table 18 - Results obtained for the mould in Phase 2, in which there was a statistically significant difference between the tested culture media and PDA.....	33
Table 19 - Qualitative results of the mold from Phase 2 obtained by comparing the morphology of the colonies with their growth in NA and with their growth in NA with the addition of one nutrient.....	33

Table 20 - Results obtained for bacteria in Phase 3, in which there was a statistically significant difference between the tested culture media and NA.	35
Table 21 - Qualitative results of bacteria from Phase 3 obtained by comparing the morphology of the colonies with their growth in NA and with their growth in the media with the addition of several components.....	35
Table 22 - Statistical Data Analysis from Phase 3 of the yeasts.	37
Table 23 - Qualitative results of yeasts from Phase 3 obtained by comparing the morphology of the colonies with their growth in PDA and with their growth in the media with the addition of several components.....	38
Table 24 - Statistical Data Analysis from Phase 3 of the mould.	40
Table 25 - Qualitative results of the mould from Phase 3 obtained by comparing the morphology of the colonies with their growth in PDA and with their growth in the media with the addition of several components.....	40
Table 26 - Results obtained in Phase 2 for the different inoculums of <i>B. cereus</i>	50
Table 27 - Results obtained in Phase 2 for the different inoculums of <i>E. coli</i>	51
Table 28 - Results obtained in Phase 2 for the different inoculums of <i>L. mesenteroides</i>	52
Table 29 - Results obtained in Phase 2 for the different inoculums of <i>P. aeruginosa</i>	54
Table 30 - Statistical Data Analysis from Phase 2 of the bacteria.	55
Table 31 - Results obtained in Phase 2 for the different inoculums of <i>S. cerevisiae</i> , when comparing PDA with PDA_GLU_4, PDA_SAC_4, PDA_FRU_4, PDA_BIO, PDA_THI, PDA_Mg_1 and PDA_Fe.....	55
Table 32 - Results obtained in Phase 2 for the different inoculums of <i>S. cerevisiae</i> , when comparing PDA with PDA_P_0,5.	57
Table 33 - Results obtained in Phase 2 for the different inoculums of <i>S. cerevisiae</i> , when comparing PDA with PDA_LYA_0,5.....	58
Table 34 - Results obtained in Phase 2 for the different inoculums of <i>C. intermedia</i>	58
Table 35 - Results obtained in Phase 2 for the different inoculums of <i>P. fermentans</i> , when comparing PDA with PDA_GLU_4, PDA_SAC_4, PDA_FRU_4, PDA_LYS_0,5, PDA_BIO, PDA_THI, PDA_Mg_1 and PDA_Fe.	60
Table 36 - Results obtained in Phase 2 for the different inoculums of <i>P. fermentans</i> , when comparing PDA with PDA_P_0,5.	62
Table 37 - Results obtained in Phase 2 for the different inoculums of <i>N. fischeri</i>	63
Table 38 - Statistical Data Analysis from Phase 2 of the mould.	65
Table 39 - Results obtained in Phase 3 for the different inoculums of <i>E. coli</i>	66
Table 40 - Results obtained in Phase 3 for the different inoculums of <i>B. cereus</i>	67
Table 41 - Results obtained in Phase 3 for the different inoculums of <i>P. aeruginosa</i>	67
Table 42 - Results obtained in Phase 3 for the different inoculums of <i>L. mesenteroides</i>	68
Table 43 - Statistical Data Analysis from Phase 3 of the bacteria.	69
Table 44 - Results obtained in Phase 3 for the different inoculums of <i>C. intermedia</i>	70
Table 45 - Results obtained in Phase 3 for the different inoculums of <i>S. cerevisiae</i>	71

Table 46 - Results obtained in Phase 3 for the different inoculums of <i>P. fermentans</i>	72
Table 47 - Results obtained in Phase 3 for the different inoculums of <i>N. fischeri</i>	74
Table 48 - Results obtained in Phase 3 for the pH measurements of the Culture Media.	75

List of abbreviations

BC	<i>Bacillus cereus</i>
CI	<i>Candida intermedia</i>
EC	<i>Escherichia coli</i>
LM	<i>Leuconostoc mesenteroides</i>
NA	Nutrient Agar
NF	<i>Neosartorya fischeri</i>
PA	<i>Pseudomonas aeruginosa</i>
PDA	Potato Dextrose Agar
PF	<i>Pichia fermentans</i>
SA	<i>Staphylococcus aureus</i>
SC	<i>Saccharomyces cerevisiae</i>
SAC	Sucrose
GLU	Glucose
FRU	Fructose
XYL	Xylose
MAL	Maltose
LYS	Lysine
BIO	Biotin
THI	Thiamine
GLI	Glycerol
PDA	Potato Dextrose Agar
NaCl	Sodium Chloride
Mg	Magnesium ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)
Ca	Calcium
P	Potassium (KH_2PO_4)
Fe	Iron III ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$)
Zn	Zinc ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$)

Introduction

This dissertation project was carried out in partnership with Escola Superior de Biotecnologia da Universidade Católica Portuguesa, Centro Regional do Porto, and the company FRULACT – Ingredientes para a Indústria Alimentar, S.A.

1. Company Presentation

The Frulact is a Business Group comprising three business units: FRULACT, FRUSENSES, and OATVITA. FRULACT is skilled in stabilised preparations of fruits and vegetables, seeds and cereals, and others. FRUSENSES specialises in food flavourings, making a difference through knowledge of the flavourings and the synergies between fruit preparations and aromas. OATVITA is dedicated to the development of value-added plant-based products.

Frulact was founded in 1987 by Commander Arménio Pinheiro Miranda and his two sons, Francisco Miranda and João Miranda. The rapid growth took the founders to invest in an industrial unit, so in 1992 the Maia Industrial Plant was inaugurated. This new unit increased the production capacity, which enabled the search for new markets. In 1998, Frulact expanded by building a new factory strategically located in Ferro, Covilhã, because of its proximity to agricultural fields.

In 1999, the Group made its first bet in the international market. It invested in a new industrial facility in Morocco, enabling the company to supply international brands in the food sector. Later, in 2006, Frulact expanded once more for Tortosendo, Covilhã, and this new facility was equipped with cutting-edge technology. Furthermore, the Business Group acquired a French company, Granger Bouguet Pau. The year 2010 was an essential mark for the company since the project FRUTECH – Centro de Investigação, Desenvolvimento e Inovação of FRULACT was launched in Maia, Portugal. FRUSENSES was born in 2013 as a reinforcement in the food sector of the food flavourings. In 2017 a new industrial facility was launched in Canada in Kingston. With its entry into North America, FRULACT has made a significant step forward on the international market, with a presence on three continents.

Thereby, FRULACT is dedicated to fruit preparations and plant-based ingredients, and is an innovative company that constantly improves its supply with added-value ingredients. The fast growth of the company is possible and continues to be because of the serious focus on producing knowledge that is necessary and central in the Development and Innovation sector (IDI). Being this always accompanied by an investment in new technologies, equipment, industrial facilities, modern technologies, and better human practices.

The high quality and safety of FRULACT products have leading customers worldwide and locally, like NESTLÉ, PASCUALS, LACTOGAL, DANONE, YOPLAIT, and others.

Over the years, Frulact's great success has made it stand out among other companies, winning several awards such as 2003 Innovation Award (Ministry of Agriculture and Fisheries); PME Innovation 2007 Prize (COTEC Portugal and BPI); Finalist with Honorable Mention – Entrepreneur of the Year Award 2008 (Ernest & Young); Innovation and Technology Award 2008 (IAPMEI – Agency for Competitiveness and Innovation); Entrepreneurship Award 2010 (INSEAD); Ruban d'honneur – European Company of the Year 2011 (European Business Awards); Leader of the year in New

Technologies 2012 (Best Leader Awards); Company of the year – Excellens Oeconomia Award 2013 (PwC and Journal de Negócios); Innovation Award Finalist | Large Companies Category 2017 (Millenium BCP); Best Digital Operational Process 2018 (Portugal Digital Awards); Export & Internationalization Award – Strategic Sector – Agro-Food 2018 and International Company of the Year 2019 (SAPCC – ABSA Business Excellence Awards).

Frulact looks the future with the ambition of always to do better. For this reason, Innovation plays a vital role since it is at the base of all processes. To the company, Innovation is essential to stand out and constantly evolve.

2. Food Safety

In many countries, one of the most common concerns about public health issues is foodborne disease. This is because foodborne diseases, in addition to affecting the health of individuals, affect society as a whole (**Schlundt, 2002**).

It is estimated that over 600 million cases of foodborne diseases occur each year, and that 420.000 die worldwide. Besides that, those most affected by foodborne illnesses are the ones with low income and children under five years of age (**WHO, 2022**). So, it is necessary to ensure that the food systems are safe and secure and that the food that goes to the consumer's houses is safe.

To achieve this, we need to understand what food safety is. According to the *Codex Alimentarius* (**FAO, WHO, n.d.**), the definition of food safety is to “guarantee that food does not cause harm to the consumer, when prepared and/or consumed according to the use for which they are intended”. The consumption of unsafe food can cause more than 200 different diseases, and prevention requires collaboration between producers, consumers, and governments to ensure well-built food systems (**WHO, 2022**).

The food can be contaminated through the introduction or presence of a contaminant. This contaminant can be chemical, physical or microbiological, or another kind of substance that is not supposed to be present in the food and compromises the safety of the consumers (**FAO, WHO, n.d.**).

2.1. Microbial Foodborne Diseases

Nowadays, it is hard to establish the incidence of microbiological foodborne diseases.

According to **WHO (2019)**, in the European Region, the number of people eating contaminated food is more than 23 million per year; further, 4700 people die. The author also states that the most common foodborne disease is provoked by diarrhoeal agents (94% of them are diarrhoeal diseases), being norovirus (15 million cases) and *Campylobacter* spp. (5 million cases) the most frequent. Deaths are caused mainly by *Listeria monocytogenes*; other agents responsible for the deaths are *Salmonella* spp, *Campylobacter* spp., norovirus, and *E. coli*.

A study carried out in the United States estimates that, per year, nearly 76 million illnesses are caused by foodborne pathogens; this results in 325 000 hospitalisations and leads to 5 000 deaths (**Mead et al., 1999**). According to **Schlundt (2002)**, if the numbers from the study held in the United

States were extrapolated to the rest of the world, it would mean that foodborne diseases affect approximately one-third of the population in developed countries.

Microbiological foodborne illnesses symptoms can vary from gastroenteritis to renal, neurological, and hepatic life-threatening syndromes. The causes of this kind of disease can be viruses, parasites, bacteria, toxins and prions (**Mead et al., 1999**).

2.2. Food Pathogen Detection

To achieve food safety, the detection methods of pathogens in foods must be sensitive, accurate, and fast. However, most currently used methods take 48 hours at best (**Batt, 2007; Habimana, Ji & Sun, 2018**). The most commonly used techniques for detecting pathogens are based on culture. Although these techniques are accurate and can differentiate between live and dead cells, this analysis requires specialised people and is time-consuming (**Habimana, Ji & Sun, 2018**).

2.3. Food Pathogen Detection: Microbiological Analysis

In the food industry, when a microbiological analysis is performed, it is generally based on the detection of microorganisms in immunological, visual, or biochemical ways, enumeration or detection methods can be performed. The former is done when it is intended to perform an enumeration or other quantitative analysis, and the latter is intended to do a qualitative analysis (presence/absence test) after an enrichment step of the food sample (**López-Campos et al., 2012**).

Microbiological analysis of food continues to be challenging for practically all technologies and assays. This problem can have different sources, like the composition of the food and its matrix, the stress on microorganisms from processing, and the natural microbiota of the food (**López-Campos et al., 2012**).

2.4. Traditional Culture Methods

Analytical methods for official controls, or standardised methods, are primarily traditional approaches using liquid or solid culture media to grow microorganisms to isolate the target and/or enumerate (**Jasson et al., 2010**). Traditional culture methods are generally divided into qualitative and quantitative methods.

Qualitative methods are used when the presence or absence of the target microorganism must be known rather than the amount. In this process, a sample with a determined weight is required. In the case of obtaining a typical colony of the target microorganism on selective media, confirmation will be performed using biochemical and/or serological tests (**López-Campos et al., 2012**).

Quantitative methods are usually based on plate count or most probable number (MPN) techniques. The plate count technique is based on performing dilutions of a sample and plating the previously selected dilutions in order to be able to count the number of colonies. To perform the MPN method it is necessary to carry out decimal dilutions of the sample; from 3 serial dilutions is necessary to transfer subsamples 9 or 15 tubes with liquid culture media. These tubes are incubated, and the ones that show turbidity are counted. The method calculates the viable microorganisms' number taking into

consideration, and the final number is compared to a standard table that will give the MPN of bacteria (López-Campos *et al.*, 2012).

3. Culture Media

According to Lagier *et al.* (2015), bacterial culture was the pioneering technique to describe the human microbiota. In the nineteenth century, Louis Pasteur developed the first culture media, which enabled the deployment of microbiology. Pasteur created a medium with different components with the primary objective of associating each fermentation with different microorganisms. This allowed him to note that some elements promote their growth and other inhibits it (Bonet *et al.*, 2020). For many years, the capability to catabolise some specific substrates allowed the identification of a particular pure culture of bacteria (Konopka, Oliver & Jr., 1998).

Nowadays, it is possible to cultivate bacteria that could not be grown before by adding different components to a culture medium (Bonet *et al.*, 2020). For microorganisms, the proliferation of cells is induced by the presence of nutrients in the medium in which they are. For example, yeast will start growth if they are in the presence of sufficient nutrients, but in the absence of nutrients, yeasts will stop proliferation and enter a stationary phase (Granot & Snyder, 1993).

Culture media are generally presented in three forms, liquid, solid and semi-solid. The liquid culture media, or culture broths, is a mixture of nutrients in water. This kind of media eases access to nutrients, especially if under agitation. Using these media, it is possible to detect microbial growth through increased turbidity. However, it is impossible to isolate bacteria directly with these culture broths (Bonet *et al.*, 2020).

Solid culture media are obtained by adding a gelling agent, usually agar, to the liquid media. These media permit isolated colonies of different microorganisms to be obtained and identified. Unlike liquid culture media, solid culture media allows one to visualise and describe the morphological characteristics of the colonies (Bonet *et al.*, 2020).

Both in solid and liquid culture media, nutrients can be added to enrich the media in order to provide microbial growth. Added nutrients can be sugars (as a carbon source), proteins or amino acids, mineral salts, vitamins, among others.

3.1. Sugars as a Carbon Source

Microorganisms can use different types of sugars as carbon sources, such as sucrose, fructose, and glucose. In cells, energy production is achieved through carbon sources, and they are also a “basic block building of cells” (Iwama & Ohsumi, 2019). According to Gorke & Stülke (2008) and Iwama & Ohsumi (2019), one of the most preferred carbon sources is glucose; many microorganisms use it as a primary carbon source. Brückner & Titgemeyer (2002) quote the example of *E. coli* as a bacterium that prefers glucose because of the glucose-lactose diauxie.

In bacteria, carbon is the most exuberant component in its constitution once they need to produce molecules such as fats, proteins, carbohydrates, and nucleic acids. Bacteria can use organic or inorganic carbon sources, such as sugars or carbon dioxide (Bonet *et al.*, 2020). Most bacteria can

use different carbon compounds as carbon sources, which can be co-metabolized, or choose a preferential carbon that allows the fastest growth (**Gorke & Stülke, 2008**).

Sucrose, commonly known as sugar, is produced from sugarcane and sugar beet processing (**Pennington & Baker, 1990**). It is a disaccharide constituted by 50% of glucose and 50% fructose (**Anton et al., 2010**). In Western, the consumption of refined sugars, like sucrose, is high, and this sugar can be found in processed foods such as cakes, cookies, chocolate, commercial cereals, puddings, and fruit juice drinks (**Whitbread, 2023**).

Regarding fructose, it is most commonly found in nature as a constituent of the disaccharide sucrose. However, the fructose monosaccharide can be found in honey and fruits, like grapes, berries, apples, dates, and figs (**Riby, Fujisawa & Kretchmer, 1993**).

Glucose is commonly found in nature. In plants, glucose is produced through photosynthesis; in some plants, like potatoes, wheat, corn, and rice, it is stored in chains (starch). It is also possible to find glucose monosaccharides in some foods, like honey and dried fruits (**Sollid, 2021**).

Many eukaryotic microorganisms assimilate glucose to produce ATP (adenosine triphosphate). This is achieved over glycolysis (**Iwama & Ohsumi, 2019; Lunt & Vander Heiden, 2011**). Glycolysis metabolises glucose to pyruvate, and in the TCA (tricarboxylic acid) cycle, pyruvate can be oxidatively metabolised to CO₂ generating a large amount of ATP over oxidative phosphorylation. This process is represented in **Figure 1** (**Lunt & Vander Heiden, 2011; Fernie, Carrari & Sweetlove, 2004**). Also, fermentation can reductively metabolise pyruvate to alcohol or organic acids. Fermentation of glucose does not require oxygen. However, it is lower efficient in generating ATP. Regardless of that, many microorganisms depend on glucose fermentation despite the presence of oxygen. For instance, *Saccharomyces cerevisiae* ferments glucose to ethanol to achieve fast growth, even in aerobic conditions. Nonetheless, in environments with poor glucose, this yeast depends upon oxidative phosphorylation (**Lunt & Vander Heiden, 2011**).

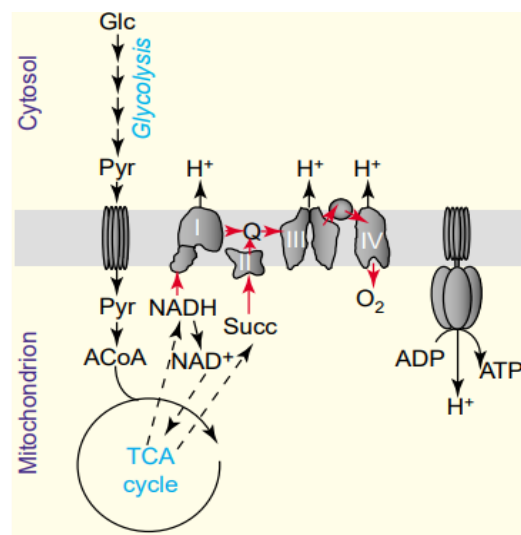


Figure 1 - ATP synthesis through glycolysis and TCA cycle (*in* **Fernie, Carrari & Heiden, 2011**).

Thereby, glucose is essential in generating energy for reproduction and growth, as glucose is the primary source of glycolysis and the pathways of anaerobic and aerobic respiration (**Galant, Kaufman & Wilson, 2015**).

To yeast that are in the stationary phase and spores, the presence of only glucose can induce growth. However, without other nutrients to support growth, the cells lose viability in a few days. Their growth is induced by physiological and morphological changes such as the dispersion of the large vacuole (stationary phase cells characteristic), and the biosynthetic pathway of adenine is induced **(Granot & Snyder, 1993)**.

Despite yeast having glucose as their favoured carbon source, they can grow through other carbon sources, like fructose, glycerol, galactose, acetate, and ethanol, so these carbon sources are expected to induce growth too **(Granot & Snyder, 1993)**.

3.2. Alcohols as a Carbon Source

Alcohol can be produced through chemical synthesis or as a byproduct of fermentation. Nonetheless, the accumulation of alcohol in the environment inhibits microbial growth. It has long been known that ethanol stops fermentation abruptly, despite the microorganism having an unlimited supply of substrate **(Ingram & Buttke, 1985)**.

However, some alcohols, like glycerol, can be used by microorganisms as a carbon source **(Wendisch et al., 2016)**. In the Food Industry, glycerol is commonly used to increase some food additives' solubility and water-coating ability **(Azelee et al., 2019)**.

According to **Wendisch et al. (2016)**, *S. cerevisiae* and *E. coli* can use glycerol as a substrate for growth. **Gancedo, Gancedo & Sols (1968)** stated that glycerol could penetrate yeast cells by simple diffusion, unlike sugars that require a transport system to penetrate the cells. **Da Silva, Mack & Continero (2009)** also mentioned that in *S. cerevisiae*, glycerol transport is mediated by passive diffusion, and in *E. coli* the transport is controlled by facilitated diffusion. This alcohol can easily penetrate the cell membrane compared to sugars. Further, some yeasts like *Candida utilis* can grow as efficiently on glycerol as glucose **(Gancedo, Gancedo & Sols, 1968)**.

The degradation of glycerol occurs in many yeasts, being *S. cerevisiae* one example of that. There are two reported pathways of glycerol degradation, via "glycerol-3-phosphate" or via "dihydroxyacetone" **(Da Silva, Mack & Continero, 2009; Klein et al., 2017)**. The first path involves a glycerol kinase which transforms glycerol into glycerol-3-phosphate. This product can be used in glycolysis or serve as a precursor for the biosynthesis of lipids **(Da Silva, Mack & Continero, 2009; Klein et al., 2017)**. In the second pathway, glycerol is oxidated to dihydroxyacetone, latter dihydroxyacetone kinase phosphorylates the dihydroxyacetone to dihydroxyacetone phosphate **(Klein et al., 2017)**. These two processes can be seen in **Figure 2**. In **2i**, it is represented the first path mentioned, and in **2ii**, the second one.

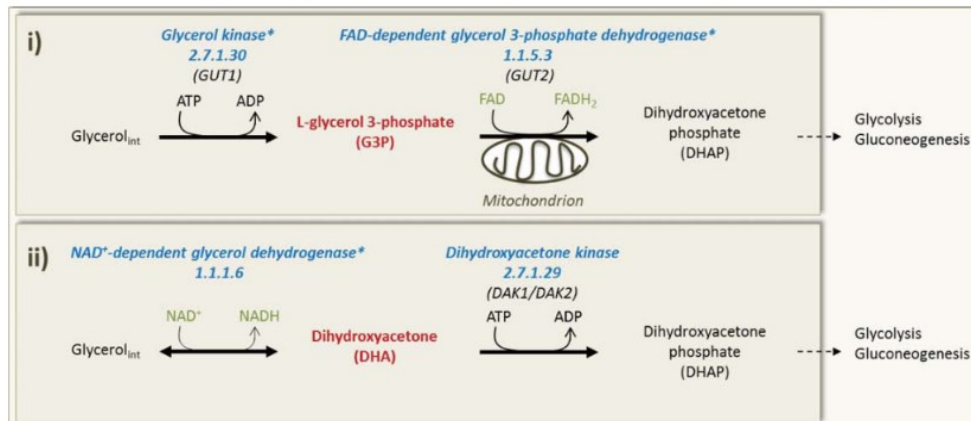


Figure 2 - Representation of the two pathways of the degradation of glycerol in yeasts. i - glycerol degradation via glycerol-3-phosphate; ii - glycerol degradation via dihydroxyacetone. (in Klein *et al.*, 2017)

Further, according to Klein *et al.* (2017), the ability of yeasts to grow in glycerol is affected by adding amino acids and other supplements.

3.3. Sodium Salts

Sodium salt can be obtained by evaporating seawater with sunlight. This kind of salt has a representative amount of mineral salt. Purified salt subsists mainly on NaCl (Lee *et al.*, 2017). With regard to microorganisms, sodium salts are known to have inhibitory properties and are used in high concentrations to select halophilic bacteria (Bonnet *et al.*, 2020). This inhibitory property can be called salt stress, which includes specific cation toxicity and osmotic stress (Goossens *et al.*, 2001).

The capacity of bacteria to grow in significant amounts of sodium chloride vary (Christian & Waltho, 1961). For example, *B. cereus* can tolerate a maximum of 7.5% of NaCl concentration; on the other hand, *E. coli* and *Salmonella* can not tolerate high concentrations of salts (Food and Drug Administration, 2012; Omotoyinbo & Omotoyinbo, 2016). When exposed to salt stress, some yeasts, such as *S. cerevisiae*, can quickly adapt (Goossens *et al.*, 2001). One mechanism proposed to explain the tolerance to high concentrations of salts was the regulation of intracellular salts concentration (Norkrans & Kylin, 1969).

3.4. Amino Acids

Amino acids are molecules comprising an α -carbon group bounded to four functional groups: hydrogen, carboxyl, amine, and a side chain (Marcone *et al.*, 2019). For microorganisms, amino acids are used to synthesise proteins; and are considered growth factors (Bonet *et al.*, 2020).

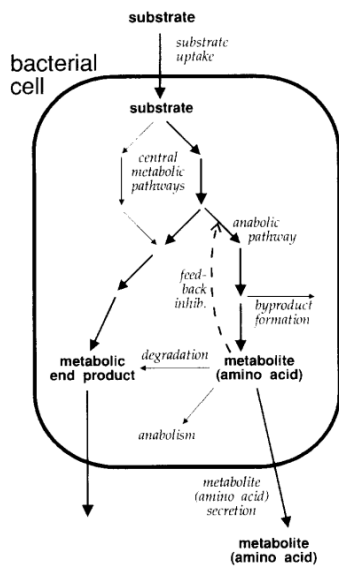


Figure 3 - Secretion of amino acids by cells. (in Krämer, 1994)

During metabolic events, the segregation of amino acids is the last step. First, the cell takes up a substrate, which begins to be transformed by one of the cell's different metabolic pathways (e.g., glycolysis). Specific biosynthetic pathways for different amino acids branch from this central metabolic branch, as shown in **Figure 3**. These pathways can lead to side reactions; for example, if the flux of a specific amino acid increases, degradation reactions can be triggered (Krämer, 1994). Finally, the amino acids cross the plasmatic membrane, and the amino acid transporters that moulds and yeasts have can be classified by their location, structure, regulation, and substrate specificity range (Krämer, 1994; McCarthy & Walsh, 2018).

Jiraneck, Langridge, and Henschke (1995) conducted a study analysing the amino acids utilisation by strains of *S. cerevisiae*.

The authors concluded that, in terms of the total amount of nitrogen required, the utilization of amino acids made a uniform contribution. Further, the most utilised amino acids were lysine, serine, arginine, aspartate, glutamate, and threonine. Lysine is an essential amino acid with a long side chain, three methyl groups, and an ϵ -amino group (Azevedo & Saiardi, 2016).

3.5. Vitamins

Vitamins are coenzymes, or their precursors, and are growth factors. There are certain vitamins that some organisms cannot synthesize and therefore need to obtain them from external sources (Bonnet *et al.*, 2020).

Vitamins from the B-group (which includes biotin, thiamine, riboflavin, pyridoxine, niacin, pantothenic acid, cobalamin, and folate) are required for microbial growth once they participate in metabolic processes such as energy production (LeBlanc *et al.*, 2015; Snell, 1948).

Koser (1948) mentioned that some moulds require vitamins for growth; for example, *Penicillium digitatum* requires thiamine and biotin is stimulatory, for *Rhizopus suinus* thiamine accelerates growth. The author also mentioned the requirements of several yeasts for thiamine, biotin, inositol, nicotinic acid, and pantothenate.

Biotin is a water-soluble vitamin, and several bacteria can synthesise it. However, several microorganisms cannot and must ingest biotin (Livaniou *et al.*, 2000). Biotin has several biological functions, some in chromatin's cell signals structure and genes' epigenetic regulations. Further, this vitamin has a vital role in carboxylases as a covalently bound coenzyme (Zempleni, Wijeratne & Hassan, 2009).

Regarding thiamine, it is a vitamin that is a cofactor for enzymes involved in carbohydrate metabolism. Many of these produce ATP, ribose-5-phosphate, and NADH, which are critical in generating cell energy and producing fatty acids, nucleic acids, and amino acids (Edwards *et al.*, 2017).

3.6. Mineral Salts

Similar to carbohydrates and amino acids, mineral salts function as a source of ions and enzyme cofactors (**Ernandes, 2006**). So, many mineral salts are essential nutrients to microorganisms. However, some are toxic. Generally, the chemical formula of the elements, which could depend on the environmental condition, determines if it is toxic to the microorganism. Some of the primary vital cations for growth are Fe^{2+} , Fe^{3+} , Mg^{2+} , Na^+ , K^+ , Zn^{2+} , Ca^{2+} , and Cu^{2+} , and anions are phosphate, sulphate, carbonate silicate, chloride, and chlorate (**Nicolau, 2014**).

For example, magnesium is a crucial mineral for yeasts like *S. cerevisiae* since it is involved in several essential functions, such as growth and cell division, “respirofermentative metabolism”, and functions and structures of mitochondria (**Birch, Ciani & Walker, 2003**).

Ernandes (2006) states that inorganic nutrients can be divided into macronutrients and micronutrients. According to the author, macronutrients include calcium, phosphorus, sodium, potassium, and magnesium, and micronutrients include iron, copper, iodine, zinc, and manganese. When used for microbiological growth, macronutrients must be used in concentrations between 0.01 g/L and 40 g/L and micronutrients between 5 mg/L and 130 mg/L.

3.7. Culture Media pH

Controlling the pH of the culture media is very important, as it can affect the growth of microorganisms and be a limiting factor (**Ernandes, 2006; Rosso et al., 1995**). Changes in the extracellular pH can affect the intracellular pH, so it is crucial to maintain an optimum intracellular pH once it regulates enzymatic reactions, calcium concentration, cell division and other cellular processes (**Gatimel et al., 2020**).

When the internal pH drops below a specific value, microorganisms die; for example, for most bacteria, if the internal pH drops below 5 to 5.5, the bacteria die. The effects of external pH extremes include the disintegration of the plasma membrane, inhibition of enzymatic activity, inhibition of membrane transporters and protein denaturation (**Nicolau, 2014**). For these reasons it is very important to guarantee that the external pH does not negatively affect microbial growth.

Ernandes (2006) stated that pH values between 6.5 to 8 have a low influence on the rate of bacteria development but can affect the total number of bacteria.

4. Microorganisms

In fruit preparations it is essential to carry out microbiological analyses, since these have a high availability of nutrients. This high availability of nutrients promotes the growth of several microorganisms. For example, as mentioned earlier fructose monosaccharide can be found in various fruits, which are rich in other nutrients, which provide the ideal conditions for yeasts to carry out fermentation.

Below, the characteristics of some of the microorganisms most commonly found in fruit preparations are described.

4.1. Bacteria

4.1.1. *Bacillus cereus*

Bacillus cereus is a facultative anaerobic and Gram-positive bacterium belonging to the *Bacillaceae* family. This microorganism can form spores and produce two toxins: diarrheal and emetic (ASAE, n.d.).

The optimum growth temperature of *B. cereus* varies between 28 to 35 °C, with a minimum growth of 4 °C and a maximum of 48 °C (Food and Drug Administration, 2012). Regarding the pH, these bacteria can grow between 5.0 to 9.3, but the optimum pH range for growth is 6.0 to 7.0. Regarding water activity, *B. cereus* can grow at minimum a_w between 0.92 and 0.95. At NaCl concentrations above 7.5%, its growth is inhibited (ASAE, n.d.; Macena, 1997).

Bacillus cereus does not ferment mannitol (Udayangani, 2021). According to Macena (1997), *B. cereus* cannot ferment lactose but can ferment sucrose in aerobic conditions. This microorganism can ferment glucose in anaerobic conditions, reduce nitrate, and break down tyrosine and citrate (Frechaut, 2014).

4.1.2. *Escherichia coli*

Escherichia coli is a Gram-negative bacterium belonging to the *Enterobacteriaceae* family and is a facultative anaerobic microorganism (Kasnowski, 2004).

E. coli can grow at the temperature ranges of 7 to 48 °C, 37 °C their optimum temperature growth, about the pH levels, this microorganism can grow in a range from 4.4 to 10, and the optimum ranges are 6 to 7. Regarding water activity, *E. coli* can grow with an a_w higher than 0.95, and as mentioned before, *E. coli* cannot tolerate high concentrations of NaCl (Kasnowski, 2004; Omotoyinbo & Omotoyinbo, 2016).

According to Kasnowski (2004), this bacterium can ferment glucose, and some strains can ferment lactose. Rossi (2001) reported that *E. coli* cannot metabolise galactose, xylose, and mannitol, and some strains require their growth medium to be given increased supplementation with thiamine (B1 vitamin) and amino acids.

4.1.3. *Leuconostoc mesenteroides*

Leuconostoc mesenteroides belongs to the lactic acid bacteria group. It is a Gram-positive microorganism, and is described as a facultative anaerobic microorganism non-spore-forming, mesophile, and immobile (Severo, 2018; Lonvaud-Funel, 1999). Mesophilic bacteria grow in moderate temperatures, typically between 20-45 °C (Wiley, Sherwood & Woolverton, 2008).

According to Lonvaud-Funel (1999), *L. mesenteroides* can ferment glucose, lactose, maltose, and fructose, and some strains can also ferment mannitol and sucrose.

4.1.4. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa belongs to the *Pseudomonadaceae* family and is a Gram-negative bacterium (Wiehlmann *et al.*, 2007; Todar, 2020). This microorganism is capable of growing at various temperatures, ranging from 25 to 55 °C, and across a pH range of 6.5 to 8.5 (Das and Mukherjee, 2007). However, the optimum temperature for growth is 37 °C (Todar, 2020).

Pseudomonas aeruginosa has straightforward nutritional requirements, which can be easily seen as they often grow in streams of water. This microorganism can use sulphate as a nitrogen source and, as a carbon source, can use acetate (Todar, 2020).

Besides that, this bacterium can resist to various temperatures and physical conditions and is also tolerant to high salt concentrations and many antibiotics (Todar, 2020).

4.2. Yeasts

4.2.1. *Candida intermedia*

Candida intermedia is a yeast with optimum growth temperature between 20 and 30 °C and growing better in neutral pH. However, it can grow over various pH values (UCDAVIS Viticulture & Enology, n.d.).

Candida intermedia can ferment sugars like glucose, sucrose, and galactose and assimilate galactose, maltose, xylose, and mannitol. However, *C. intermedia* cannot grow in media free of vitamins, and this yeast requires biotin to grow (UCDAVIS Viticulture & Enology, n.d.).

4.2.2. *Pichia fermentans*

Pichia fermentans are noted as moderate ethanol and low acetic acid producers (Vicente *et al.*, 2021).

This yeast has optimum growth temperature between 24-27 °C and cannot grow above 40 °C (Salvadó i Belart, 2013). Prabhu *et al.* (2020) stated that *P. fermentans* presented a good pH tolerance and showed that the microorganism can grow between the pH levels of 4.5 to 7.0.

Pichia fermentans is a yeast fermenting glucose and assimilating glycerol, xylose, and ethanol. *Pichia fermentans* cannot grow in media free of vitamins, requires thiamine, and some strains require biotin (UCDAVIS Viticulture & Enology, n.d.).

4.2.3. *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a facultative aerobic yeast (Paixão, 2018; Salari & Salari, 2017). This microorganism has a minimum water activity of 0.89 (Santin, 1996). The yeast has optimum pH values between 4 and 6 (Narendranath and Power, 2005).

The yeast can ferment glucose and fructose (Santin, 1996). According to Paixão (2018), the central carbon compounds used by *S. cerevisiae* are monosaccharides (fructose, glucose, and galactose) and disaccharides (sucrose and maltose). Furthermore, the author stated that the yeast, for industrial processes, needs the culture medium to contain a nitrogen source, vitamins, and mineral salts.

In addition, **Salari and Salari (2017)** noted that all strains of *S. cerevisiae*, in order to grow, need phosphorus and nitrogen sources, and for optimum growth, the yeast needs various metals (like magnesium) and sulphur.

4.3. Molds

4.3.1. *Neosartorya fischeri*

Neosartorya fischeri are heat-resistant moulds that produce ascospores and mycotoxins (**Nielsen, Beuchat & Frisvad, 1988; Leal, 2007**). This mould can grow between 10 to 52 °C, and the optimum temperature for growth is around 26-45 °C (**Nielsen, Beuchat & Frisvad, 1988**). Regarding the pH, *N. fischeri* can grow between 3 to 7.95 (**Tournas, 1994**). Concerning water activity, *N. fischeri* has a minimum water activity for growth of 0.915 (**Leal, 2007**).

According to **Nielsen, Beuchat, and Frisvad (1988)**, adding glucose, sucrose, and fructose intensified the production of fumitremorgin, one of the mycotoxins produced by this mould.

Work Objectives

The main objective of this study is to develop an optimum culture media for the growth of yeasts, bacteria and moulds. These culture media intend to mimic the fruit preparations and guarantee that if the samples are contaminated, the microorganism will grow in the media and grow faster and more prominent to quickly identify the microorganism.

For this, adding sugars, alcohols, sodium salt, amino acids, vitamins and mineral salts in simple media will be tested. In the first step of the study, will be added one nutrient alone to see if that nutrient improves the size of the colonies formed by the different microorganisms. In the study, the carbon sources tested will be glucose, fructose, sucrose, maltose and xylose. The first three carbon sources will be tested in three different concentrations: 20 g/L, 40 g/L and 60 g/L, for maltose and xylose the concentration that will be tested will be 20 g/L. Glycerol will be added to culture media to only grow yeasts to perceive if their growth is affected and, if so, how it is affected, and it will be used in 3 different concentrations: 2 g/L, 5 g/L, and 10 g/L. About sodium salt, it will be used to understand whether low NaCl concentrations positively affect microorganisms' growth. About the amino acids, lysine will be used in two concentrations, 5 mg/L and 0.5 mg/L, to see if positively affects microbial growth. Regarding vitamins, in this study it will be tested the addition of biotin and thiamine, as previously mentioned, belonging to the B-group of vitamins, in culture media for the growth of yeasts and moulds. Finally, the study will use calcium, sodium chloride, magnesium, and potassium in two concentrations, 10 g/L and 1 g/L, also, will be tested zinc and iron using a concentration of 0.1 g/L, to see if the addition of mineral salts affects the growth of the microorganisms.

For the second test, will be performed a confirmation, three inoculums of each microorganism will be plated in the media with the best results. Finally, will be added a mix of the different compounds in the simple media will be tested according to the results obtained. For this second step, the pH will also be adjusted to favour the growth of the microorganism.

The microorganism that will be used for the study, will be the previously mentioned: *Bacillus cereus*, *Escherichia coli*, *Leuconostoc mesenteroides*, *Pseudomonas aeruginosa*, *Candida intermedia*, *Pichia fermentans*, *Saccharomyces cerevisiae* and *Neosartorya fischeri*.

Material and Methods

1. Preparation of Culture Media

1.1. Preparation of Culture Media for Yeasts and Molds

1.1.1. Preparation of the Control Culture Media

21 g of potato dextrose agar (PDA) (Infusion from potatoes 200.0 g/L; Dextrose 20.0 g/L; Agar 17.0 g/L; pH 5.6 ± 0.2 at 25 °C) and 500 g of distilled water were added to a 500 ml glass flask. The mixture was then sterilised in an autoclave (Panasonic, MLS-3781L) at 125°C for 25 minutes.

1.1.2. Preparation of the Culture Media With the addition of Sugars

The following components were weighed as indicated on **Table 1** and placed in a 500 ml flask:

Table 1 - Preparation of the Culture Media for yeasts and molds with the addition of Sugars.

	PDA (g)	Glucose (g)	Sucrose (g)	Fructose (g)	Maltose (g)	Xylose (g)	Water (g)
<i>PDA_GLU</i>	21	10	-	-	-	-	500
<i>PDA_GLU_4</i>	21	20	-	-	-	-	500
<i>PDA_GLU_6</i>	21	30	-	-	-	-	500
<i>PDA_SAC</i>	21	-	10	-	-	-	500
<i>PDA_SAC_4</i>	21	-	20	-	-	-	500
<i>PDA_SAC_6</i>	21	-	30	-	-	-	500
<i>PDA_FRU</i>	21	-	-	10	-	-	500
<i>PDA_FRU_4</i>	21	-	-	20	-	-	500
<i>PDA_FRU_6</i>	21	-	-	30	-	-	500
<i>PDA_MAL</i>	21	-	-	-	10	-	500
<i>PDA_XYL</i>	21	-	-	-	-	10	500

Each mixture was then sterilised in an autoclave (Panasonic, MLS-3781L) at 125 °C for 25 minutes.

1.1.3. Preparation of the Culture Media With the addition of Alcohols

The following components were weighed as indicated on **Table 2** and placed in a 500 ml flask:

Table 2 - Preparation of the Culture Media for yeasts and molds with the addition of Alcohol.

	PDA (g)	Glycerol (g)	Water (g)
<i>PDA</i>	21	-	500
<i>PDA_GLY_0,2</i>	21	1	500
<i>PDA_GLY_0,5</i>	21	2.5	500
<i>PDA_GLY_1</i>	21	5	500

Each mixture was then sterilised in an autoclave (Panasonic, MLS-3781L) at 125 °C for 25 minutes.

1.1.4. Preparation of the Culture Media With the addition of Vitamins

PDA_BIO: To glass flask of 500 mL weight 21 g of PDA, 0.01 g of biotin and 500 g of distilled water. Then the mixture was sterilised using an autoclave (Panasonic, MLS-3781L); the temperature and sterilisation time were programmed to 125°C and 25 minutes.

PDA_THI: To glass flask of 490 mL weight 21 g of PDA and 500 g of distilled water. Then the mixture was sterilised using an autoclave (Panasonic, MLS-3781L); the temperature and sterilisation time were programmed to 125°C and 25 minutes. Before using the media and after melting it, mix 0.002 g of thiamine in a vertical air chamber in 10 mL of distilled water, then, with the help of a syringe and a disposable membrane filter unit (25 mm, with a pore size of 0.20 µm, from Frilabo), pour the mix into the glass flask.

1.1.5. Preparation of the Culture Media With the addition of Proteins

PDA_LYS_0,5: To glass flask of 490 mL weight 21 g of PDA and 500 g of distilled water. Then the mixture was sterilised using an autoclave (Panasonic, MLS-3781L); the temperature and sterilisation time were programmed to 125°C and 25 minutes. Before using the media and after melting it, in a vertical air chamber, mix 0.25 g of lysine in 10 mL of distilled water, then, with the help of a syringe and a disposable membrane filter unit (25 mm, with a pore size of 0.20 µm, from Frilabo), pour the mix in the glass flask.

PDA_LYS_1: To glass flask of 490 mL weight 21 g of PDA and 500 g of distilled water. Then the mixture was sterilised using an autoclave (Panasonic, MLS-3781L); the temperature and sterilisation time were programmed to 125°C and 25 minutes. Before using the media and after melting it in a vertical air chamber, mix 0.5 g of lysine in 10 mL of distilled water, then, with the help of a syringe and a disposable membrane filter unit (25 mm, with a pore size of 0.20 µm, from Frilabo), pour the mix in the glass flask.

1.1.6. Preparation of the Culture Media With the addition of Mineral Salts

The following components were weighed as indicated on **Table 3** and placed in a 500 ml flask:

Table 3 - Preparation of the Culture Media for yeasts and molds with the addition of Mineral Salts.

	PDA (g)	NaCl (g)	Calcium (g)	Magnesium (g)	Potassium (g)	Iron III (g)	Zinc (g)	Water (g)
<i>PDA_NaCl_0,1</i>	21	0,5	-	-	-	-	-	500
<i>PDA_NaCl_1</i>	21	5	-	-	-	-	-	500
<i>PDA_Ca_0,1</i>	21	-	0.5	-	-	-	-	500
<i>PDA_Ca_1</i>	21	-	5	-	-	-	-	500
<i>PDA_Mg_0,1</i>	21	-	-	0.5	-	-	-	500
<i>PDA_Mg_1</i>	21	-	-	5	-	-	-	500
<i>PDA_P_0,5</i>	21	-	-	-	0.5	-	-	500
<i>PDA_P_1</i>	21	-	-	-	5	-	-	500
<i>PDA_Fe</i>	21	-	-	-	-	0.05	-	500
<i>PDA_Zn</i>	21	-	-	-	-	-	0.05	500

Each mixture was then sterilised in an autoclave (Panasonic, MLS-3781L) at 125 °C for 25 minutes.

1.2. Preparation of Culture Media for Bacteria

1.2.1. Preparation of the Control Culture Media

14 g of Nutrient Agar (NA) (Meat Extract 1.0 g/L Yeast Extract 2.0 g/L Peptone 5.0 g/L; Sodium Chloride 5.0 g/L; Agar 15.0 g/L; pH 7.4 ± 0.2 at 25 °C) and 500 g of distilled water were added to a 500 ml glass flask. The mixture was then sterilised in an autoclave (Panasonic, MLS-3781L) at 125 °C for 25 minutes.

1.2.2. Preparation of the Culture Media With the addition of Sugars

The following components were weighed as indicated on **Table 4** and placed in a 500 ml flask:

Table 4 - Preparation of the Culture Media for bacteria with the addition of Sugars.

	NA (g)	Glucose (g)	Sucrose (g)	Fructose (g)	Maltose (g)	Xylose (g)	Water (g)
NA_GLU	14	10	-	-	-	-	500
NA_GLU_4	14	20	-	-	-	-	500
NA_GLU_6	14	30	-	-	-	-	500
NA_SAC	14	-	10	-	-	-	500
NA_SAC_4	14	-	20	-	-	-	500
NA_SAC_6	14	-	30	-	-	-	500
NA_FRU	14	-	-	10	-	-	500
NA_FRU_4	14	-	-	20	-	-	500
NA_FRU_6	14	-	-	30	-	-	500
NA_MAL	14	-	-	-	10	-	500
NA_XYL	14	-	-	-	-	10	500

Each mixture was then sterilised in an autoclave (Panasonic, MLS-3781L) at 125 °C for 25 minutes.

1.2.3. Preparation of the Culture Media With the addition of Proteins

PDA_LYS_0,5: To glass flask of 490 mL weight 14 g of NA and 500 g of distilled water. Then the mixture was sterilised using an autoclave (Panasonic, MLS-3781L); the temperature and time of sterilization were programmed to 125°C and 25 minutes. Before using the media and after melting it, in a vertical air chamber, mix 0.25 g of lysine in 10 mL of distilled water, then, with the help of a syringe and a disposable membrane filter unit (25 mm, with a pore size of 0.20 µm, from Frilabo), pour the mix in the glass flask.

PDA_LYS_1: To glass flask of 490 mL weight 21 g of NA and 500 g of distilled water. Then the mixture was sterilised using an autoclave (Panasonic, MLS-3781L); the temperature and time of sterilization were programmed to 125°C and 25 minutes. Before using the media and after melting it, in a vertical air

chamber, mix 0.5 g of lysine in 10 mL of distilled water, then, with the help of a syringe and a disposable membrane filter unit (25 mm, with a pore size of 0.20 µm, from Frilabo), pour the mix in the glass flask.

1.2.4. Preparation of the Culture Media With the addition of Mineral Salts

The following components were weighed as indicated on **Table 5** and placed in a 500 ml flask:

Table 5 - Preparation of the Culture Media for bacteria with the addition of Mineral Salts.

	NA (g)	NaCl (g)	Calcium (g)	Magnesium (g)	Potassium (g)	Iron III (g)	Zinc (g)	Water (g)
<i>NA_NaCl_0,1</i>	14	0.5	-	-	-	-	-	500
<i>NA_NaCl_1</i>	14	5	-	-	-	-	-	500
<i>NA_Ca_0,1</i>	14	-	0.5	-	-	-	-	500
<i>NA_Ca_1</i>	14	-	5	-	-	-	-	500
<i>NA_Mg_0,1</i>	14	-	-	0.5	-	-	-	500
<i>NA_Mg_1</i>	14	-	-	5	-	-	-	500
<i>NA_P_0,5</i>	14	-	-	-	0.5	-	-	500
<i>NA_P_1</i>	14	-	-	-	5	-	-	500
<i>NA_Fe</i>	14	-	-	-	-	0.05	-	500
<i>NA_Zn</i>	14	-	-	-	-	-	0.05	500

Each mixture was then sterilised in an autoclave (Panasonic, MLS-3781L) at 125 °C for 25 minutes.

2. Preparation of Culture Media: Synergism

2.1. Preparation of Culture Media for Yeasts

2.1.1. Preparation of the Control Culture Media

21 g of potato dextrose agar (PDA) (Infusion from potatoes 200.0 g/L; Dextrose 20.0 g/L; Agar 17.0 g/L; pH 5.6 ± 0.2 at 25 °C) and 500 g of distilled water were added to a 500 ml glass flask. The mixture was then sterilised in an autoclave (Panasonic, MLS-3781L) at 125°C for 25 minutes.

2.1.2. Preparation of the Culture Media

The following components were weighed as indicated on **Table 6** and placed in a 500 ml flask:

Table 6 - Preparation of the Culture Media for yeasts with the addition of sugars, protein, vitamins and mineral salts to test the synergism between them.

	PDA (g)	GLU (g)	SAC (g)	FRU (g)	BIO (g)	Mg (g)	P (g)	Fe (g)	Water (g)
<i>401</i>	21	20	-	-	0.01	5	-	0.05	490
<i>402</i>	21	20	-	-	0.01	-	0.5	0.05	490
<i>501</i>	21	-	20	-	0.01	5	-	0.05	490
<i>502</i>	21	-	20	-	0.01	-	0.5	0.05	490
<i>601</i>	21	-	-	20	0.01	5	-	0.05	490

602	21	-	-	20	0.01	-	0.5	0.05	490
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The pH of each medium was measured using a potentiometer and was adjusted to a pH of 6 using sodium ascorbate.

Each mixture was then sterilised in an autoclave (Panasonic, MLS-3781L) at 125 °C for 25 minutes. After melting, in a vertical chamber and before use, each medium was supplemented with a solution containing 0.002 g thiamine and 0.25 g lysine in 10 ml distilled water, with a syringe and a disposable membrane filter unit (25 mm, with a pore size of 0.20 µm, from Frilabo), the solution was poured to the mediums.

2.2. Preparation of Culture Media for Molds

2.2.1. Preparation of the Control Culture Media

21 g of potato dextrose agar (PDA) (Infusion from potatoes 200.0 g/L; Dextrose 20.0 g/L; Agar 17.0 g/L; pH 5.6 ± 0.2 at 25 °C) and 500 g of distilled water were added to a 500 ml glass flask. The mixture was then sterilised in an autoclave (Panasonic, MLS-3781L) at 125°C for 25 minutes.

2.2.2. Preparation of the Culture Media

The following components were weighed as indicated on **Table 7** and placed in a 500 ml flask:

Table 7 - Preparation of the Culture Media for molds with the addition of sugars, protein, vitamins and mineral salts to test the synergism between them.

	PDA (g)	GLU (g)	SAC (g)	FRU (g)	BIO (g)	NaCl (g)	Mg (g)	P (g)	Water (g)
701	21	30	-	-	0.01	0.5	5	0.5	500
702	21	30	-	-	0.01	-	5	0.5	500
801	21	-	30	-	0.01	0.5	5	0.5	500
802	21	-	30	-	0.01	-	5	0.5	500
901	21	-	-	20	0.01	0.5	5	0.5	500
902	21	-	-	20	0.01	-	5	0.5	500

The pH of each medium was measured using a potentiometer and was adjusted to a pH of 6 using sodium ascorbate.

The mixtures were then sterilised in an autoclave (Panasonic, MLS-3781L) at 125 °C for 25 minutes.

2.3. Preparation of Culture Media for Bacteria

2.3.1. Preparation of the Control Culture Media

14 g of Nutrient Agar (NA) (Meat Extract 1.0 g/L Yeast Extract 2.0 g/L Peptone 5.0 g/L; Sodium Chloride 5.0 g/L; Agar 15.0 g/L; pH 7.4 ± 0.2 at 25 °C) and 500 g of distilled water were added to a 500 ml glass flask. The mixture was then sterilised in an autoclave (Panasonic, MLS-3781L) at 125°C for 25 minutes.

2.3.2. Preparation of the Culture Media

The following components were weighed as indicated on **Table 8** and placed in a 500 ml flask:

Table 8 - Preparation of the Culture Media for bacteria with the addition of sugars, protein and mineral salts to test the synergism between them.

	NA (g)	SAC (g)	Mg (g)	Fe(g)	Water (g)
201	14	20	5	-	490
202	14	20	-	0.05	490
203	14	20	5	0.05	490

The pH of each medium was measured using a potentiometer and was adjusted to a pH of 7 using sodium ascorbate.

The mixtures were then sterilised in an autoclave (Panasonic, MLS-3781L) at 125 °C for 25 minutes.

After melting in a vertical chamber and before use, each medium was supplemented with a solution containing 0.25 g lysine in 10 ml distilled water, with a syringe and a disposable membrane filter unit (25 mm, with a pore size of 0.20 µm, from Frilabo), the solution was poured to the mediums.

3. Microorganisms Plating

3.1. Microbial Cultures

In the study, the microbial cultures of *Escherichia coli*, *Pseudomonas aeruginosa* and *Leuconostoc mesenteroides* used were provided by Escola Superior de Biotecnologia da Universidade Católica Portuguesa, Centro Regional do Porto. The microbial cultures of *Bacillus cereus*, *Saccharomyces cerevisiae*, *Pichia fermentans*, *Candida intermedia* and *Neosartotya fischeri* were provided by Frulact.

3.2. Inoculum Preparation

20 g of Buffered Peptone Water (BPW) and 500 g of distilled water were added to a 500 ml glass flask. The mixture was then sterilised in an autoclave (Panasonic, MLS-3781L) at 125 °C for 25 minutes.

Using a loop and flame, the culture of each selected microorganism was transferred from the Frulact's culture collection into a glass flask containing 450 mL of pre-prepared buffered peptone water (BPW). The flask was then placed in an incubator at 30 °C and allowed to incubate for three days, except for *L. mesenteroides*. In the case of *L. mesenteroides*, the glass flask was opened and placed in an airtight plastic box under anaerobic conditions at 30 °C for 3 days.

3.3. Yeasts, Moulds, and *Pseudomonas aeruginosa* Plating

The previously prepared culture media were melted and poured into Petri dishes. After the media solidified, the Petri dishes were correctly marked with the microorganism and the corresponding media.

The inoculum was transferred from the incubator to the laminar flow cabinet, the content was transferred to 10 Falcon tubes of 50 mL and centrifuged (Hettich, ROTOFIX 32 A) at 40 RPM/RCPx100 for 10 minutes. Afterwards, the supernatant liquid was placed again in the flask of 500 mL where previously the inoculum was, leaving in each Falcon tube 10 mL. The remaining liquid, about 10 mL in each Falcon tube, was mixed and placed in 2 Falcon tubes. These two tubes were placed in a water bath at 40 °C for 5 minutes.

After that, the content of the two tubes was placed in a plastic flask of 100 mL, and the dilutions were carried out. With a micropipette, 1 mL was taken from the plastic flask of 100 mL and placed on a test tube, and the content was mixed. This step was repeated until the desired dilution was reached. *Candida intermedia* required six dilutions, for *P. fermentans* five, *S. cerevisiae* four, *N. fischeri* five, and *P. aeruginosa* seven.

After performing the dilutions with a micropipette, 0.2 mL of each dilution was added to separate Petri dishes, except for *P. aeruginosa*, which received 0.1 mL of each dilution. Then, with a spreader, the liquid was spread in the media. Lastly, all the Petri dishes were incubated at 30 °C for three days.

3.4. Bacteria Plating

Petri dishes were identified correctly with the microorganism and the proper media.

The inoculum was brought from the incubator to the laminar flow cabinet and its content was placed into 10 Falcon tubes of 50 mL. Then the Falcon tubes were placed in a centrifuge (Hettich, ROTOFIX 32 A) at 40 RPM/RCPx100 and 10 minutes. The supernatant was then transferred back into the 500 mL flask in which the inoculum had previously been placed. The remaining liquid, about 10 mL in each Falcon tube, was mixed and placed in two Falcon tubes of 50 mL. These two tubes were placed in a water bath at 40 °C for 5 min.

After that, the content of the two tubes was placed in a plastic flask of 100 mL and the dilutions were carried out. With a micropipette, 1 mL was taken from the plastic flask of 100 mL and placed in a test tube and the content was mixed. This step was repeated until the desired dilution was reached. For *B. cereus*, seven dilutions were required; for *E. coli* eight; and for *L. mesenteroides* seven.

Following the micropipette dilutions, 1 mL of each dilution was transferred to individual Petri dishes and the appropriate agar medium indicated on each Petri dish was added. To ensure thorough mixing, all Petri dishes were gently swirled using an '8' motion to distribute the contents evenly. After the culture media solidified, the dishes were incubated at 30 °C for 3 days, except for *L. mesenteroides*. For *L. mesenteroides*, the Petri dishes were instead put inside a sealed plastic container with anaerobic conditions maintained and kept at 30 °C for 3 days.

4. Qualitative Analysis

The qualitative analysis was performed by comparing the sizes of the colonies that developed on the simple media (PDA and NA) with the sizes of the colonies that developed on the media with different components added.

Example of a positive impact on cell growth:

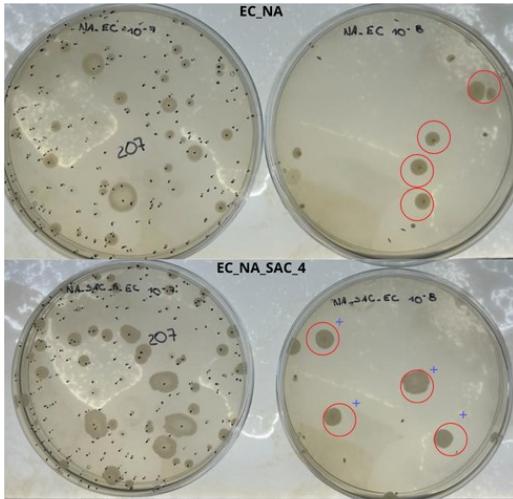


Figure 4 - An example of a positive impact on the growth of *E. coli* when grown in NA_SAC_4, compared to NA.

As it is possible to see in **Figure 4**, when comparing the occupied area by the colonies in the red circles on both media, the area higher in the medium NA_SAC_4, meaning that the colony has a higher number of cells or the cells are bigger.

So, when comparing both media, it was considered that the NA_SAC_4 had a positive impact in the cell growth.

Example of a negative impact on cell growth:

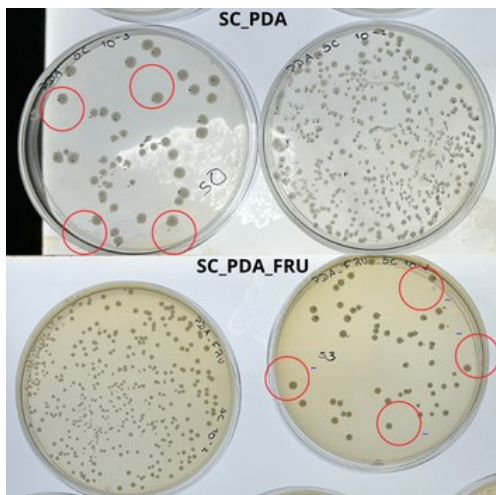


Figure 5 - An example of a negative impact on the growth of *S. cerevisiae* in PDA_FRU compared to PDA.

As it is possible to see in the **Figure 5**, when comparing the occupied area by the colonies in the red circles in both media, the area is smaller in the medium PDA_FRU, which means that a smaller number of cells were able to grow or the cells grew in a smaller size.

So, when comparing both media, it was considered that the PDA_FRU had a negative impact in the cell growth.

Example of no impact on cell growth:

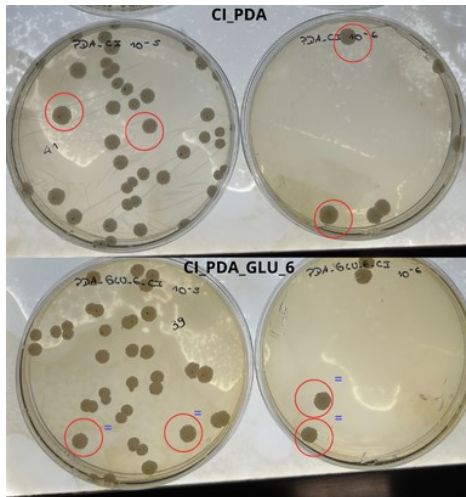


Figure 6 - An example of no impact on the growth of *C. intermedia*, when grown in PDA_GLU_6 compared to PDA.

As it is possible to see in the **Figure 6**, when comparing the occupied area by the colonies in the red circles in both media, the area is equal.

So, when comparing both media, it was considered that the PDA_GLU_6 did not result in an improved growth.

5. Statistical Analysis

To perform the data analysis was used IBM SPSS Statistics.

First, the normality of the data was tested using the Shapiro-Wilk test with a confidence level of 95%. Then it was used the ANOVA-ONE WAY to compare the means, also with a confidence level of 95%, were used POST HOC Multiple Comparison tests of LSD, Bonferroni and Tukey to compare the means between all the media.

If the data did not follow normality, various transformations were tested, such as the base 10 logarithm, the inverse, and the Napierian logarithm. If the data still did not follow the normality, the Mann-Whitney test was used instead of the ONE-WAY ANOVA.

Results and Discussion

1. Phase 1: Overview

To determine the compounds that would benefit microbial growth when added to a culture medium, various compounds were tested. The different sugars, proteins, and minerals were tested for all the microorganisms, and the vitamins and the alcohol were tested only for the yeasts.

After these were tested, a qualitative analysis was made to verify the morphology of the different microbial colonies.

For this study five different sugars were tested: glucose, fructose, sucrose, xylose, and maltose. The first three sugars were tested in three different concentrations in the media: 20 g/L, 40 g/L, and 60 g/L; the other two sugars were tested in only one concentration: 20 g/L. The results from the qualitative analysis are shown in **Table 9**.

Table 9 - Results from microorganisms' growth in NA (for bacteria) and PDA (for yeast and moulds) with the addition of different sugars.

	BC	EC	PA	LM	SC	CI	PF	NF
GLU	-	+	-	+	-	-	-	=
GLU_4	-	+	-	+	=/+	+	+	+
GLU_6	-	=	-	+	+	=	+	+
SAC	=	+	+	+	-	-	-	=
SAC_4	+	+	+	+	=	+	+	=
SAC_6	=	+	=/+	+	=	=	+	+
FRU	=	=/+	-	+	-	-	-	+
FRU_4	=/-	-	-	+	=	=/+	+	+
FRU_6	X	-	X	+	=	+	=	=/+
XYL	X	X	X	+	=	=	+	-
MAL	=/-	=/+	=	+	=	=	=	=

- the microbial colonies' size was smaller when compared with NA; =: the microbial colonies' size was equal when compared with NA; +: the microbial colonies' size was better when compared with NA; X: no microbial growth occurred.

BC: *B. cereus*; **EC:** *E. coli*; **PA:** *P. aeruginosa*; **LM:** *L. mesenteroides*; **SC:** *S. cerevisiae*; **CI:** *C. intermedia*; **PF:** *P. fermentans*; **NF:** *N. fischeri*.

GLU: Glucose at 20 g/L; **GLU_4:** Glucose at 40 g/L; **GLU_6:** Glucose at 60 g/L; **SAC:** Sucrose at 20 g/L; **SAC_4:** Sucrose at 40 g/L; **SAC_6:** Sucrose at 60 g/L; **FRU:** Fructose at 20 g/L; **FRU_4:** Fructose at 40 g/L; **FRU_6:** Fructose at 60 g/L; **XYL:** Xylose at 20 g/L; **MAL:** Maltose at 20 g/L.

Regarding the impact of glucose on the size of bacterial colonies, it is evident from **Table 9** that both glucose concentrations, 20 g/L and 40 g/L, yielded identical results. Thus, the first concentration was chosen for the next step in bacteria because it spent fewer resources. Concerning the yeast, better results were shown for the concentrations of 40 g/L, which is why this was the concentration chosen to continue. Respecting the mould, the concentration chosen to continue the study was the highest because it was the one that conducted to better results.

Concerning sucrose, for bacteria, this was the sugar that presented the better result, as it is possible to see in **Table 9**. As it is possible to see in **Figure 7**, all the colonies from the different bacteria were larger in size when sucrose was added to the medium, and the concentration chosen for the next step was the lowest for the same reason as glucose. The same that happened with glucose for the yeast occurred to sucrose, and the concentration that will be used is 40 g/L. For *N. fischeri*, the best result appears to be with the highest sucrose concentration. Therefore, this will be the concentration used for the mould. One of the reasons sucrose conducted to good results is probably because this sugar is the

most abundant disaccharide worldwide and probably because of its beneficial effect on cells, such as protection from oxidation, heat and acids (Lee *et al.*, 2010). And this beneficial effect probably played an important role in the cells growth after they had been exposed to heat in a water bath at 40 °C for 5 minutes.

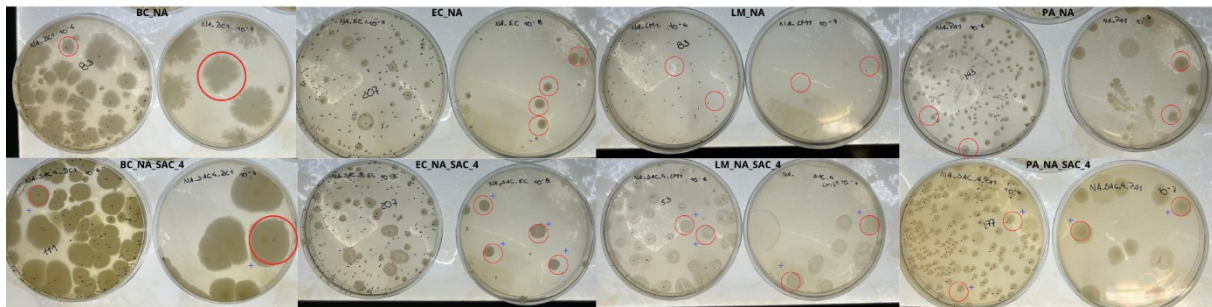


Figure 7 - Comparison of the bacterial growth between Nutrient Agar and Nutrient Agar with 40 g/L of Sucrose. BC_NA: *B. cereus* on Nutrient Agar; BC_NA_SAC_4: *B. cereus* on Nutrient Agar with 40 g/L of sucrose; EC_NA: *E. coli* on Nutrient Agar; EC_NA_SAC_4: *E. coli* on Nutrient Agar with 40 g/L of sucrose; LM_NA: *L. mesenteroides* on Nutrient Agar; LM_NA_SAC_4: *L. mesenteroides* on Nutrient Agar with 40 g/L of sucrose; PA_NA: *P. aeruginosa* on Nutrient Agar; PA_NA_SAC_4: *P. aeruginosa* on Nutrient Agar with 40 g/L of sucrose.

With regard to the effect of fructose on bacteria, at concentrations of 20 g/L and 40 g/L, the only microorganism for which a positive effect was observed was *L. mesenteroides*; for the others, the results were similar to those obtained in the NA. For the concentration of 60 g/L of fructose, only *L. mesenteroides* and *E. coli* could grow. However, the size of *E. coli* colonies was negatively affected by the high concentration of fructose in the medium, but for the *L. mesenteroides*, the opposite was observed. Therefore, fructose was not chosen to continue to be used in the media for bacteria. When cultivated in a medium containing 40 g/L of fructose, similar to the results observed in the presence of glucose and fructose, positive results were obtained with yeasts. Therefore, this concentration will continue to be used in the media for yeasts. For the mould, both 20 g/L and 40 g/L fructose concentrations presented positive results, so to decide which concentration would be chosen for the second phase, these results were compared with the results obtained for the other microorganism, and between the two. The 40 g/L of fructose was the one which showed better results.

Xylose was the sugar with the worst results. Regarding the bacteria, it is possible to see that only *L. mesenteroides* could grow in the presence of xylose in the medium, and the size of its colonies was positively affected by the sugar. This may be because, despite xylose being the second most abundant sugar in nature, only a small percentage of microorganisms possess the metabolic pathway of xylose (Zhao *et al.*, 2020). For moulds and yeast, all of them grew in the presence of xylose. However, positive results were only observed for *P. fermentans*. This may be explained by the fact that most fermenting microorganisms cannot efficiently use xylose as a carbon source. For these reasons, xylose was not used to continue the study.

In relation to maltose, as can be seen in **Table 9**, only one microorganism was positively affected, with the others showing equal results when comparing growth on NA or PDA. This was considered insufficient to continue using maltose in the media' preparations.

Has mentioned before, *P. fermentans* can assimilate glycerol, so this alcohol was tested to see if it positively affected the growth of the yeasts. Three different concentrations were tested (2 g/L, 5 g/L,

and 10 g/L) to see how the yeast growth and the colony size were affected. The results are shown in **Table 10**.

Table 10 - Results from yeast growth in PDA with the addition of different glycerol concentrations.

	SC	CI	PF
GLI_0,2	=	-	=
GLI_0,5	=	-	-
GLI_1	-	-	=

- the microbial colonies' size was smaller when compared with NA; =: the microbial colonies' size was equal when compared with NA; +: the microbial colonies' size was better when compared with NA; X: no microbial growth occurred.

SC: *S. cerevisiae*; CI: *C. intermedia*; PF: *P. fermentans*;

GLI_0,2: Glycose at 2 g/L; GLI_0,5: Glycose at 5 g/L; GLI_1: Glycose at 10 g/L.

Is it possible to see in **Table 10** that none of the tested glycerol concentrations positively affected the colony size of the different yeasts. Therefore, the utilisation of glycerol in the culture media was not pursued further in the study. Although glycerol could be used as a carbon source, some yeasts, like *S. cerevisiae*, grow poorly (Klein *et al.*, 2016) and this is probably the reason for not obtaining favourable results.

As mentioned before, *C. intermedia* cannot grow in media free of vitamins; this yeast requires biotin to grow; *P. fermentans* cannot grow in media free of vitamins, requires thiamine, and some strains require biotin to grow (UCDAVIS Viticulture & Enology, n.d.). So, two different vitamins, biotin and thiamine, were tested to see if they positively affected yeast and mould growth. As previously mentioned, only thiamine was added after media sterilisation because it has already been reported that biotin can resist pasteurisation. At the same time, thiamine losses in thermal processing are significant (Sucupira, Xerez & de Sousa, 2012). Because of that, thiamine was only added to the media after sterilization. For biotin, the concentration was 0.02 g/L, and for thiamine was 0.004 g/L. The results are presented in the **Table 11**.

Table 11 - Results from yeast and mold growth in PDA with the addition of biotin and thiamine.

	SC	CI	PF	NF
BIO	+	+	+	=
THI	+	+	+	=

- the microbial colonies' size was smaller when compared with NA; =: the microbial colonies' size was equal when compared with NA; +: the microbial colonies' size was better when compared with NA; X: no microbial growth occurred.

SC: *S. cerevisiae*; CI: *C. intermedia*; PF: *P. fermentans*; NF: *N. fischeri*.

BIO: Biotin at 0.02 g/L; THI: Thiamine at 0.004 g/L.

As expected, higher colonies were observed for *S. cerevisiae*, *C. intermedia* and *P. fermentans* when the vitamins were added to the media, as it is possible to confirm in **Figure 8**. Both of the vitamins tested were used in the next step of the study.

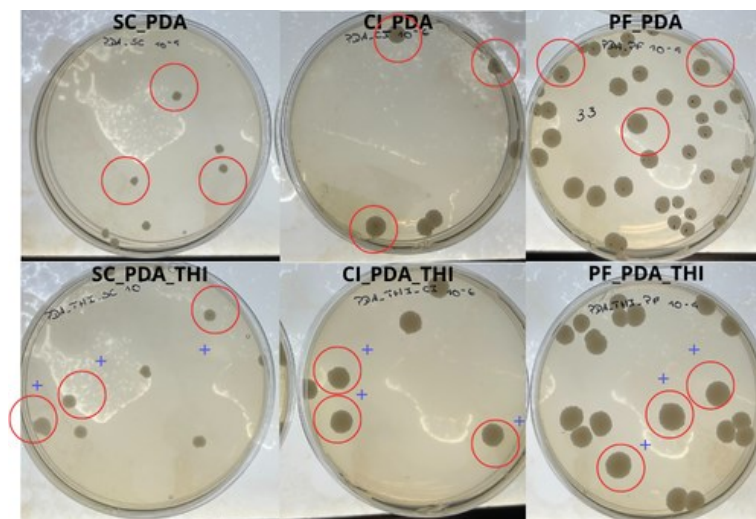


Figure 9 - Comparison of the yeast growth between PDA and PDA with thiamine. SC_PDA: *S. cerevisiae* on PDA; SC_PDA_THI: *S. cerevisiae* on PDA with thiamine; CI_PDA: *C. intermedia* on PDA; CI_PDA_THI: *C. intermedia* on PDA with thiamine; PF_PDA: *P. fermentans* on PDA; PF_PDA_THI: *P. fermentans* on PDA with thiamine.

Lysine was the only amino acid tested. It was added after sterilisation since significant lysine losses occur when subjected to heat treatments (Bianchini, Beleia & Bianchini, 2014). Two concentrations were used for the study: 0.5 g/L and 1 g/L. The results obtained are shown in **Table 12**.

Table 12 – Qualitative results from microorganisms growth on NA, for bacteria, and PDA, for yeast and moulds, with the addition of different concentrations of lysine.

	BC	EC	PA	LM	SC	CI	PF	NF
LYS_0,5	+	=	=/+	=	=	+	+	=
LYS_1	=/+	=	=	=	-	=	+	-

- the microbial colonies' size was smaller when compared with NA; =: the microbial colonies' size was equal when compared with NA; +: the microbial colonies' size was better when compared with NA; X: no microbial growth occurred.

BC: *B. cereus*; EC: *E. coli*; PA: *P. aeruginosa*; LM: *L. mesenteroides*; SC: *S. cerevisiae*; CI: *C. intermedia*; PF: *P. fermentans*; NF: *N. fischeri*.

LYS_0,5: Lysine at 0.5 g/L; LYS_1: Lysine at 1 g/L.

Bacillus cereus was the only bacterium positively affected at both concentrations. For yeasts, the best results were obtained at the lowest concentration, with *S. cerevisiae* being the only one not positively affected by lysine. For mould, no positive results were obtained at the lowest concentration. Lysine will be used in the next stage at a concentration of 0.5 g/L for bacteria and yeasts. It will not be used in the preparation of media for mould as it did not give a positive result. Rhodes *et al.* (1961) studied the lysine content in microorganisms and observed that the lysine:nitrogen ratio was higher in yeasts when compared to moulds and bacteria. Furthermore, the authors also observed that the bacteria had more variability in lysine content than the fungi. These observations are in line with the results obtained since the yeasts were the ones that obtained the best results.

Six different mineral salts were tested, being them sodium chloride (NaCl), magnesium (MgSO₄.7H₂O), calcium, potassium (KH₂PO₄), iron III (FeCl₃.6H₂O), and zinc (ZnSO₄.7H₂O). For yeasts and moulds, NaCl was tested at two different concentrations: 1 g/L and 10 g/L, and for bacteria it was tested at 10 g/L. For magnesium, calcium, and potassium, the concentrations tested were 1 g/L and 10 g/L; for iron III and zinc were 0.1 g/L. The results obtained are presented in **Table 13**.

Table 13 - Results from microorganisms growth in NA, for bacteria, and PDA, for yeast and moulds, with the addition of different minerals.

	BC	EC	PA	LM	SC	CI	PF	NF
NaCl_0,1					-	-	-	+
NaCl_1	=	-	=	=	-	-	-	=
Mg_0,1	=	=/-	=/+	=/+	+	=	=	=
Mg_1	=/+	=	=/+	=	=	+	+	=
Ca_0,1	-	-	-	=	-	-	-	=
Ca_1	-	-	-	=	=	-	=	+
P_0,5	-	-	-	=	=	+	+	+
P_1	-	-	-	=	+	+	+	+
Fe	+	=/-	=/-	=/+	=	+	+	+
Zn	-	=	-	=	=	+	=	=

- the microbial colonies' size was smaller when compared with NA; =: the microbial colonies' size was equal when compared with NA; +: the microbial colonies' size was better when compared with NA; X: no microbial growth occurred.

BC: *B. cereus*; EC: *E. coli*; PA: *P. aeruginosa*; LM: *L. mesenteroides*; SC: *S. cerevisiae*; CI: *C. intermedia*; PF: *P. fermentans*; NF: *N. fischeri*.

NaCl_0,1: Sodium salt at 1 g/L; NaCl_1: Sodium salt at 10 g/L; Mg_0,1: Magnesium at 1 g/L; Mg_1: Magnesium 10 g/L; Ca_0,1: Calcium at 1 g/L; Ca_1: Calcium at 10 g/L; P_0,5: Potassium at 1 g/L; P_1: Potassium at 10 g/L; Fe: Iron III at 0.1 g/L; Zn: Zinc at 0.1 g/L.

It is known that many microorganisms cannot tolerate high salt concentrations, however many of them can tolerate small salt concentrations, as mentioned before. The objective of the study, when adding salt to the media, was to understand whether or not it could positively affect the growth of the colonies. However, *N. fischeri* was the only microorganism to present good results for sodium chloride as it is possible to see in **Table 13**, so 1 g/L of NaCl will continue to be used for *N. fischeri*.

Magnesium showed better results for the concentration of 10 g/L both for bacteria and yeasts. For the fungi, magnesium showed an equal result when compared to PDA. Has mentioned before, magnesium plays a vital role in the growth and functions of microorganisms. In the case of bacteria, the absence of magnesium in the culture media inhibits growth, and the increase of the magnesium in the medium, until a certain point, also increases bacterial growth (**Webb, 1953**). Yeasts' growth is also improved by adding magnesium in the media once it is involved in cell division (**Birch, Ciani & Walker, 2003**). Therefore, the results obtained were in line with those predicted in the literature, and this mineral salt will continue to be used for all microorganisms in the concentration of 10 g/L.

About calcium, *N. fischeri* was the only microorganism with a positive result for the higher concentration, and all bacteria showed a negative outcome for both concentrations. This result may be because countless cells secrete calcium actively to maintain the intracellular concentration at low levels (**Birch, Ciani & Walker, 2003**). So, for Phase 2, calcium will only be used in preparing the media for *N. fischeri* in the concentration of 10 g/L.

Regarding potassium, its addition in media for bacteria showed negative results, but on others, it showed positive results for yeasts and the mould, mainly when used at 10 g/L. Potassium is one of the minerals required for several biochemical processes in many organisms. However, its accumulation can be toxic, and the concentrations used for bacteria were probably toxic (**Yenush, 2016**). Nonetheless, it showed a positive outcome in the colony's size for the fungi and yeasts, so the concentration of 10 g/L of this mineral will be continued in the next phase in yeasts. For the mould, it will be used at a concentration of 1 g/L.

Iron (III) showed positive results for one bacteria, two yeasts, and the mould. This was expected once iron (III) is an essential nutrient. For this reason, iron (III) will continue to be used in Phase 2 for all the microorganisms.

Finally, the only positive result for zinc was observed with *C. intermedia*; the other microorganisms exhibited similar outcomes when comparing colony sizes to those that grew in simpler media, with the exception of *B. cereus* and *P. aeruginosa*, both of which yielded negative results. Probably that happened for *B. cereus* and *P. aeruginosa* because, according to some studies, high concentrations of Zn²⁺ can have antibacterial properties (ATMACA, GÜL & Cicek, 1998). Thus, this mineral will not be used further in the study.

2. Phase 2: Confirmation

In this phase, some tested media were selected to confirm if the number of colony-forming units (CFU) was affected. Has mentioned before, the inoculums were submitted to light thermal shocks of 40 °C for 5 min. This can damage part of the cells, but not all of them, allowing growth in all cases. However, after this light thermal shock, the inoculums will not be placed in enrichment media, and they may or may not be able to grow in the different culture media. Moreover, to understand if they can grow in the different media, the colonies will be counted and will be run a statistical analysis to see the results. For this, it was necessary to grow three different inoculums of each microorganism, plate three replicates of each, and count the colonies after three days of growth. Further, the qualitative analysis was repeated for each of the three inoculums of each microorganism to confirm previously obtained results.

For bacteria, the media tested were NA_GLU, NA_SAC_4, NA_LYS_0,5, NA_Mg1 and NA_P_0,5, for yeasts were PDA_GLU_4, PDA_SAC_4, PDA_FRU_4, PDA_LYS_0,5, PDA_BIO, PDA_THI, PDA_Mg_1, PDA_Fe and PDA_P_0,5, and for the mold were PDA_GLU_6, PDA_SAC_6, PDA_FRU_4, PDA_BIO, PDA_THI, PDA_NaCl_0,1, PDA_Ca_1, PDA_Fe and PDA_P_0,5.

2.1. Bacteria

The data obtained for bacteria is presented in the **Appendix, section A.1** in **Tables 26, 27, 28, 29** and **30**. In **Table 14** is presented the results obtained in Phase 2 for bacteria in which there was a statistically significant difference between the tested media and NA.

Table 14 - Results obtained for bacteria in Phase 2, in which there was a statistically significant difference between the tested culture media and NA.

Microorganism	Culture Media	Average (CFU/mL)	Standard Deviation	Test Analysis	Sig.
<i>B. cereus</i>	NA	9,59E+07	1,45E+07	One-Way ANOVA	0,03
	NA_Mg_1	7,54E+07	2,30E+07		
<i>L. mesenteroides</i>	NA	1,03E+08	3,69E+07	One-Way ANOVA	0,03
	NA_SAC_4	6,79E+07	1,51E+07		

SAC_4: Sucrose at 40 g/L; **Mg_1**: Magnesium at 10 g/L.

Has it is possible to confirm in the **Table 14**, except for *B. cereus* in NA_Mg_1 and *L. mesenteroides* in NA_SAC_4, counts in all the other media were similar ($p > 0.05$) to those on NA. This

means that the different media used did not significantly affect the number of cells that were able to grow.

In the case of *B. cereus*, as it is possible to see in **Table 14** and in **Table 26** in the **Appendix on section A.1**, the average obtained for the CFU in NA was 9.59E+07 CFU/mL and the average obtained for NA_Mg_1 was 7.54E+07 CFU/mL. As the *p-value* obtained was 0.03, the average obtained for NA_Mg_1 was significantly lower than that obtained for NA. Has previously stated, magnesium has a vital role in cell division, however, according to **Robinson et al. (2010)**, an increase of a simple amount of the ion Mg²⁺ had no effect, but the increase of magnesium in the medium had an antibacterial effect in some bacteria. This could explain why this magnesium concentration had a negative effect on *B. cereus*.

In the case of *L. mesenteroides*, has it is possible to see in **Table 14** and **Table 28** in the **Appendix in section A.1**, the average obtained for the CFU in NA was 1.03E+08 CFU/mL, and the average obtained for NA_SAC_4 was 6.79E+07 CFU/mL. As the *p-value* obtained was 0.03, that means that the average obtained for NA_SAC_4 was significantly lower than the one obtained for NA. This outcome was not expected since previously has been noted that *L. mesenteroides* had a positive result in the qualitative analysis for NA_SAC_4, and according to literature, this microorganism can grow at high concentrations of sucrose and even prefers sucrose to glucose or fructose (**Dols, Remaud-Simeon & Monsan, 1997**; **Reid & Abratt, 2005**). Further, **Santos, Teixeira & Rodrigues (2000)** conducted a study to study the production of dextran, dextransucrose and fructose using *L. mesenteroides* and used concentrations of sucrose between 10 g/dm³ and 120 g/dm³. The authors concluded that the higher sucrose concentrations did not affect the bacteria's growth. The concentrations used in the study of **Santos, Teixeira & Rodrigues (2000)** were higher than the concentrations used in this study, so it is possible to conclude that the sucrose concentrations were not the factor that made the CFU obtained for NA_SAC_4 significantly lower than the one obtained for NA. So, this could be because the colonies presented in the Petri dishes were large enough to cause human errors in plate counts, as it is possible to see in **Figure 9**.

Despite of the statistically significant differences shown in **Table 14**, as these differences do not have a logarithmic unit of difference, in biological terms these differences in these results may not be relevant.

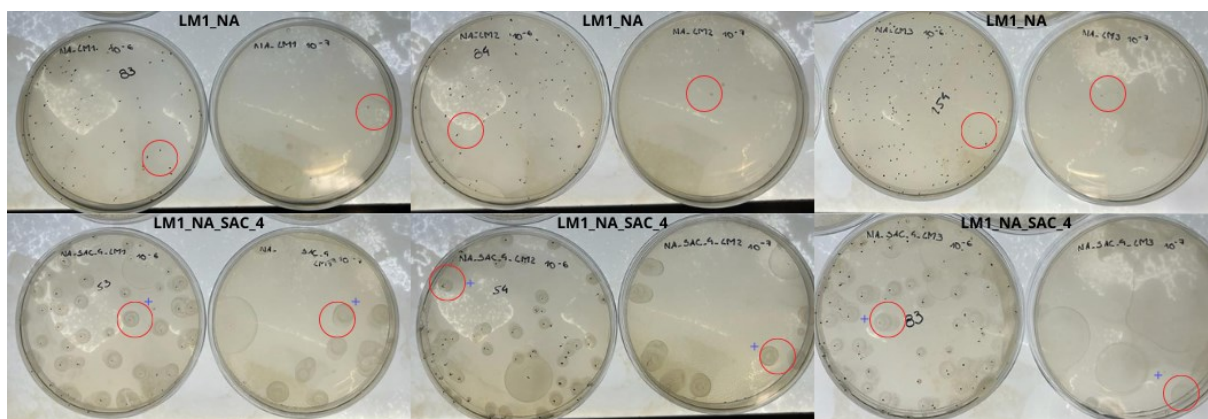


Figure 10 - Results obtained in Phase 2 for the different inoculums of *L. mesenteroides* in the culture media with 40 g/L of sucrose (NA_SAC_4).

Has mentioned before, the qualitative analysis was performed by comparing the sizes of the colonies that grew on NA with the colonies that grew in the media that are being studied. The qualitative analysis from Phase 2 of bacteria is presented in **Table 15**.

Table 15 - Qualitative results of bacteria from Phase 2 obtained by comparing the morphology of the colonies with their growth in NA and with their growth in NA with the addition of one nutrient.

Culture Media	BC1	BC2	BC3	EC1	EC2	EC3	LM1	LM2	LM3	PA1	PA2	PA3
NA_GLU	+	+	=	=	+	=	=	=	=	-	-	-
NA_SAC_4	+	+	+	=	+	+	+	+	+	+	+	+
NA_LYS_0,5	+	+	+	=	=	=	=	=	=	+	=	=
NA_Mg_1	=	=	+	=	=	=	=	=	=	+	=	=
NA_Fe	+	+	+	+	=	=	=	=	=	=	=	+

- the microbial colonies' size was smaller when compared with NA; =: the microbial colonies' size was equal when compared with NA; +: the microbial colonies' size was better when compared with NA; X: no microbial growth occurred.

BC: *B. cereus*; **EC:** *E. coli*; **PA:** *P. aeruginosa*; **LM:** *L. mesenteroides*;

GLU: Glucose at 20 g/L; **SAC_4:** Sucrose at 40 g/L; **LYS_0,5:** Lysine at 0.5 g/L; **Mg_1:** Magnesium at 10 g/L; **Fe:** Iron III at 0.1 g/L.

It is possible to confirm in **Table 15** that only the three inoculums of *P. aeruginosa* had a negative outcome for one of the media, for NA_GLU. Except for that, all the media had a positive or equal result.

NA_SAC_4 was the culture medium with the best outcome; it had a positive result for the three inoculums of *B. cereus*, *L. mesenteroides* and *P. aeruginosa*. Regarding *B. cereus*, the result was not expected once that had been reported that this bacterium prefers glucose over sucrose (**Shukor et al., 2009**). However, the result could be that in this stage of the study, the amount of sucrose used was higher than the amount of glucose. For *L. mesenteroides*, as mentioned earlier, this microorganism prefers sucrose over glucose (**Dols, Remaud-Simeon & Monsan, 1997; Reid & Abratt, 2005**), so this outcome was already expected. Concerning *P. aeruginosa*, there is little literature about glucose preference over sucrose or otherwise. However, there are reported that their preferred carbon source is organic acids (**Karishma et al., 2015**).

For the next part of the study, as sucrose had the best results, it will only be used sucrose as a carbon source, and in the media, it will be mixed with lysine, magnesium and iron (III). This will be made to understand if the use of the components will have synergetic effects on the growth of the bacteria.

2.2. Yeasts

The data obtained for the yeasts are presented in the **Appendix, section A.2** in **Tables 30, 31, 32, 33, 34** and **35**. The statistical data analysis from Phase 2 of the bacteria is presented in **Table 16**.

Table 16 - Statistical Data Analysis from Phase 2 of the yeasts.

<i>Microorganism</i>	Culture Media	Test Analysis	Sig.
<i>S. cerevisiae</i>	PDA_GLU_4	Mann-Whitney	0.596
	PDA_SAC_4	Mann-Whitney	0.691
	PDA_FRU_4	One-Way ANOVA	0.923
	PDA_LYS_0,5	Mann-Whitney	1
	PDA_BIO	One-Way ANOVA	1
	PDA_THI	One-Way ANOVA	1
	PDA_Mg_1	One-Way ANOVA	0.981
	PDA_Fe	One-Way ANOVA	1
	PDA_P_0,5	One-Way ANOVA	0.683
<i>C. intermedia</i>	PDA_GLU_4	One-Way ANOVA	1
	PDA_SAC_4	One-Way ANOVA	1
	PDA_FRU_4	One-Way ANOVA	1
	PDA_LYS_0,5	One-Way ANOVA	1
	PDA_BIO	One-Way ANOVA	1
	PDA_THI	One-Way ANOVA	1
	PDA_Mg_1	Mann-Whitney	0.750
	PDA_Fe	One-Way ANOVA	0.997
	PDA_P_0,5	One-Way ANOVA	1
<i>P. fermentans</i>	PDA_GLU_4	One-Way ANOVA	1
	PDA_SAC_4	One-Way ANOVA	0.407
	PDA_FRU_4	Mann-Whitney	0.691
	PDA_LYS_0,5	One-Way ANOVA	1
	PDA_BIO	One-Way ANOVA	1
	PDA_THI	One-Way ANOVA	1
	PDA_Mg_1	One-Way ANOVA	1
	PDA_Fe	One-Way ANOVA	1
	PDA_P_0,5	One-Way ANOVA	0.427

GLU_4: Glucose at 40 g/L; **SAC_4:** Sucrose at 40 g/L; **FRU_4:** Fructose at 40 g/L; **LYS_0,5:** Lysine at 0.5 g/L; **BIO:** Biotin at 0.02 g/L; **THI:** Thiamine at 0.002 g/L; **Mg_1:** Magnesium at 10 g/L; **Fe:** Iron III at 0.1 g/L; **P_0,5:** Potassium at 1 g/L.

As it is possible to confirm in **Table 16**, counts in all the media were similar ($p > 0.05$) to those on PDA. This means that the different media used did not significantly affect the number of cells that were able to grow.

As mentioned earlier, a qualitative analysis was performed by comparing the sizes of the colonies that grew on PDA with the colonies that grew on the other media. This analysis is presented in **Table 17**.

Table 17 - Qualitative results of yeasts from Phase 2 obtained by comparing the morphology of the colonies with their growth in NA and with their growth in NA with the addition of one nutrient.

Culture Media	SC1	SC2	SC3	CI1	CI2	CI3	PF1	PF2	PF3
PDA_GLU_4	=	+	=	=	+	=	+	=	=
PDA_SAC_4	=	=	=	-	+	=	=	=	+
PDA_FRU_4	=	+	+	-	+	-	+	+	+
PDA_LYS_0,5	=	=	=	=	+	+	=	+	+
PDA_BIO	=	=	+	+	=	=	=	+	+
PDA_THI	=	=	=	-	+	+	+	+	+
PDA_Mg_1	=	=	=	+	+	+	+	+	+
PDA_Fe	=	=	+	=	+	+	=	+	+
PDA_P_0,5	=	+	=	+	+	=	+	+	+

- the microbial colonies' size was smaller when compared with NA; =: the microbial colonies' size was equal when compared with NA; +: the microbial colonies' size was better when compared with NA; X: no microbial growth occurred.

SC: *S. cerevisiae*; CI: *C. intermedia*; PF: *P. fermentans*;

GLU_4: Glucose at 40 g/L; SAC_4: Sucrose at 40 g/L; FRU_4: Fructose at 40 g/L; LYS_0,5: Lysine at 0.5 g/L; BIO: Biotin at 0.02 g/L; THI: Thiamine at 0.002 g/L; Mg_1: Magnesium at 10 g/L; Fe: Iron III at 0.1 g/L; P_0,5: Potassium at 1 g/L.

The media with the best result was PDA_Mg_1, which had a positive result in the three inoculums of *C. intermedia* and *P. fermentans*. This positive result has been expected once, has mentioned magnesium is a critical mineral salt since it is involved in growth and cell division (Birch, Ciani & Walker, 2003). Regarding the media PDA_FRU_4 and PDA_THI, both had positive results for the three inoculums of *P. fermentans*. This result was also expected for thiamine since *P. fermentans* cannot grow on media free of vitamins (UCDAVIS Viticulture & Enology, n.d.). Regarding the positive results of fructose over glucose and sucrose, the literature mentions both cases, the preference of *P. fermentans* for fructose over glucose and the preference of glucose over fructose (da Silva Vale et al., 2019; Liu et al., 2019).

The yeast *C. intermedia* had two inoculums with a negative result for fructose and one positive. The outcome obtained is contradictory. There is no evidence in the literature that fructose is the minor carbon source preferred by the yeast; on the contrary, it is indicated that glucose and fructose are the most consumed by the yeast (Pérez-Cadena et al., 2018). Nonetheless, that did not show for both sugars in the qualitative analysis.

Even though fructose did not show any remarkable results, it will still be used in the next stage of the study since, in the company, fructose is one of the primary carbon sources of the products. Furthermore, yeasts are the microorganisms most used in the company, so studying the synergetic effects of fructose with the other components is of greater interest.

So, in the next stage, the synergetic effects of combining each of the sugars with the lysine, the vitamins and the minerals salts used in Phase 2 will be studied.

2.3. Mould

The data obtained for the mould is presented in the Appendix, section A.3 in Table 37 and 38. In Table 18 is presented the results obtained in Phase 2 for the mould in which there was a statistically significant difference between the tested media and PDA.

Table 18 - Results obtained for the mould in Phase 2, in which there was a statistically significant difference between the tested culture media and PDA.

<i>Microorganism</i>	Culture Media	Average (CFU/mL)	Standard Deviation	Test Analysis	Sig.
<i>N. fischeri</i>	PDA	3,98E+06	4,86E+05	One-Way ANOVA	0,018
	PDA_Mg_1	3,20E+06	5,18E+05		
	PDA	3,98E+06	4,86E+05	One-Way ANOVA	0,0002
	PDA_Ca_1	2,93E+06	4,32E+05		
	PDA	3,98E+06	4,86E+05	One-Way ANOVA	0,001
	PDA_P_0,5	3,04E+06	3,84E+05		

GLU_6: Glucose at 60 g/L; **SAC_6:** Sucrose at 60 g/L; **FRU_4:** Fructose at 40 g/L; **BIO:** Biotin at 0.02 g/L; **THI:** Thiamine at 0.002 g/L; **NaCl_0,1:** Sodium salt at 1 g/L; **Mg_1:** Magnesium 10 g/L; **Ca_1:** Calcium at 10 g/L; **Fe:** Iron III at 0.1 g/L; **P_0,5:** Potassium at 1 g/L.

Has it is possible to confirm in **Table 18** and **Table 38** in the **Appendix, section A.3**, except for PDA_Mg_1, PDA_Ca_1 and PDA_P_0,5, counts on all the other media were similar ($p > 0.05$), to those obtained on PDA.

In the case of magnesium, as it is possible to see in **Table 37** on the **Appendix, section A.3**, the average obtained for the CFU in PDA was 3.98E+06 CFU/mL and the average obtained for PDA_Mg_1 was 3.20E+06 CFU/mL. As the *p-value* obtained was 0.018, that means that the average obtained for PDA_Mg_1 was significantly lower than the one obtained for PDA. Unfortunately, there is not much information in the literature about the magnesium uptake by *N. fischeri*.

Regarding the calcium, it is possible to confirm on the **Appendix, section A.3** in **Table 37** and in **Table 18**, that the average obtained for PDA_Ca_1 was 2.93E+06 CFU/mL, and the average obtained for PDA is the same mentioned before. As before, as the *p-value* obtained was lower than 0.05, that means that the average obtained for PDA_Ca_1 is significantly lower than the obtained for PDA. This could be explained by the fact that mitochondria can absorb significant amounts of calcium, changing the cytoplasmatic Ca^{2+} , which could lead to cell death (**Nguyen et al., 2018**).

About potassium, as the average obtained for PDA_P_0,5 was 3.04E+06 CFU/mL and the *p-value* obtained was 0.001, that means that the average obtained for PDA_P_0,5 was significantly lower than the one obtained for PDA. This outcome was not expected once potassium is essential for some processes in cell metabolism (**Urik et al., 2010**). As mentioned earlier, potassium is required for several biochemical processes, however, its accumulation can be toxic, and the concentrations used for the mould was probably toxic (**Yenush, 2016**).

As mentioned earlier, despite of the statistically significant differences shown in Table 14, as these differences do not have a logarithmic unit of difference, in biological terms these differences in these results may not be relevant.

A qualitative analysis was also done and is presented in **Table 19**.

Table 19 - Qualitative results of the mold from Phase 2 obtained by comparing the morphology of the colonies with their growth in NA and with their growth in NA with the addition of one nutrient.

Culture Media	NF1	NF2	NF3
PDA_GLU_6	+	+	+
PDA_SAC_6	+	+	+
PDA_FRU_4	+	+	+

<i>PDA_BIO</i>	+	+	+
<i>PDA_THI</i>	=	=	+
<i>PDA_NaCl_0,1</i>	+	=	+
<i>PDA_Mg_1</i>	=	+	+
<i>PDA_Ca_1</i>	-	+	-
<i>PDA_Fe</i>	=	+	=
<i>PDA_P_0,5</i>	+	+	+

- the microbial colonies' size was smaller when compared with NA; =: the microbial colonies' size was equal when compared with NA; +: the microbial colonies' size was better when compared with NA; X: no microbial growth occurred.

NF: *N. fischeri*.

GLU_6: Glucose at 60 g/L; **SAC_6:** Sucrose at 60 g/L; **FRU_4:** Fructose at 40 g/L; **BIO:** Biotin at 0.02 g/L; **THI:** Thiamine at 0.002 g/L; **NaCl_0,1:** Sodium salt at 1 g/L; **Mg_1:** Magnesium 10 g/L; **Ca_1:** Calcium at 10 g/L; **Fe:** Iron III at 0.1 g/L; **P_0,5:** Potassium at 1 g/L.

For the qualitative analysis of *N. fischeri*, the best outcomes were for the sugars (sucrose, glucose and fructose), biotin and potassium, since the three inoculums had a positive result. Sucrose, glucose and fructose are commonly used to enhance microorganisms' growth, so the results obtained were expected. The result of biotin could be explained by the fact that biotin plays a role as a cofactor for enzymes (**Magliano et al., 2011**), thus promoting the fast growth of fungi. The outcome obtained for potassium in the utilized methodology goes against the expected results. This could be because the number of colonies in the countable Petri dishes was very high, and the colonies presented were large enough to cause a human error in plate counting.

Calcium had two negative results in the qualitative utilized methodology, which are in agreement with the results obtained in the statistical data analysis. So it is possible to assume that calcium, at the concentration used, inhibits the growth of *N. fischeri*. Thiamine and iron (III) did not significantly impact the qualitative or statistical analysis, so the three components (calcium, thiamine and iron (III)) were not used in the next stage to prepare the culture media for the mould.

Therefore, the components used to grow *N. fischeri* in the next stage were the sugars (sucrose, glucose and fructose), biotin and some mineral salts (NaCl, magnesium and potassium).

3. Phase 3: Synergism Between Nutrients

In Phase 3 of the study, the components added to the media for Phase 2 with better results were placed together to understand if they had a synergetic effect between them and positively affected the growth of the microorganisms. As before, the microorganisms were also subjected to a light thermal shock.

The results of the pH measurements are presented in **Appendix section B.4**, in **Table 48**. The pH level has been set in all the media used to ensure that the pH value would not interfere with microbial growth. The pH levels for bacteria were 7 and 7.3, for yeasts between 5.9 and 6.2 and for mould between 5.8 and 6.1.

3.1. Bacteria

In Phase 3 of the study, three different media, 201, 202 and 203, were tested for bacteria. For the first culture media, NA was mixed with sucrose (40 g/L), lysine (0,5 g/L) and magnesium (10 g/L);

the second culture media was composed of NA with a mixture of sucrose (40 g/L), lysine (0,5 g/L) and iron III (0,1 g/L), the last media culture were composed by sucrose (40 g/L), lysine (0,5 g/L), magnesium (10 g/L) and iron III (0,1 g/L).

As mentioned before, the pH levels were adjusted, and the media used in bacteria had a pH between 7 and 7.3.

The data obtained for bacteria is presented in the **Appendix, section B.1** in **Tables 39, 40, 41, 42** and **43**. In **Table 20** is presented the results obtained in Phase 3 for bacteria in which there was a statistically significant difference between the tested media and NA.

Table 20 - Results obtained for bacteria in Phase 3, in which there was a statistically significant difference between the tested culture media and NA.

Microorganism	Culture Media	Average (CFU/mL)	Standard Deviation	Test Analysis	Sig.
<i>L. mesenteroides</i>	NA	1,55E+09	2,59E+08	One-Way ANOVA	6.79E-06
	202	7,44E+08	2,99E+08		
	NA	1,55E+09	2,59E+08	One-Way ANOVA	0.0004
	203	9,32E+08	3,83E+08		

As it is possible to see in **Table 20** and **Table 43** in the **Appendix section B.1**, except for *L. mesenteroides* in 202 and in 203, all media had a *p-value* superior to 0.05, which means that the number of CFU in the different media was not significantly different from the one obtained in NA. This means that the different media used did not had a significant negative effect on the number of cells that were able to grow.

In the case of *L. mesenteroides*, as it is possible to see in **Table 20**, the average obtained for the CFU in NA was 1.55E+09 CFU/mL and the average obtained for 202 was 7.44E+08 CFU/mL and for the medium 203 was 9.32E+08 CFU/mL. As the *p-value* obtained was lower than 0.05, the average obtained for 202 and 203 was significantly lower than that obtained for NA. Despite of the statistically significant differences shown in Table 14, as these differences do not have a logarithmic unit of difference, in biological terms these differences in these results may not be relevant.

The qualitative analysis made for Phase 3 for the study's bacteria is presented in **Table 21**.

Table 21 - Qualitative results of bacteria from Phase 3 obtained by comparing the morphology of the colonies with their growth in NA and with their growth in the media with the addition of several components.

Culture Media	BC1	BC2	BC3	EC1	EC2	EC3	PA1	PA2	PA3	LM1	LM2	LM3
201	+	+	+	+	+	+	+	+	=	+	+	+
202	=	+	+	+	+	+	=	+	=	+	+	+
203	+	+	+	+	+	+	=	+	+	+	+	+

- the microbial colonies' size was smaller when compared with NA; =: the microbial colonies' size was equal when compared with NA; +: the microbial colonies' size was better when compared with NA; X: no microbial growth occurred.

BC: *B. cereus*; EC: *E. coli*; PA: *P. aeruginosa*; LM: *L. mesenteroides*;

As it is possible to confirm in **Table 21**, *P. aeruginosa* and one of the *B. cereus* inoculums were the only bacteria that did not have a positive result for the three inoculums in all of the media. The three inoculums of *B. cereus* had a positive result for media 201 and 203, and the three inoculums of *E. coli* and *L. mesenteroides* had a positive result for the three different media.

Regarding *B. cereus*, when comparing **Table 15** with **Table 21**, it is possible to see that in Phase 2, the bacteria did not have a positive result for the three inoculums in the media that contained magnesium. However, in Phase 3, the media that contained magnesium were the ones that had a positive outcome for the three different inoculums. So, in this case, it is possible to assume that the magnesium alone negatively affected the growth of *B. cereus*. However, when magnesium was present in the medium with other components, they had a positive effect between them, which led to a positive outcome.

In the case of *E. coli*, in Phase 2, the bacteria did not have a positive result for any of the media in any of the three inoculums (**Table 15**); however, in Phase 3, all the media tested had a positive outcome in all three inoculums, as it is possible to see in **Table 21** and the **Figure 10**. So, the same can be said about all the components added to the medium regarding this bacterium, and it is possible to assume that the components alone did significantly impact the growth of *E. coli*. However, the components together had a synergetic effect between them and positively impacted the growth of the bacteria.

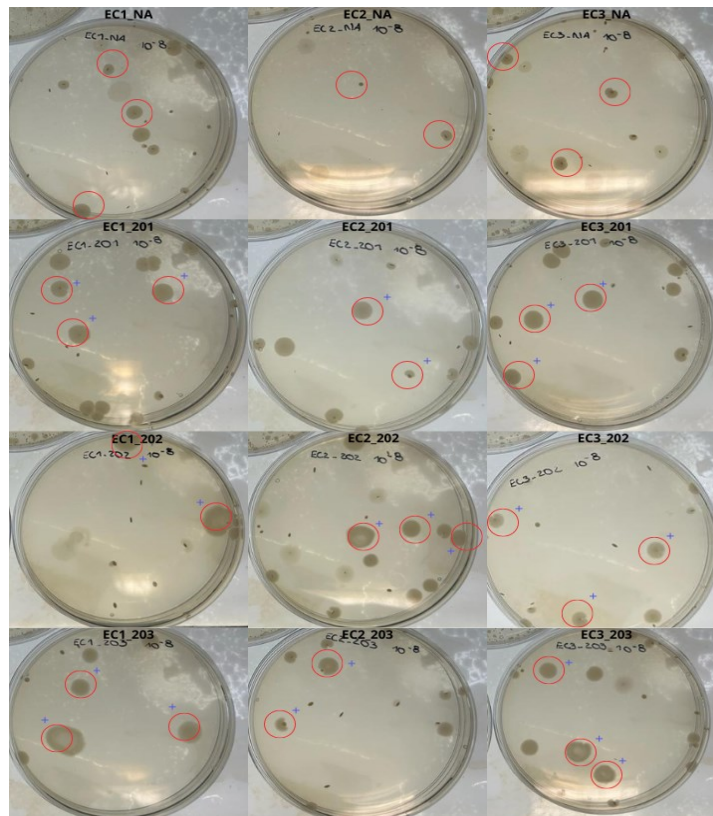


Figure 11 - Results obtained in Phase 3 for the different inoculums of *E. coli* in all the different culture media tested.

For *L. mesenteroides*, sucrose had already shown to have a positive effect on their growth in Phase 2, as it is possible to see in **Figure 9** and **Table 15**. Therefore, as in all media tested in Phase 3, sucrose was added, it is impossible to see if the components together had a synergetic effect between them or if the growth of the bacteria was only affected by sucrose. The outcome obtained for *L. mesenteroides* in mediums 202 and 203 in the utilized methodology goes against the expected results.

This could be because the number of colonies in the countable Petri dishes was very high, and the colonies presented were large enough to cause a human error in plate counting.

Concerning bacteria, media 201 and 203 had the best results; three bacteria had positive outcomes in their three inoculums for both culture media. In both of them was added magnesium, unlike the media 202, which confirms that magnesium is an essential component of the growth of microorganisms.

3.2. Yeasts

In Phase 3 of the study, six different media were tested for yeasts. All the media had lysine (0.5 g/L), biotin (0.02 g/L), thiamine (0,004 g/L) and iron (III) (0.1 g/L). In the media labelled as 40x, the added sugar was glucose; in the media labelled as 50x, the sugar used was sucrose, and for the media labelled as 60x, fructose was included. Additionally, in the media where x=1, magnesium (10 g/L) was introduced, while in the media where x=2, potassium (5 g/L) was added.

As mentioned before, the pH levels were adjusted, and the media used in yeasts had a pH between 5.9 and 6.2.

The data obtained for yeasts is presented in the **Appendix, section B.2** in **Tables 44, 45** and **46**. The statistical data analysis from Phase 3 of the yeasts is presented in **Table 22**.

Table 22 - Statistical Data Analysis from Phase 3 of the yeasts.

Microorganism	Culture Media	Test Analysis	Sig.
<i>S. cerevisiae</i>	401	One-Way ANOVA	1
	402	One-Way ANOVA	1
	501	One-Way ANOVA	1
	502	One-Way ANOVA	1
	601	One-Way ANOVA	1
	602	One-Way ANOVA	1
<i>C. intermedia</i>	401	One-Way ANOVA	1
	402	One-Way ANOVA	1
	501	One-Way ANOVA	0.951
	502	One-Way ANOVA	1
	601	One-Way ANOVA	1
	602	One-Way ANOVA	1
<i>P. fermentans</i>	401	One-Way ANOVA	1
	402	One-Way ANOVA	1
	501	One-Way ANOVA	0.971
	502	One-Way ANOVA	0.982
	601	One-Way ANOVA	0.999
	602	One-Way ANOVA	1

Has it is possible to see in **Table 22** counts on all media were similar ($p > 0.05$) to those obtained on PDA. This means that the different media used did not had a significant effect on the number of cells that were able to grow.

The qualitative analysis made for Phase 3 for the study's bacteria is presented in **Table 23**.

Table 23 - Qualitative results of yeasts from Phase 3 obtained by comparing the morphology of the colonies with their growth in PDA and with their growth in the media with the addition of several components.

Culture Media	SC1	SC2	SC3	PF1	PF2	PF3	CI1	CI2	CI3
401	+	=	=	+	=	+	+	+	+
402	=	=	=	+	=	-	+	+	+
501	+	+	=	+	=	=	+	+	+
502	+	=	=	=	=	-	+	+	+
601	+	+	+	+	+	+	=	=	=
602	+	-	=	+	+	-	+	+	=

- the microbial colonies' size was smaller when compared with NA; =: the microbial colonies' size was equal when compared with NA; +: the microbial colonies' size was better when compared with NA; X: no microbial growth occurred.
SC: *S. cerevisiae*; **CI:** *C. intermedia*; **PF:** *P. fermentans*;

As it is possible to confirm in **Table 23**, none of the yeasts had a negative result in the three inoculums, so all the media had a positive or equal effect on their colonies size.

In the case of *C. intermedia*, the yeast had a positive outcome in the three inoculums for the media 401, 402, 501 and 502. These results mean that the mixture of the components in the culture media had a synergetic effect between them since *C. intermedia* in Phase 2 only had a positive result for the three inoculums in the media with magnesium (as it is possible to see in **Table 17**).

Regarding *S. cerevisiae* and *P. fermentans*, both microorganisms had a positive result in the three inoculums for media 601, as it is possible to see in **Table 23** and **Figure 11**. For *S. cerevisiae*, the yeast did not have any positive result in Phase 2 of the study, so it is possible to assume that the combination of the different components positively affected the growth and the size of the colonies. However, in the case of *P. fermentans*, the yeast had previously had a positive outcome for some of the components used in the composition of the culture media 601. So, in this second case is not possible to affirm with certainty if the combination of the different components had a synergetic effect and positively influenced the growth of *P. fermentans*.

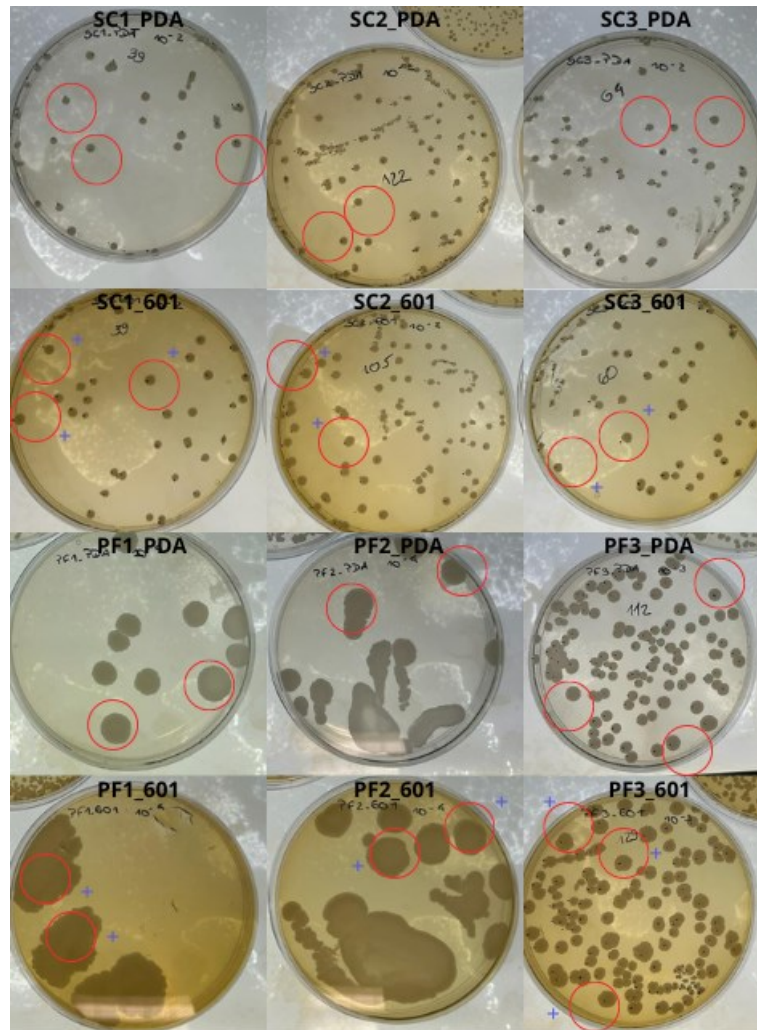


Figure 12 - Results obtained for the culture media 601 for the growth of the 3 inoculums of *S. cerevisiae* and *P. fermentans*.

Concerning yeasts, as mentioned before, the culture medium that presented the best results was the medium 601. This medium may not have had a positive outcome for *C. intermedia*, but it did not also have a negative outcome, which means that it does not negatively compromise the yeast's growth.

3.3. Mould

In Phase 3 of the study, six different media were tested for the mould. All the media had biotin (0.02 g/L), magnesium (10 g/L) and potassium (5 g/L). In the media coded as 70x, the sugar added was glucose, the media coded as 80x the sugar was sucrose and the media coded as 90x the sugar was fructose. Additionally, in the media where $x=1$, sodium chloride (5 g/L) was added, and sodium chloride was not added for the media where $x=2$.

As mentioned before, the pH levels were adjusted, and the media used in the mould had a pH between 5.8 and 6.1.

The data obtained for *N. fischeri* is presented in the **Appendix, section B.3** in **Table 47**. The statistical data analysis from Phase 3 of the mould is presented in **Table 24**.

Table 24 - Statistical Data Analysis from Phase 3 of the mould.

Microorganism	Culture Media	Test Analysis	Sig.
<i>N. fischeri</i>	701	Mann-Whitney	0.305
	702	One-Way ANOVA	0.953
	801	One-Way ANOVA	0.995
	802	One-Way ANOVA	0.423
	901	One-Way ANOVA	0.325
	902	One-Way ANOVA	0.764

As it is possible to see in **Table 24**, counts on all media were similar ($p > 0.05$) to those PDA. This means that the different media used did not had a significant effect on the number of cells that were able to grow.

The qualitative analysis made for Phase 3 for the study's mould, it is presented in **Table 25**.

Table 25 - Qualitative results of the mould from Phase 3 obtained by comparing the morphology of the colonies with their growth in PDA and with their growth in the media with the addition of several components.

Culture Media	NF1	NF2	NF3
701	-	-	-
702	=	=	-
801	-	-	-
802	-	-	-
901	-	-	-
902	=	-	-

- the microbial colonies' size was smaller when compared with NA; =: the microbial colonies' size was equal when compared with NA; +: the microbial colonies' size was better when compared with NA; X: no microbial growth occurred.
NF: *N. fischeri*.

As it is possible to confirm in **Table 25**, *N. fischeri* did not have a positive result in any of the three inoculums for none of the media. Since the mould had positive outcomes regarding the testing of

the components alone (see **Table 19**), this could mean that the mixture of the different components had a synergetic effect and negatively affected the growth of the fungi.

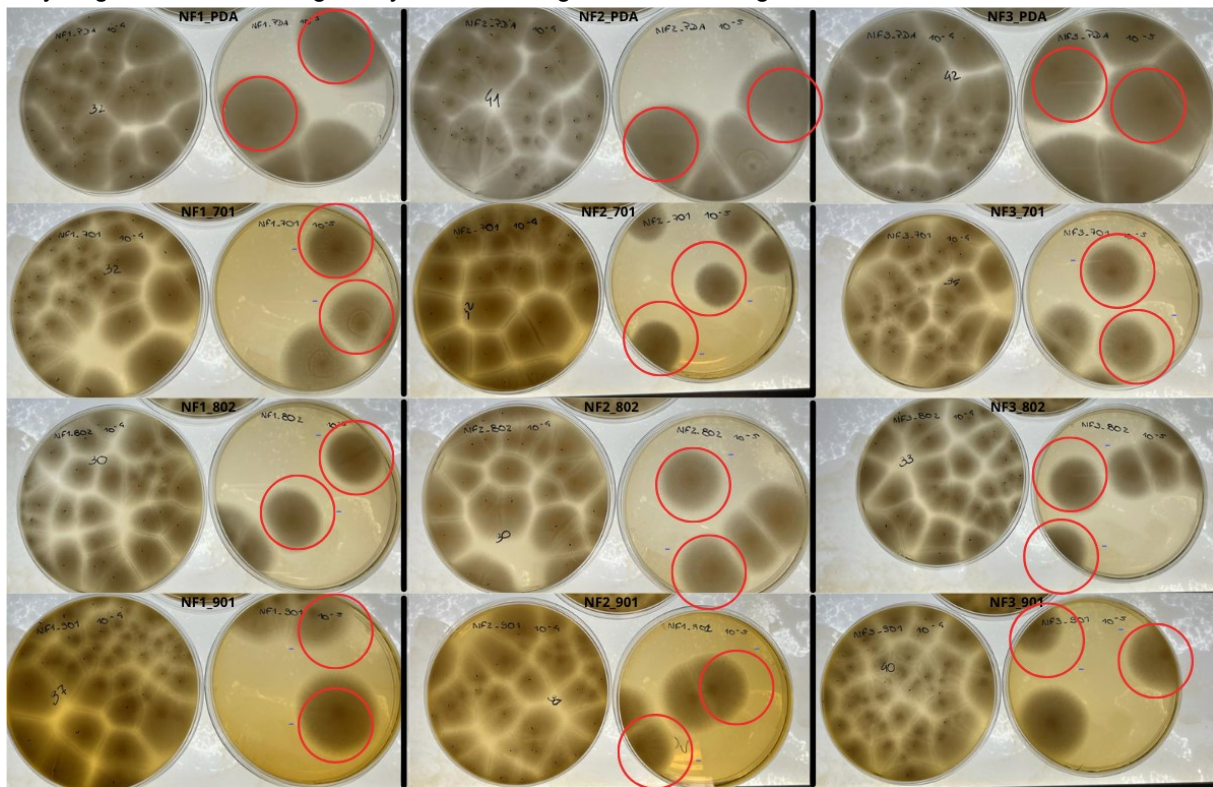


Figure 13 - Results obtained for the culture media 701, 802 and 901 for the 3 inoculums of *N. fischeri*.

As it is possible to see in **Figure 12**, *N. fischeri* had better growth in PDA than in the media tested, so it is possible to assume that for this mould, simpler culture media, like PDA, are better for growth. To understand if the same happens for other moulds, it is necessary to test the media in more species of moulds.

Conclusions

The primary aim of the conducted study was to formulate an optimal culture medium for the growth of yeasts, bacteria and moulds. These culture media are intended to mimic the fruit preparations and to guarantee that if the samples are contaminated the microorganism will grow in the media and it will grow faster and more significantly to quickly detect the microorganism.

To achieve this goal, an initial assessment was undertaken by supplementing basic culture media like NA and PDA with diverse constituents, to understand how each component affected the growth of the different microorganisms. The microorganisms used throughout the study were *B. cereus*, *E. coli*, *L. mesenteroides*, *P. aeruginosa*, *S. cerevisiae*, *P. fermentans*, *C. intermedia* and *N. fischeri*. Later, a confirmation phase was performed to validate the results obtained and to see if the components negatively or positively affected the growth of the microorganisms at a statistical level. In this second phase of the study, the components with better results for bacteria were sucrose, lysine, magnesium and iron (III); for yeasts, were glucose, sucrose, fructose, lysine, biotin, thiamine, magnesium, potassium and iron (III), for the mould were glucose, sucrose, fructose, biotin sodium chloride, magnesium and potassium. Finally, the components that yielded the better results were mixed in the media. In the last stage of the study, three different media were tested for bacteria and six different culture media for yeasts and moulds.

For bacteria, the media with better results in the qualitative analysis were 201 (NA with 40 g/L of sucrose, lysine and magnesium) and 203 (NA with 40 g/L of sucrose, lysine, magnesium and iron III); since three different bacteria had positive outcomes, in their 3 inoculums for both culture media. Further, with a *p-value* superior to 0.05 when comparing the average of CFU obtained in the medium with the average obtained in NA.

Regarding yeasts, the culture media with the best result in the qualitative analysis was 601 (PDA with 40 g/L of fructose, lysine, biotin, thiamine, magnesium and iron III). This media had a positive outcome for *S. cerevisiae* and *P. fermentans*. Concerning *C. intermedia*, the media may not have had a positive outcome but did not also have a negative result, which means that does not negatively compromise the growth of the yeast. Further, in the statistical data analysis the *p-value* obtained for the media was superior to 0.05 in all the yeasts when comparing the average of CFU obtained in the medium with the average obtained in PDA.

Lastly, for the mould all the culture media tested had a *p-value* superior to 0.05. However, *N. fischeri* had a better growth in PDA than in the media tested, so it is possible to assume that simpler culture media, like PDA, are better for growth for this mould.

Thus, it is possible to state that, although not all the results obtained were the desired ones, the study went well since it was possible to perceive that which mixture of components enhanced the growth of bacteria and yeasts and that for *N. fischeri* the growth was better in simpler media.

During the training placement, it was possible to acquire much experience in the microbiology laboratory, visit the company's largest production plant in Tortosendo, Covilhã, and it was also possible to get to know the sector better.

In conclusion, the training placement gave me much experience in the microbiology lab, extended know-how and networking. In practical terms, it allowed me to understand some of the necessary components for microorganisms' growth and their role in their growth.

Future Work

The following steps of the study would be to see if there is any fruit preparation in the company which contains the components that had good results. After identifying these, their addition to culture media could be tested to see how the microorganisms behaved. Unfortunately, this cannot be done due to the limited time of the internship.

In the future, it would also be interesting to collect all the microorganisms that tend to be more frequent in microbiological analyses of fruit preparations and cultivate them in these culture media obtained to understand whether the use of these media provides good results at a general level. This would be very interesting to do with more fungi since only one was used throughout the study.

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Appendix

A. Results obtained for Phase 2

A.1. Results obtained in Phase 2 for Bacteria

Table 26 - Results obtained in Phase 2 for the different inoculums of *B. cereus*.

Culture Media	CFU/mL	Average	Standard Deviation
NA	8.80E+07	9.59E+07	1.45E+07
	8.30E+07		
	1.09E+08		
	9.30E+07		
	7.40E+07		
	9.60E+07		
	9.00E+07		
	1.12E+08		
	1.18E+08		
NA_GLU	9.30E+07	9.72E+07	1.07E+07
	8.90E+07		
	1.01E+08		
	8.70E+07		
	9.20E+07		
	9.10E+07		
	9.80E+07		
	1.22E+08		
	1.02E+08		
NA_SAC_4	1.28E+08	1.12E+08	1.45E+07
	1.11E+08		
	8.80E+07		
	1.16E+08		
	1.07E+08		
	9.60E+07		
	1.25E+08		
	1.30E+08		
	1.05E+08		
NA_LYS_0,5	7.60E+07	9.12E+07	1.72E+07
	9.00E+07		
	8.90E+07		
	6.00E+07		
	8.50E+07		
	1.00E+08		
	9.60E+07		
	1.05E+08		
	1.20E+08		
NA_Mg_1	7.10E+07	7.54E+07	2.30E+07
	5.70E+07		

	7.00E+07		
	5.50E+07		
	6.70E+07		
	4.70E+07		
	1.03E+08		
	9.70E+07		
	1.12E+08		
NA_Fe	9.00E+07	9.70E+07	1.16E+07
	1.05E+08		
	7.80E+07		
	9.70E+07		
	9.30E+07		
	1.00E+08		
	1.11E+08		
	8.60E+07		
	1.13E+08		

GLU: Glucose at 20 g/L; **SAC_4:** Sucrose at 40 g/L; **LYS_0,5:** Lysine at 0.5 g/L; **Mg_1:** Magnesium at 10 g/L; **Fe:** Iron III at 0.1 g/L.

Table 27 - Results obtained in Phase 2 for the different inoculums of *E. coli*.

Culture Media	CFU/mL	Average	Standard Deviation
NA	1.72E+09	1.64E+09	2.02E+08
	1.61E+09		
	1.65E+09		
	1.56E+09		
	1.47E+09		
	1.24E+09		
	1.84E+09		
	1.79E+09		
	1.89E+09		
NA_GLU	1.86E+09	1.63E+09	2.08E+08
	1.70E+09		
	1.75E+09		
	1.40E+09		
	1.62E+09		
	1.35E+09		
	1.35E+09		
	1.85E+09		
	1.76E+09		
NA_SAC_4	1.76E+09	1.59E+09	1.91E+08
	1.65E+09		
	1.76E+09		
	1.35E+09		
	1.31E+09		
	1.43E+09		
	1.78E+09		

	1.75E+09		
	1.51E+09		
NA_LYS_0,5	1.90E+09	1.70E+09	1.80E+08
	1.77E+09		
	1.91E+09		
	1.54E+09		
	1.48E+09		
	1.44E+09		
	1.69E+09		
	1.87E+09		
	1.73E+09		
	1.78E+09		
1.69E+09			
1.72E+09			
1.66E+09			
1.51E+09			
1.29E+09			
1.61E+09			
1.69E+09			
1.69E+09			
NA_Fe	1.73E+09	1.60E+09	1.91E+08
	1.64E+09		
	1.39E+09		
	1.45E+09		
	1.45E+09		
	1.39E+09		
	1.64E+09		
	1.79E+09		
	1.92E+09		

GLU: Glucose at 20 g/L; **SAC_4:** Sucrose at 40 g/L; **LYS_0,5:** Lysine at 0.5 g/L; **Mg_1:** Magnesium at 10 g/L; **Fe:** Iron III at 0.1 g/L.

Table 28 - Results obtained in Phase 2 for the different inoculums of *L. mesenteroides*

Culture Media	CFU/mL	Average	Standard Deviation
NA	8.30E+07	1.03E+08	3.69E+07
	8.30E+07		
	6.30E+07		
	8.00E+07		
	8.40E+07		
	7.90E+07		
	1.54E+08		
	1.56E+08		
	1.43E+08		
	NA_GLU		

	9.00E+07		
	7.20E+07		
	6.70E+07		
	8.10E+07		
	6.40E+07		
	1.27E+08		
	1.48E+08		
	1.37E+08		
<i>NA_SAC_4</i>	5.30E+07	6.79E+07	1.51E+07
	5.30E+07		
	6.70E+07		
	5.40E+07		
	6.10E+07		
	6.40E+07		
	8.10E+07		
	9.50E+07		
	8.30E+07		
<i>NA_LYS_0,5</i>	7.80E+07	1.05E+08	3.50E+07
	8.80E+07		
	8.40E+07		
	8.20E+07		
	7.60E+07		
	8.20E+07		
	1.54E+08		
	1.49E+08		
	1.51E+08		
<i>NA_Mg_1</i>	9.20E+07	1.02E+08	3.03E+07
	9.80E+07		
	8.70E+07		
	8.10E+07		
	8.60E+07		
	5.80E+07		
	1.33E+08		
	1.37E+08		
	1.49E+08		
<i>NA_Fe</i>	6.10E+07	7.59E+07	1.47E+07
	7.60E+07		
	6.50E+07		
	6.60E+07		
	8.20E+07		
	5.60E+07		
	9.80E+07		
	9.00E+07		
	8.90E+07		

GLU: Glucose at 20 g/L; **SAC_4:** Sucrose at 40 g/L; **LYS_0,5:** Lysine at 0.5 g/L; **Mg_1:** Magnesium at 10 g/L; **Fe:** Iron III at 0.1 g/L.

Table 29 - Results obtained in Phase 2 for the different inoculums of *P. aeruginosa*.

Culture Media	CFU/mL	Average	Standard Deviation
NA	1.43E+09	1.87E+09	1.04E+09
	1.50E+09		
	1.34E+09		
	1.23E+09		
	8.60E+08		
	8.40E+08		
	3.00E+09		
	3.60E+09		
	3.00E+09		
	3.00E+09		
NA_GLU	1.32E+09	1.90E+09	1.09E+09
	1.27E+09		
	9.10E+08		
	1.15E+09		
	9.30E+08		
	3.00E+09		
	3.20E+09		
	3.40E+09		
NA_SAC_4	1.41E+09	1.80E+09	8.96E+08
	1.82E+09		
	1.77E+09		
	9.80E+08		
	8.00E+08		
	9.50E+08		
	2.68E+09		
	2.32E+09		
	3.48E+09		
NA_LYS_0,5	1.46E+09	1.89E+09	1.13E+09
	1.79E+09		
	1.17E+09		
	9.20E+08		
	1.01E+09		
	7.50E+08		
	3.00E+09		
	3.00E+09		
	3.90E+09		
	3.90E+09		
NA_Mg_1	1.88E+09	1.31E+09	5.94E+08
	1.81E+09		
	1.52E+09		
	9.60E+08		
	1.11E+09		
	9.60E+08		

	2.40E+08		
	1.96E+09		
NA_Fe	1.66E+09	1.72E+09	7.03E+08
	1.45E+09		
	1.17E+09		
	8.70E+08		
	1.08E+09		
	2.52E+09		
	2.40E+09		
	2.64E+09		

GLU: Glucose at 20 g/L; **SAC_4:** Sucrose at 40 g/L; **LYS_0,5:** Lysine at 0.5 g/L; **Mg_1:** Magnesium at 10 g/L; **Fe:** Iron III at 0.1 g/L.

Table 30 - Statistical Data Analysis from Phase 2 of the bacteria.

Microorganism	Culture Media	Test Analysis	Sig.
<i>B. cereus</i>	NA_GLU	One-Way ANOVA	1
	NA_SAC_4	One-Way ANOVA	0.459
	NA_LYS_0,5	One-Way ANOVA	0.985
	NA_Mg_1	One-Way ANOVA	0.030
	NA_Fe	One-Way ANOVA	1
<i>E. coli</i>	NA_GLU	One-Way ANOVA	1
	NA_SAC_4	One-Way ANOVA	0.979
	NA_LYS_0,5	One-Way ANOVA	0.960
	NA_Mg_1	Mann-Whitney	0.825
	NA_Fe	One-Way ANOVA	0.991
<i>L. mesenteroides</i>	NA_GLU	One-Way ANOVA	0.991
	NA_SAC_4	One-Way ANOVA	0.030
	NA_LYS_0,5	Mann-Whitney	0.965
	NA_Mg_1	One-Way ANOVA	1
	NA_Fe	One-Way ANOVA	0.324
<i>P. aeruginosa</i>	NA_GLU	Mann-Whitney	0.923
	NA_SAC_4	One-Way ANOVA	1
	NA_LYS_0,5	One-Way ANOVA	1
	NA_Mg_1	One-Way ANOVA	0.691
	NA_Fe	One-Way ANOVA	1

A.2. Results obtained in Phase 2 for Yeasts

Table 31 - Results obtained in Phase 2 for the different inoculums of *S. cerevisiae*, when comparing PDA with PDA_GLU_4, PDA_SAC_4, PDA_FRU_4, PDA_BIO, PDA_THI, PDA_Mg_1 and PDA_Fe.

Culture Media	CFU/mL	Average	Standard Deviation
PDA	3.20E+06	9,91E+05	1.42E+06
	2.90E+06		
	2.50E+06		

	3.10E+04		
	3.95E+04		
	3.20E+04		
	7.50E+04		
	7.65E+04		
	6.80E+04		
<i>PDA_GLU_4</i>	3.40E+06	9.73E+05	1.42E+06
	2.85E+06		
	2.25E+06		
	3.00E+03		
	4.05E+04		
	2.95E+04		
	6.80E+04		
	5.80E+04		
	5.70E+04		
<i>PDA_SAC_4</i>	3.05E+06	8.28E+05	1.19E+06
	1.90E+06		
	2.15E+06		
	2.95E+04		
	2.35E+04		
	2.50E+04		
	9.00E+04		
	1.10E+05		
	7.35E+04		
<i>PDA_FRU_4</i>	1.75E+06	7.69E+05	1.10E+06
	2.45E+06		
	2.45E+06		
	2.30E+04		
	1.55E+04		
	1.95E+04		
	7.00E+04		
	7.05E+04		
	7.10E+04		
<i>PDA_BIO</i>	3.10E+06	9.61E+05	1.37E+06
	2.80E+06		
	2.40E+06		
	3.40E+04		
	2.70E+04		
	3.55E+04		
	7.35E+04		
	9.10E+04		
	8.55E+04		
<i>PDA_THI</i>	1.95E+06	6.87E+05	9.48E+05
	1.90E+06		
	2.00E+06		
	3.30E+04		

	2.75E+04		
	4.10E+04		
	7.85E+04		
	8.05E+04		
	6.85E+04		
<i>PDA_Mg_1</i>	2.15E+06	8.23E+05	1.18E+06
	2.60E+06		
	2.40E+06		
	2.05E+04		
	2.80E+04		
	2.00E+04		
	6.70E+04		
	5.70E+04		
<i>PDA_Fe</i>	6.80E+04	8.36E+05	1.22E+06
	2.65E+06		
	2.15E+06		
	2.20E+04		
	3.75E+04		
	7.95E+04		
7.95E+04			

GLU_4: Glucose at 40 g/L; **SAC_4:** Sucrose at 40 g/L; **FRU_4:** Fructose at 40 g/L; **BIO:** Biotin at 0.02 g/L; **THI:** Thiamine at 0.002 g/L; **Mg_1:** Magnesium at 10 g/L; **Fe:** Iron III at 0.1 g/L.

Table 32 - Results obtained in Phase 2 for the different inoculums of *S. cerevisiae*, when comparing PDA with PDA_P_0,5.

Culture Media	CFU/mL	Average	Standard Deviation
<i>PDA</i>	5.70E+04	4.26E+04	1.85E+04
	6.35E+04		
	7.15E+04		
	4.00E+04		
	3.80E+04		
	4.45E+04		
	1.75E+04		
	2.30E+04		
	2.80E+04		
<i>PDA_P_0,5</i>	6.20E+04	4.66E+04	2.26E+04
	7.65E+04		
	7.85E+04		
	4.00E+04		
	4.95E+04		
	4.70E+04		
	2.85E+04		
	2.15E+04		
	1.60E+04		

P_0,5: Potassium at 1 g/L.

Table 33 - Results obtained in Phase 2 for the different inoculums of *S. cerevisiae*, when comparing PDA with PDA_LYA_0,5.

Culture Media	CFU/mL	Average	Standard Deviation
<i>PDA</i>	3.20E+06	1.07E+06	1.36E+06
	2.90E+06		
	2.50E+06		
	7.50E+04		
	7.65E+04		
	6.80E+04		
	2.55E+05		
	3.05E+05		
	2.65E+05		
<i>PDA_LYS_0,5</i>	2.65E+05	1.04E+06	1.14E+06
	3.30E+05		
	3.50E+05		
	2.40E+06		
	2.35E+06		
	2.50E+06		
	7.10E+04		

LYS_0,5: Lysine at 0.5 g/L;

Table 34 - Results obtained in Phase 2 for the different inoculums of *C. intermedia*.

Culture Media	CFU/mL	Average	Standard Deviation
<i>PDA</i>	4.85E+07	5.16E+07	1.18E+07
	3.65E+07		
	4.90E+07		
	4.45E+07		
	7.45E+07		
	5.35E+07		
	5.50E+07		
<i>PDA_GLU_4</i>	4.70E+07	4.91E+07	1.01E+07
	4.65E+07		
	4.60E+07		
	3.85E+07		
	4.20E+07		
	3.70E+07		
	6.45E+07		
	6.20E+07		
	5.85E+07		
<i>PDA_SAC_4</i>	5.55E+07	5.09E+07	1.36E+07
	5.35E+07		
	6.00E+07		
	3.60E+07		
	2.85E+07		
	3.75E+07		

	6.90E+07		
	5.70E+07		
	6.10E+07		
<i>PDA_FRU_4</i>	4.00E+07	5.01E+07	1.11E+07
	5.30E+07		
	5.00E+07		
	3.80E+07		
	3.60E+07		
	4.60E+07		
	6.10E+07		
	6.85E+07		
	5.80E+07		
<i>PDA_LYS_0,5</i>	5.20E+07	4.93E+07	1.07E+07
	4.50E+07		
	4.50E+07		
	3.80E+07		
	4.10E+07		
	3.75E+07		
	6.10E+07		
	5.55E+07		
6.85E+07			
<i>PDA_BIO</i>	5.70E+07	5.16E+07	7.82E+06
	4.60E+07		
	5.45E+07		
	4.65E+07		
	3.95E+07		
	4.45E+07		
	5.75E+07		
	5.45E+07		
	6.40E+07		
<i>PDA_THI</i>	5.00E+07	4.83E+07	6.95E+06
	4.20E+07		
	5.15E+07		
	4.30E+07		
	4.25E+07		
	4.75E+07		
	6.15E+07		
<i>PDA_Mg_1</i>	5.35E+07	4.94E+07	9.19E+06
	5.50E+07		
	5.65E+07		
	3.70E+07		
	3.90E+07		
	3.60E+07		
	5.40E+07		
	5.85E+07		
	5.50E+07		

<i>PDA_Fe</i>	6.40E+07	5.62E+07	1.43E+07
	5.00E+07		
	3.85E+07		
	3.75E+07		
	5.00E+07		
	4.90E+07		
	7.00E+07		
	7.35E+07		
	7.30E+07		
	7.30E+07		
<i>PDA_P_0,5</i>	5.05E+07	5.09E+07	1.27E+07
	5.25E+07		
	5.30E+07		
	4.50E+07		
	2.90E+07		
	3.70E+07		
	5.65E+07		
	6.85E+07		
	6.60E+07		
	6.60E+07		

GLU_4: Glucose at 40 g/L; **SAC_4:** Sucrose at 40 g/L; **FRU_4:** Fructose at 40 g/L;
LYS_0,5: Lysine at 0.5 g/L; **BIO:** Biotin at 0.02 g/L; **THI:** Thiamine at 0.002 g/L; **Mg_1:**
Magnesium at 10 g/L; **Fe:** Iron III at 0.1 g/L; **P_0,5:** Potassium at 1 g/L.

Table 35 - Results obtained in Phase 2 for the different inoculums of *P. fermentans*, when comparing PDA with PDA_GLU_4, PDA_SAC_4, PDA_FRU_4, PDA_LYS_0,5, PDA_BIO, PDA_THI, PDA_Mg_1 and PDA_Fe.

Culture Media	CFU/mL	Average	Standard Deviation
<i>PDA</i>	3.55E+05	8.21E+05	5.96E+05
	2.25E+05		
	3.60E+05		
	5.65E+05		
	5.10E+05		
	5.75E+05		
	1.65E+06		
	1.50E+06		
	1.65E+06		
	1.65E+06		
<i>PDA_GLU_4</i>	3.55E+05	9.47E+05	8.30E+05
	4.65E+05		
	3.40E+05		
	4.85E+05		
	4.55E+05		
	4.25E+05		
	2.50E+06		
	1.50E+06		
	2.00E+06		
	2.00E+06		
<i>PDA_SAC_4</i>	3.75E+05	5.91E+05	5.57E+05
	3.75E+05		
	4.30E+05		

	2.70E+05		
	1.20E+05		
	5.00E+05		
	1.50E+05		
	1.55E+06		
	1.55E+06		
<i>PDA_FRU_4</i>	3.75E+05	1.08E+06	1.04E+06
	3.65E+05		
	3.90E+05		
	4.30E+05		
	4.70E+05		
	4.75E+05		
	1.80E+06		
	2.40E+06		
	3.00E+06		
<i>PDA_LYS_0,5</i>	4.15E+05	8.36E+05	5.74E+05
	6.85E+05		
	2.95E+05		
	3.50E+05		
	4.90E+05		
	5.40E+05		
	1.50E+06		
	1.70E+06		
	1.55E+06		
<i>PDA_BIO</i>	4.95E+05	9.09E+05	7.25E+05
	4.30E+05		
	3.85E+05		
	5.40E+05		
	3.65E+05		
	4.70E+05		
	1.50E+06		
	1.70E+06		
	2.30E+06		
<i>PDA_THI</i>	4.00E+05	8.16E+05	6.45E+05
	4.10E+05		
	4.70E+05		
	3.60E+05		
	2.85E+05		
	4.15E+05		
	1.80E+06		
	1.50E+06		
	1.70E+06		
<i>PDA_Mg_1</i>	3.75E+05	8.36E+05	6.06E+05
	3.45E+05		
	3.45E+05		
	5.45E+05		

	5.25E+05		
	4.90E+05		
	1.75E+06		
	1.65E+06		
	1.50E+06		
<i>PDA_Fe</i>	3.15E+05	1.07E+06	1.01E+06
	4.55E+05		
	4.15E+05		
	3.40E+05		
	3.35E+05		
	9.85E+05		
	3.20E+06		
	1.55E+06		
	2.00E+06		

GLU_4: Glucose at 40 g/L; **SAC_4:** Sucrose at 40 g/L; **FRU_4:** Fructose at 40 g/L; **LYS_0,5:** Lysine at 0.5 g/L; **BIO:** Biotin at 0.02 g/L; **THI:** Thiamine at 0.002 g/L; **Mg_1:** Magnesium at 10 g/L; **Fe:** Iron III at 0.1 g/L.

Table 36 - Results obtained in Phase 2 for the different inoculums of *P. fermentans*, when comparing PDA with PDA_P_0,5.

<i>Culture Media</i>	CFU/mL	Average	Standard Deviation
<i>PDA</i>	6.05E+05	4.60E+05	6.80E+04
	4.70E+05		
	4.55E+05		
	4.75E+05		
	3.95E+05		
	4.80E+05		
	4.30E+05		
	3.60E+05		
	4.70E+05		
<i>PDA_P_0,5</i>	5.25E+05	4.87E+05	7.08E+04
	4.05E+05		
	5.25E+05		
	5.30E+05		
	3.80E+05		
	5.25E+05		
	5.90E+05		
	4.85E+05		
	4.15E+05		

P_0,5: Potassium at 1 g/L.

A.3. Results obtained in Phase 2 for the Mold

Table 37 - Results obtained in Phase 2 for the different inoculums of *N. fischeri*.

Culture Media	CFU/mL	Average	Standard Deviation
<i>PDA</i>	3.35E+06	3.98E+06	4.86E+05
	4.45E+06		
	4.55E+06		
	3.95E+06		
	3.60E+06		
	4.05E+06		
	4.45E+06		
	3.40E+06		
<i>PDA_GLU_6</i>	3.45E+06	3.67E+06	4.07E+05
	3.65E+06		
	4.10E+06		
	3.15E+06		
	3.50E+06		
	3.70E+06		
	4.25E+06		
	4.10E+06		
3.15E+06			
<i>PDA_SAC_6</i>	4.00E+06	3.68E+06	3.22E+05
	3.15E+06		
	3.80E+06		
	3.80E+06		
	3.50E+06		
	3.30E+06		
	4.10E+06		
	3.55E+06		
3.90E+06			
<i>PDA_FRU_4</i>	3.85E+06	3.84E+06	3.24E+05
	4.20E+06		
	4.15E+06		
	3.20E+06		
	3.50E+06		
	3.75E+06		
	4.10E+06		
	3.90E+06		
3.95E+06			
<i>PDA_BIO</i>	4.25E+06	3.96E+06	3.59E+05
	4.10E+06		
	4.00E+06		
	3.80E+06		
	4.10E+06		
	4.45E+06		
	4.05E+06		

	3.25E+06		
	3.60E+06		
<i>PDA_THI</i>	4.40E+06	4.18E+06	4.21E+05
	4.70E+06		
	3.85E+06		
	3.95E+06		
	3.70E+06		
	4.35E+06		
	3.90E+06		
	3.90E+06		
	4.90E+06		
<i>PDA_NaCl_0,1</i>	4.15E+06	4.03E+06	5.24E+05
	4.95E+06		
	3.80E+06		
	4.55E+06		
	3.35E+06		
	3.40E+06		
	4.30E+06		
	4.00E+06		
	3.75E+06		
<i>PDA_Mg_1</i>	3.85E+06	3.20E+06	5.18E+05
	2.95E+06		
	3.15E+06		
	3.00E+06		
	2.60E+06		
	2.85E+06		
	4.10E+06		
	2.75E+06		
	3.55E+06		
<i>PDA_Ca_1</i>	2.30E+06	2.93E+06	4.32E+05
	3.25E+06		
	2.95E+06		
	3.70E+06		
	3.15E+06		
	2.95E+06		
	2.35E+06		
	2.90E+06		
	2.80E+06		
<i>PDA_Fe</i>	3.30E+06	3.39E+06	5.57E+05
	3.80E+06		
	4.10E+06		
	3.30E+06		
	3.10E+06		
	2.35E+06		
	3.75E+06		
	2.90E+06		

	3.90E+06		
<i>PDA_P_0,5</i>	3.65E+06	3.04E+06	3.84E+05
	3.45E+06		
	2.80E+06		
	2.60E+06		
	3.15E+06		
	2.45E+06		
	3.00E+06		
	3.05E+06		
	3.20E+06		

GLU_6: Glucose at 60 g/L; **SAC_6:** Sucrose at 60 g/L; **FRU_4:** Fructose at 40 g/L; **BIO:** Biotin at 0.02 g/L; **THI:** Thiamine at 0.002 g/L; **NaCl_0,1:** Sodium salt at 1 g/L; **Mg_1:** Magnesium 10 g/L; **Ca_1:** Calcium at 10 g/L; **Fe:** Iron III at 0.1 g/L; **P_0,5:** Potassium at 1 g/L.

Table 38 - Statistical Data Analysis from Phase 2 of the mould.

Microorganism	Culture Media	Test Analysis	Sig.
<i>N. fischeri</i>	PDA_GLU_6	One-Way ANOVA	0.938
	PDA_SAC_6	One-Way ANOVA	0.945
	PDA_FRU_4	One-Way ANOVA	1
	PDA_BIO	One-Way ANOVA	1
	PDA_THI	One-Way ANOVA	0.996
	PDA_NaCl_0,1	One-Way ANOVA	1
	PDA_Mg_1	One-Way ANOVA	0.018
	PDA_Ca_1	One-Way ANOVA	0.0002
	PDA_Fe	One-Way ANOVA	0.191
	PDA_P_0,5	One-Way ANOVA	0.001

GLU_6: Glucose at 60 g/L; **SAC_6:** Sucrose at 60 g/L; **FRU_4:** Fructose at 40 g/L; **BIO:** Biotin at 0.02 g/L; **THI:** Thiamine at 0.002 g/L; **NaCl_0,1:** Sodium salt at 1 g/L; **Mg_1:** Magnesium 10 g/L; **Ca_1:** Calcium at 10 g/L; **Fe:** Iron III at 0.1 g/L; **P_0,5:** Potassium at 1 g/L.

B. Results obtained for Phase 3

B.1. Results obtained in Phase 3 for the Bacteria

Table 39 - Results obtained in Phase 3 for the different inoculums of *E. coli*.

Culture Media	CFU/mL	Average	Standard Deviation
NA	2.11E+09	1.98E+09	1.12E+08
	1.96E+09		
	1.98E+09		
	2.02E+09		
	1.96E+09		
	1.97E+09		
	1.79E+09		
	2.16E+09		
	1.87E+09		
201	2.00E+09	1.81E+09	1.17E+08
	1.94E+09		
	1.85E+09		
	1.69E+09		
	1.68E+09		
	1.80E+09		
	1.67E+09		
	1.82E+09		
	1.88E+09		
202	1.93E+09	1.86E+09	1.84E+08
	2.07E+09		
	2.15E+09		
	1.67E+09		
	1.95E+09		
	1.86E+09		
	1.70E+09		
	1.60E+09		
	1.85E+09		
203	1.88E+09	1.86E+09	1.99E+08
	1.77E+09		
	1.74E+09		
	1.69E+09		
	1.69E+09		
	1.64E+09		
	2.10E+09		
	2.12E+09		
	2.11E+09		

Table 40 - Results obtained in Phase 3 for the different inoculums of *B. cereus*.

Culture Media	CFU/mL	Average	Standard Deviation
NA	1.07E+08	9.38E+07	2.53E+07
	8.50E+07		
	8.00E+07		
	1.20E+08		
	1.30E+08		
	1.18E+08		
	7.50E+07		
	6.10E+07		
	6.80E+07		
201	8.60E+07	9.19E+07	2.65E+07
	8.50E+07		
	7.20E+07		
	1.20E+08		
	1.25E+08		
	1.32E+08		
	6.30E+07		
	7.40E+07		
	7.00E+07		
202	9.00E+07	9.30E+07	2.11E+07
	9.20E+07		
	7.10E+07		
	1.14E+08		
	1.21E+08		
	1.21E+08		
	8.10E+07		
	8.20E+07		
	6.50E+07		
203	1.08E+08	1.03E+08	2.84E+07
	9.50E+07		
	7.90E+07		
	1.38E+08		
	1.28E+08		
	1.44E+08		
	7.50E+07		
	9.00E+07		
	6.70E+07		

Table 41 - Results obtained in Phase 3 for the different inoculums of *P. aeruginosa*.

Culture Media	CFU/mL	Average	Standard Deviation
NA	2.26E+09	2.12E+09	2.12E+08
	1.83E+09		
	2.38E+09		
	1.84E+09		

	2.11E+09		
	2.25E+09		
	2.18E+09		
201	2.48E+09	2.08E+09	3.05E+08
	2.45E+09		
	2.09E+09		
	2.05E+09		
	1.73E+09		
	1.54E+09		
	2.19E+09		
	2.20E+09		
	1.99E+09		
202	2.24E+09	2.21E+09	2.98E+08
	2.63E+09		
	2.55E+09		
	1.82E+09		
	1.72E+09		
	2.22E+09		
	2.17E+09		
	2.36E+09		
	2.18E+09		
203	2.60E+09	2.24E+09	2.22E+08
	2.32E+09		
	2.34E+09		
	1.90E+09		
	1.97E+09		
	2.20E+09		
	2.26E+09		
	2.33E+09		

Table 42 - Results obtained in Phase 3 for the different inoculums of *L. mesenteroides*.

Culture Media	CFU/mL	Average	Standard Deviation
NA	1.28E+09	1.55E+09	2.59E+08
	1.76E+09		
	1.61E+09		
	1.26E+09		
	1.21E+09		
	1.40E+09		
	1.73E+09		
	1.85E+09		
	1.82E+09		
201	1.15E+09	1.24E+09	1.46E+08
	1.31E+09		
	1.22E+09		
	1.32E+09		

	1.00E+09		
	1.07E+09		
	1.31E+09		
	1.32E+09		
	1.47E+09		
202	1.15E+09	7.44E+08	2.99E+08
	1.06E+09		
	1.05E+09		
	3.80E+08		
	5.10E+08		
	4.00E+08		
	7.20E+08		
	8.70E+08		
203	5.60E+08	9.32E+08	3.83E+08
	1.38E+09		
	1.27E+09		
	1.56E+09		
	5.70E+08		
	5.70E+08		
	5.20E+08		
	8.30E+08		
7.80E+08			
9.10E+08			

Table 43 - Statistical Data Analysis from Phase 3 of the bacteria.

Microorganism	Culture Media	Test Analysis	Sig.
<i>B. cereus</i>	201	One-Way ANOVA	0.999
	202	One-Way ANOVA	1
	203	One-Way ANOVA	0.880
<i>E. coli</i>	201	One-Way ANOVA	0.141
	202	One-Way ANOVA	0.398
	203	One-Way ANOVA	0.357
<i>L. mesenteroides</i>	201	One-Way ANOVA	0.125
	202	One-Way ANOVA	6.79E-06
	203	One-Way ANOVA	0.0004
<i>P. aeruginosa</i>	201	One-Way ANOVA	0.990
	202	One-Way ANOVA	0.912
	203	One-Way ANOVA	0.827

B.2. Results obtained in Phase 3 for the Yeasts

Table 44 - Results obtained in Phase 3 for the different inoculums of *C. intermedia*.

Culture Media	CFU/mL	Average	Standard Deviation
PDA	2.55E+07	2.74E+07	3.98E+06
	2.80E+07		
	3.15E+07		
	3.25E+07		
	2.80E+07		
	3.05E+07		
	2.15E+07		
	2.80E+07		
	2.15E+07		
	2.15E+07		
401	2.60E+07	2.78E+07	4.41E+06
	3.35E+07		
	3.15E+07		
	3.05E+07		
	2.75E+07		
	2.95E+07		
	2.95E+07		
	2.05E+07		
	2.15E+07		
	2.15E+07		
402	3.60E+07	2.76E+07	6.37E+06
	2.55E+07		
	3.25E+07		
	3.00E+07		
	3.65E+07		
	2.00E+07		
	2.25E+07		
	2.10E+07		
	2.40E+07		
	2.40E+07		
501	2.65E+07	2.52E+07	2.24E+06
	2.95E+07		
	2.25E+07		
	2.40E+07		
	2.25E+07		
	2.60E+07		
	2.50E+07		
	2.65E+07		
	2.40E+07		
	2.40E+07		
502	3.00E+07	2.79E+07	5.13E+06
	3.45E+07		
	2.80E+07		
	2.40E+07		
	3.45E+07		
	3.25E+07		

	2.25E+07		
	2.25E+07		
	2.30E+07		
601	3.00E+07	2.70E+07	5.24E+06
	2.50E+07		
	2.95E+07		
	3.35E+07		
	3.35E+07		
	2.45E+07		
	1.70E+07		
	2.40E+07		
	2.60E+07		
	2.60E+07		
602	3.30E+07	2.70E+07	5.26E+06
	2.65E+07		
	2.45E+07		
	3.45E+07		
	2.90E+07		
	2.85E+07		
	2.55E+07		
	1.65E+07		
	2.50E+07		
	2.50E+07		

Table 45 - Results obtained in Phase 3 for the different inoculums of *S. cerevisiae*.

Culture Media	CFU/mL	Average	Standard Deviation
PDA	4.40E+04	7.38E+04	2.02E+04
	6.00E+04		
	6.10E+04		
	1.02E+05		
	9.80E+04		
	6.30E+04		
	7.95E+04		
	8.30E+04		
401	5.55E+04	7.27E+04	1.90E+04
	4.65E+04		
	5.50E+04		
	9.85E+04		
	8.40E+04		
	9.95E+04		
	6.45E+04		
	7.50E+04		
	7.60E+04		
402	3.40E+04	6.97E+04	2.46E+04
	4.35E+04		
	4.50E+04		
	9.50E+04		

	9.35E+04		
	1.01E+05		
	6.55E+04		
	7.25E+04		
	7.75E+04		
501	4.90E+04	7.70E+04	2.69E+04
	4.40E+04		
	5.05E+04		
	9.35E+04		
	1.25E+05		
	9.55E+04		
	6.60E+04		
	8.00E+04		
	8.95E+04		
502	4.95E+04	7.17E+04	2.27E+04
	4.75E+04		
	5.05E+04		
	1.02E+05		
	8.95E+04		
	1.06E+05		
	6.00E+04		
	7.65E+04		
	6.45E+04		
601	4.70E+04	7.25E+04	1.93E+04
	5.25E+04		
	5.80E+04		
	8.10E+04		
	1.02E+05		
	1.00E+05		
	7.45E+04		
	6.55E+04		
	7.25E+04		
602	4.30E+04	7.36E+04	2.52E+04
	5.20E+04		
	4.85E+04		
	9.05E+04		
	1.11E+05		
	1.10E+05		
	6.40E+04		
	7.00E+04		
	7.45E+04		

Table 46 - Results obtained in Phase 3 for the different inoculums of *P. fermentans*.

Culture Media	CFU/mL	Average	Standard Deviation
PDA	4.30E+05	4.86E+05	1.32E+05

	4.30E+05		
	4.05E+05		
	3.70E+05		
	4.75E+05		
	3.65E+05		
	7.85E+05		
	5.60E+05		
	5.50E+05		
	5.55E+05		
	5.65E+05		
	4.00E+05		
	4.15E+05		
401	3.80E+05	5.01E+05	9.05E+04
	4.40E+05		
	5.70E+05		
	6.15E+05		
	5.70E+05		
	5.60E+05		
	4.80E+05		
	4.55E+05		
	3.50E+05		
402	4.95E+05	5.06E+05	1.51E+05
	2.45E+05		
	6.15E+05		
	7.55E+05		
	6.00E+05		
	5.80E+05		
	4.80E+05		
501	4.90E+05	5.30E+05	4.80E+04
	5.80E+05		
	5.20E+05		
	4.70E+05		
	5.40E+05		
	5.45E+05		
	4.15E+05		
502	3.60E+05	5.22E+05	8.90E+04
	6.10E+05		
	5.90E+05		
	6.15E+05		
	5.55E+05		
	3.85E+05		
	3.75E+05		
	5.50E+05		
601	4.00E+05	4.59E+05	1.02E+05
	3.80E+05		
	3.70E+05		

	5.60E+05		
	6.45E+05		
	4.70E+05		
602	5.45E+05	4.82E+05	9.13E+04
	6.05E+05		
	5.35E+05		
	3.95E+05		
	3.35E+05		
	4.25E+05		
	5.85E+05		
	4.40E+05		
	4.75E+05		

B.3. Results obtained in Phase 3 for the Mold

Table 47 - Results obtained in Phase 3 for the different inoculums of *N. fischeri*.

Culture Media	CFU/mL	Average	Standard Deviation
PDA	1.50E+06	1.91E+06	3.18E+05
	1.60E+06		
	2.20E+06		
	2.05E+06		
	1.55E+06		
	1.75E+06		
	2.10E+06		
	2.00E+06		
	2.40E+06		
	701		
1.60E+06			
1.50E+06			
1.50E+06			
1.50E+06			
1.60E+06			
2.70E+06			
1.70E+06			
1.60E+06			
702		1.85E+06	1.75E+06
	1.75E+06		
	1.50E+06		
	1.55E+06		
	1.65E+06		
	1.85E+06		
	1.85E+06		
	2.00E+06		
801	2.05E+06	1.99E+06	3.22E+05
	1.60E+06		

	1.70E+06		
	1.50E+06		
	2.00E+06		
	2.15E+06		
	2.40E+06		
	2.35E+06		
	2.15E+06		
802	1.50E+06	1.63E+06	1.82E+05
	1.60E+06		
	1.35E+06		
	1.95E+06		
	1.65E+06		
	1.50E+06		
	1.65E+06		
	1.85E+06		
901	1.85E+06	1.66E+06	3.25E+05
	1.50E+06		
	1.70E+06		
	1.50E+06		
	1.50E+06		
	1.10E+06		
	2.20E+06		
	1.55E+06		
	2.00E+06		
902	1.50E+06	1.72E+06	2.96E+05
	1.65E+06		
	1.65E+06		
	1.50E+06		
	1.50E+06		
	1.55E+06		
	2.40E+06		
	1.95E+06		
	1.75E+06		

B.4. Results obtained in Phase 3 for the pH

Table 48 - Results obtained in Phase 3 for the pH measurements of the Culture Media.

Media Culture	pH	Average	Standard Deviation	T (°C)
201	7,23	7,230	0,000	23,1
	7,23			23,3
	7,23			23,2
	7,23			23,3
202	7,19	7,213	0,036	23,3
	7,26			23,4

	7,18			23,4
	7,22			23,6
203	7,05	7,085	0,044	23,3
	7,07			23,6
	7,15			23,3
	7,07			23,3
401	5,99	6,007	0,015	22,7
	6,02			22,1
	6,01			22,2
402	6,18	6,180	0,030	22,2
	6,21			22,5
	6,15			22,5
501	6,04	6,047	0,040	23,2
	6,01			22,9
	6,09			23
502	6,13	6,137	0,012	24
	6,15			24,2
	6,13			24,3
601	6,04	6,027	0,015	24,9
	6,03			25
	6,01			24,7
602	6,14	6,143	0,006	25
	6,14			25
	6,15			25
701	5,94	5,930	0,010	23,6
	5,92			23,6
	5,93			23,6
702	5,98	5,967	0,015	23,8
	5,95			23,8
	5,97			23,8
801	5,88	5,873	0,006	23,5
	5,87			23,5
	5,87			23,5
802	6,01	5,990	0,017	23,9
	5,98			24
	5,98			24
901	5,92	5,920	0,000	23,5
	5,92			23,5
	5,92			23,5
902	6,01	6,000	0,010	23,7
	5,99			23,7
	6			23,6