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MICROORGANISM ANALYSIS IN ENSURING THE QUALITY AND SAFETY OF PHARMACEUTICAL AND NUTRACEUTICAL PRODUCTS

Relatório de estágio no âmbito do Mestrado em Bioquímica orientado pela Engenheira Sandra Sousa e coorientado pela Professora Doutora Paula Morais e apresentado ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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Resumo

Este projeto foi realizado no departamento de Controlo de Qualidade da Labialfarma, uma empresa farmacêutica e nutracêutica pertencente ao Grupo Ferraz, especificamente na área de Microbiologia.

Foi analisada a presença de microrganismos em múltiplas amostras de medicamentos, suplementos alimentares, matérias-primas, extratos alcoólicos e testes de estabilidade. Para garantir a qualidade e segurança dos produtos farmacêuticos não é suficiente fazer apenas a análise à presença de microrganismos, pois há vários fatores que podem influenciar o produto final. Consequentemente, o projeto em questão também inclui uma análise ao sistema de água purificada da empresa, controlo ambiental em todas as áreas (incluindo salas de produção, salas de higienização, salas de embalamento, etc.) e monitorização dos operadores diretamente envolvidos no fabrico do produto, bem como aqueles responsáveis pela pesagem de matérias-primas e embalamento do produto final. Todos os procedimentos foram baseados nos procedimentos internos do laboratório, desenvolvidos de acordo com a Farmacopeia Europeia.

Todos os produtos analisados estavam dentro das especificações estabelecidas e, portanto, foram aprovados. O sistema de água purificada, bem como o controlo ambiental das salas e a monitorização dos operadores, também apresentaram resultados positivos, concluindo que não afetam negativamente a qualidade do produto final.

Palavras-chave: Microbiologia; Microrganismos; Produtos Farmacêuticos; Controlo de Qualidade

Abstract

This project was conducted within the Quality Control department of Labialfarma, a pharmaceutical and nutraceutical company owned by the Ferraz Group, specifically in the area of Microbiology.

The presence of microorganisms was analyzed in multiple samples of drugs, nutritional supplements, raw materials, alcoholic extracts, and stability tests. To ensure the quality and safety of pharmaceutical products, it is insufficient to simply verify the presence of microorganisms as multiple factors can influence the final product. Consequently, the current project also includes an analysis of the company's purified water system, environmental control of all areas (including production rooms, sanitation rooms, packaging rooms, etc.), and monitoring of operators directly involved in product manufacturing, as well as those responsible for weighing raw materials and packaging the final product. All procedures were based on laboratory internal procedures, developed according to the European Pharmacopoeia.

All analyzed products were found to be within the established specifications and were therefore approved. The purified water system, as well as the environmental control of the rooms and the monitoring of the operators, also granted positive results, concluding that they did not adversely affect the quality of the final product.

Keywords: Microbiology; Microorganisms; Pharmaceutical products; Quality control

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List of Abbreviations

AC- Accelerated conditions

ALOA- Listeria agar acc. Ottaviani and Agosti

ATCC- American Type Culture Collection

CA- Columbia Agar

CFU- Colony-Forming Units

CTA- Cetrimide Agar

GC- Gas Chromatography

GI- Growth Inhibition

GLP- Good Laboratory Practices

GMLP- Good Microbiological Laboratory Practices

GMP- Good Manufacturing Practices

GP- Growth Promoting

GPhP- Good Pharmacopoeial Practices

HFB/FB- Fraser Broth

HPLC- High-Performance Liquid Chromatography

IgG- Immunoglobulin G

INT- Intermediate conditions

IP- Indicative Properties

ISO- International Organization for Standardization

mAb- Monoclonal Antibodies

MB- Enterobacteria Enrichment Mossel

MCA- MacConkey Agar

MCB- MacConkey Broth

MSA- Mannitol Salt Agar

NL- Number of Sampling Points

NW/AF- Net Water

OA- Oxford Agar Base

PEP- Buffered Sodium Chloride- Peptone Solution

PEP- Peptone Water

Ph. Eur- European Pharmacopoeia

QC- Quality Control

RCM- Reinforced Clostridial Medium

RH- Relative Humidity

RT- Real Time

RVB- Rappaport Vassiliadis Salmonella Enrichment Broth

SDA- Sabouraud Dextrose Agar

SDB- Sabouraud Dextrose Broth

SS- Stock Solution

STEC- Shiga toxin-producing *E. coli*

TAMC- Total Aerobic Microbial Count

TSA- Tryptic Soy Agar

TSB- Tryptic Soy Broth

TYMC- Yeast and Mold Count

VRB- Violet Red Bile Agar

VRBD- Violet Red Bile Dextrose Agar

WHO- World Health Organization

XLD- X.L.D Medium

Chapter I - Introduction

1. Labialfarma

Labialfarma, founded in 1981 and headquartered in Mortágua is a pharmaceutical and nutraceutical company owned by the Ferraz Group. At the facility, a diverse range of products, including pharmaceutical products, nutritional supplements, medical devices, cosmetic products, and teas are manufactured.

The current internship was carried out at Labialfarma, specifically in the quality control department.

1.1 Quality Control (QC)

Quality control (QC) is a critical process that involves systematically examining pharmaceutical products to ensure their safety, efficacy, and reliability. The QC is divided into 5 sectors : Physical-Chemical analysis of raw materials, IPC (In Process Control), Analytical Chromatography, Packaging Material, and Microbiology.

- Physical-Chemical analysis of Raw Materials – By performing various physical and chemical methods, this sector analyzes the raw materials, plants and extracts used in the manufacturing process of the products. It is also analyzed micronized plants used in the tea production.
- In Process Control – this sector operates within the production area and is responsible for ensuring that the product meets the specification while being produced during various phases of the manufacturing process.
- Analytical Chromatography – it is responsible for the analysis of raw materials, finished products and stability tests by High-Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) to identify and quantitate active substances, detect impurities and/or degradation products.
- Packaging Material - The primary and secondary packaging materials are verified in this sector. The difference between primary and secondary packaging is that primary packaging refers to the material that is in direct contact with the product (bottles, blisters, ampoules, etc.). On the other

hand, secondary packaging refers to the external material (labels, boxes, etc.). This verification guarantees the integrity of primary packaging. Moreover, it verifies the accuracy and clarity of the information written on the secondary packaging.

- Microbiology - it is responsible for the microorganism analysis in drugs, nutritional supplements, raw materials, alcoholic extracts, stability tests, and teas.

All QC sectors work together to achieve the same goal. This goal involves testing and validating both the raw materials used in the production of a drug, nutritional supplement or cosmetic product as well as analyzing and approving the finished products.

By adhering to quality guidelines and good practices, pharmaceutical companies can ensure the quality and safety of their products.

1.1.1 Good Manufacturing Practices (GMP)

Good Manufacturing Practices (GMP) is a set of guidelines defined by the World Health Organization (WHO) that comprehend all aspects of the manufacturing, testing and quality control of pharmaceutical products.

It involves the control over the facility, production stages, testing, labeling, storage, and documentation management. The aim of the GMP's is ensuring the quality of a product throughout the manufacturing process, rather than only at the end [1], [2].

Labialfarma is certified in GMP which ensures the compliance with all international and national quality standards providing a quality mark for clients and underscoring the company's commitment to delivering only high-quality products.

1.1.2 Good Pharmacopoeial Practices (GPhP)

Good Pharmacopoeial Practices (GPhP) are designed to ensure the quality, reliability, and consistency of pharmacopoeial standards. These practices aim to balance the development and maintain pharmacopoeial standards globally. By setting forth detailed and scientifically based specifications, the European

Pharmacopoeia (Ph. Eur) ensures consistency and quality in the manufacturing, control, and use of pharmaceuticals.

It is established in the pharmacopoeia acceptance criteria, including numerical limits, ranges, or other suitable measures, to determine to pass/fail outcomes when testing, thereby ensuring the integrity and efficacy of the products [3]. Throughout the development of the current project , the Ph. Eur is used as a guide in all of the analyses made [4].

1.1.3 Good Microbiological Laboratory Practices (GMLP)

Good Microbiological Laboratory Practices (GMLP) cover a range of measures aimed at maintaining a clean and controlled environment to prevent contamination and ensure accurate results. The WHO outlines the “Good practices for pharmaceutical quality control laboratories”, providing comprehensive guidelines for ensuring quality in pharmaceutical laboratories globally. It covers essential aspects such as laboratory organization, facilities, equipment, documentation, and personnel. It emphasizes adherence to Good Laboratory Practices (GLP) and Good Manufacturing Practices (GMP) to guarantee accurate and reliable testing of pharmaceutical products [5].

All analysis are conducted under aseptic conditions, a sterile environment, within a laminar flow hood, with appropriate clothing and thorough sterilization. Laminar flow hoods create a unidirectional airflow that sweeps away airborne contaminants, minimizing the risk of microbial contamination.

To stabilize the flow, ultraviolet (UV) lights should be turned on for one hour to ensure the sterilization of the working bench. Only sterile materials should be used, such as pipettes, petri dishes, and culture media. By following stringent aseptic techniques, contaminations from human sources should be minimized.

Nevertheless, while working on a laminar flow hood there are specific sterile techniques that one must follow. Before entering , the appropriate equipment should be in use (mask, gloves, hairnet). Prior to beginning work, the interior surfaces of the flow hood should be properly disinfected with 70% ethanol to eliminate potential contaminants.

Furthermore, controlling the quality and expiration date of media, reagents, and test strains, as well as regularly calibrating equipment (scale, pH meter, microscopes), are essential aspects of maintaining laboratory integrity [6].

1.2 Microbiology

The work was developed within the Microbiology department, where a wide range of microbiological analyses are conducted to detect the presence of common human pathogenic organisms, ensuring the safety and quality of the products. This department assumes the great responsibility to detect and identify microorganisms that could potentially contaminate consumer-oriented products as microorganism-contaminated products can pose significant health risk to consumer [4].

A critical component of microbiology is the stringent maintenance of aseptic conditions. By upholding the principles of GMLP and adhering to good practices, this department ensures high standards of quality and integrity, as well as guarantees the safety of the products that are analyzed. It is mandatory the use of a hairnet and uniform at all times, and gloves and mask when entering the airflow chamber to prevent contamination when performing the analysis.

All the necessary steps for ensuring the quality and microorganism-free products are carried out in the microbiology laboratory. These steps involve the preparation of culture media, the preparation of microorganism strains, and growth promotion as a positive control for the methods used.

In addition to the analysis of the products, there is a stability program of each product being conducted. Its aim is to monitor the shelf life, ensuring that it remains within specifications when stored under the recommended conditions. Acceptance criteria, quantities, and time points to consider are defined in the specification for each finished product [7].

Stability testing of drugs and nutritional supplements encompasses the systematic evaluation of their microbiological attributes and stability profiles over extended periods and under different storage conditions. There are three different conditions that the products are subjected to : Real Time (RT); Accelerated conditions (AC); Intermediate conditions (INT). The following table (Table 1)

presents the different conditions (Temperature and Relative Humidity (RH)), the duration and the different sampling times [8].

Table 1- Different conditions, duration, and sampling times for stability tests.

Study	Storage conditions	Example	
		Duration	Sampling time
Real Time	25 ºC ± 2 ºC 60% RH ± 5% RH	36 months	0, 3, 6, 9, 12, 18, 24 and 36 months
Intermediate Conditions	30 ºC ± 2 ºC 65% RH ± 5% RH	12 months	0, 6, 9, 12 months
Accelerate Conditions	40 ºC ± 2 ºC 75% RH ± 5% RH	6 months	0, 3, 6 months

Additionally, in this sector is also monitored all of the facility's rooms to analyze the presence of microorganisms as well as the purified water system. By applying such a meticulous analysis of all factors that could compromise the product and the company's integrity and safety, the microorganism contamination can be constantly monitored.

When analyzing the products, a broad list of microorganisms is assessed to guarantee thorough evaluation of microbial content: Total Aerobic Microbial Count (TAMC), Yeast and Mold Count (TYMC), Gram-Negative Bacteria, *Escherichia Coli, Salmonella spp., Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans, Clostridium,* and *Listeria monocytogenes.*

2. Microorganism contamination: Health concerns

Microbial contamination of pharmaceutical products can compromise their safety and efficacy, and for that reason, it is crucial to understand their impact on human health and well-being. The presence of microorganisms, such as bacteria, fungi, or viruses, in pharmaceuticals can pose serious health risks to consumers. These contaminants may compromise the therapeutic effectiveness of the products and, in some cases, lead to adverse effects.

Total Aerobic Microbial Count (TAMC) and Yeast and Mold Count (TYMC) are parameters to quantify microbial burden in samples. In regards to the TAMC analysis, it quantifies the total number of aerobic bacteria while the TYMC analysis assesses the levels of yeasts and molds. Finding high values of TAMC and TYMC on pharmaceuticals poses a significant health concern which can lead to adverse reactions from consumers.

Gram-negative bacteria are characterized by their distinct cell wall structure. These bacteria have a peptidoglycan cell wall and an outer membrane with lipopolysaccharides [9]. These lipopolysaccharides present in the outer membrane can cause endotoxin-related reactions in humans that can induce fever, chills, and hypotension in some cases [10].

Escherichia coli and *Salmonella spp.* are both gram-negative bacteria known for their rod-shaped morphology, that belong to the Enterobacteriaceae family . Both are facultative anaerobes and, therefore are capable of surviving in both aerobic and anaerobic environments. *E. coli* is commonly found in the intestines of humans and warm-blooded animals , while *Salmonella* is a foodborne pathogen [11], [12]. Some *E. coli* strains can cause urinary tract infections, pneumonia, blood infection, among other illnesses. The strain Shiga toxin-producing *E. coli* (STEC) can cause food poisoning, and it is usually transmitted through raw or undercooked food, raw milk, etc. [13]. *Salmonella* infection remains a major public health concern globally, as it is one of the most frequently isolated foodborne pathogens generally found in eggs, and dairy products [11]. The WHO identifies *Salmonella* as one of four leading causes of diarrhea worldwide, accentuating its significant impact on global health [14].

Staphylococcus aureus is a gram-positive bacterium with a spherical shape [15]. Unlike the microorganisms mentioned before, *S. aureus* lacks flagella and is non-motile. It is a pathogen responsible for infectious illnesses in humans. These can range from minor skin infections to more dangerous and potentially life-threatening health problems (e.g. bloodstream infection). This microorganism poses serious health challenges due to its ability to develop resistance to antibiotics. *S.aureus* biofilm creates a protective barrier around bacterial cells, making drugs partially or fully inactive against this pathogen. Besides that, biofilm

also plays a crucial role in its resilience against different environments, such as temperature changes, nutrient limitations, etc. [15], [16].

Pseudomonas aeruginosa is a gram-negative bacterium that belongs to the Pseudomonadaceae family, and it is known for its versatility in diverse environments especially in clinical settings. Therefore, this pathogen has become a concern in hospital-acquired infections [17]. As well as *S. aureus*, *Pseudomonas* can form biofilms that protect the bacteria from disinfectants and antibiotics. Pharmaceutical products contaminated with this pathogen can lead to pneumonia, urinary tract infections, etc.

Candida albicans is a fungus that is present in the human body (intestines, mouth, skin) in small amounts. Its presence is regulated by the microbiome, however when an imbalance occurs it leads to overgrowth, and it causes an infection known as Candidiasis [18].

Clostridium are strictly anaerobic, spore-forming bacilli. Spore formation allows *Clostridium* to persist in aerobic environments, in environments with low nutrient availability, and even under extreme conditions (high temperatures, UV radiation, chemical disinfectants, etc.). The spore-forming ability poses a challenge in healthcare because this resistance can lead to persistent contamination. This pathogen is responsible for gastrointestinal infections [19].

Listeria monocytogenes are gram-positive bacteria that can grow in a wide range of temperatures (1 ºC to 45º C), meaning they can survive and proliferate even in refrigeration temperatures. Listeriosis, an illness caused by these bacteria, is one of the most severe foodborne diseases. According to the WHO, despite the low incidence, the high mortality rate associated with this disease makes it a critical public health concern [20].

This enhances the importance of the meticulous microbiological analysis that is conducted to detect, identify, and quantify microorganisms to avoid contamination that could lead to severe health risks to consumers.

By implementing stringent microbial control measures, pharmaceutical companies demonstrate their commitment to product safety and adhere to GMPs. This commitment is vital for building and sustaining trust among consumers, healthcare professionals, and regulatory authorities, thereby ensuring the continued success and reputation of the pharmaceutical industry [21].

3. Aim

This project has the primary goal of safeguarding the safety and quality of the final product. Therefore, the aim is to analyze the presence of microorganisms in samples of drugs, nutritional supplements, alcoholic extracts, raw materials, and stability tests.

The final product can contain microbial load from multiple sources, including processing equipment, water used in production, the manufacturing environment, and the operators involved in the process [22]. Consequently, to deliver a safe, trustworthy, and high-quality product it will be also monitored the environmental conditions of all the rooms, the company's purified water system, and the operators involved in the manufacturing process.

The analytical framework is designed to encompass a broad spectrum of microorganisms, ensuring a comprehensive assessment of microbial presence.

Chapter II – Culture Media

1. Introduction

In pharmaceutical microbiology, culture media plays a crucial role by providing optimal conditions for microorganisms to grow and reproduce, enabling the identification and isolation of specific microorganisms [23].

Culture media formulations meet specific requirements based on nutritional needs, physiological characteristics, and growth preferences of the target microorganism. This process involves the measurement of the dehydrated media, the addition of a certain amount of purified water, the proper sterilization, and the pH adjustment. A crucial step in media preparation is the sterilization process which ensures that microbial contaminants are eliminated while preserving the integrity of the medium for the growth of target microorganisms.

2. Materials and Methods

2.1 Culture Media Preparation

The process of culture media preparation demands attention to detail, sterilization techniques and follow aseptic principles to prevent contamination.

On a calibrated and verified balance, it is measured the quantity of dehydrated culture medium specified by the manufacturer according to the desired volume to be prepared. Factors such as pH, temperature, and oxygen availability are optimized. Additionally, some culture media may be supplemented with specific nutrients. Afterward, it is added the corresponding volume of purified water and subsequently, sterilized.

2.2 Sterilization Process

Autoclaving is a common method for sterilizing culture media in microbiology laboratories. Before autoclaving, all material goes must be cleaned and washed on a specific program on the material washing machine [24].

Autoclaving involves subjecting the media to high-pressure steam at 121 ^{o}C $\pm$ 2 ^{o}C for 15 $\pm$ 1 minutes, generally. It is crucial to avoid approaches that can

compromise the efficacy of this method. Over-sterilization should be avoided, leading to precipitation, pH changes or even media component destruction. Equally important is to prevent under-sterilization, which could result in contamination of the media compromising experimental outcomes. To ensure effective sterilization, it is essential that the lids or caps are not fully tightened, allowing for proper circulation of steam. Furthermore, when verifying pH, it is crucial to calibrate the pH meter regularly and measure on media that is not excessively hot, yielding inaccurate results [25]. All disposable material (tips for micropipettes, etc.) and recoverable material (test tubes, tweezers, etc.) should be wrapped in sterilization bags with an autoclave tape. An autoclave tape must be placed over each piece of material, with the sterilization date, that should change color if the sterilization was successful [24].

This method is suitable for sterilizing liquid and solid media, however certain solid media, such as ALOA,XLD and VRBD, cannot be autoclaved. Instead, they are subjected to a water bath method instead at 100 ^{o}C for 10 minutes.

The method employed to confirm the success of the autoclaving process involves the utilization of a biological indicator which is introduced inside the autoclave in every cycle. This indicator has a purple stripe. If the sterilization process is successful, the stripe should remain purple. However, if the sterilization process fails to achieve efficacy, the stripe transitions to yellow, indicating the presence of viable spores of *Geobacillus stearothermophilus* [26]*:*

“ For steam sterilization or moist heat sterilization, the test organisms employed can be spores of a suitable strain of Geobacillus Stearothermophilus”.

- ISO 11138-7:2019

After the media sterilization by autoclaving or by a water bath method, the solid melted media, when cooled down, is dispensed onto an empty petri dish under aseptic conditions. When the media solidifies, each petri dish is labeled with the media abbreviation, batch and shelf life. In the context of microbiological control within various industries such as food, cosmetics, and pharmaceuticals, the pour plate method stands as a fundamental technique used on solid media. When inoculated, this method enables the growth and visualization of individual colonies .

Liquid media is maintained on the glass bottles followed by proper identification by labeling with the abbreviation of the medium, along with details regarding the shelf life and batch. The shelf life of both liquid and solid media is one month, less than one day [27].

2.3 Media Required

The following table (Table 2) specifies the culture media required for the analysis of each microorganism as well as the pH value.

Table 2- Media required for the analysis of each microorganism.

Microorganism	Media		
TAMC	Buffered Sodium Chloride-peptone Solution (PEP) pH: 7.0	Tryptic Soy Agar (TSA) pH: 7.3	––
TYMC	Buffered Sodium Chloride-peptone Solution (PEP) pH: 7.0	Sabouraud Dextrose Agar (SDA) pH: 5.6	––
Gram- negative bacteria	Tryptic Soy Broth (TSB) pH: 7.3	EE Enterobacteria Enrichment Mossel (MB) pH: 7.2	Violet Red Bile Dextrose Agar (VRBD) pH: 7.4
Escherichia coli	Tryptic Soy Broth (TSB) pH: 7.3	MacConkey Broth (MCB) pH: 7.4	MacConkey Agar (MCA) pH: 7.1
Salmonella spp.	Tryptic Soy Broth (TSB) pH: 7.3	Rappaport Vassiliadis Salmonella Enrichment Broth (RVB) pH: 5.2	X.L.D Medium (XLD) pH: 7.4
Staphylococcus aureus	Tryptic Soy Broth (TSB) pH: 7.3	Mannitol Salt Agar (MSA) pH: 7.4	––
Pseudomonas aeruginosa	Tryptic Soy Broth (TSB) pH: 7.3	Cetrimide Agar (CTA) pH: 7.2	––
Candida albicans	Sabouraud Dextrose Broth (SDB) pH: 5.6	Sabouraud Dextrose Agar (SDA) pH: 5.6	––
Clostridia	Reinforced Clostridial Medium (RCM) pH: 6.8	Columbia Agar (CA) pH: 7.3	––
Listeria monocytogenes	Fraser Broth	Fraser Broth	Listeria agar acc. Ottaviani and Agosti (ALOA) pH: 7.2

	(HFB) pH: 7.2	(FB) pH: 7.2	Oxford Agar Base (OA) pH: 7.0

Each microorganism necessitates an initial incubation in a liquid medium (Broth) to facilitate its growth and a subsequent transfer to a solid medium (Agar) for the enumeration of characteristic colonies. That's because liquid and solid media have different properties that are useful in distinct phases of microbial cultivation.

Liquid media serves as a crucial initial step, it provides a homogeneous environment that supports the rapid and uniform growth of microorganisms. Solid media, typically in the form of agar plates, provide a supportive matrix for the growth of microbial colonies. This enables the visualization and isolation of individual colonies, which are crucial for accurate enumeration and identification of microorganisms. This sequential transfer from liquid to solid media is fundamental in microbiological techniques such as serial dilution and colony counting for quantifying microbial populations.

The measurement of pH in culture media is an important step for optimizing growth conditions. This importance is due to the influence of pH on the growth and metabolic activities of microorganisms. Consequently, the precise measurement and subsequent adjustment of the pH within the culture media is crucial to align with the specific optimal range of the target microorganism [28].

Chapter III- Strain Preparation

1. Introduction

The primary aim of microbial strain preparation in a microbiology laboratory is to establish and maintain a repository of well-characterized, viable microbial cultures.

The preparation and maintenance of microorganism strains is important because they are used as a positive control for culture media . This ensures that culture media consistently supports the growth of microorganisms, affirming the reliability of experimental results. Reference strains serve as points of reference for procedures, certifying the sensitivity and specificity of microbial detection [29].

2. Materials and Methods

2.1 Strain Preparation

The strains are maintained in vials containing lyophilized support and stored in a freezer (-20 °C) to maintain their integrity. Within a controlled laminar airflow environment, the content of the vial of each microorganism strain is dissolved in 20 mL of sterile water, ensuring complete dissolution. In a test tube, 100 µL of this solution is combined with 9 mL of TSB, as this medium can be used for every strain. This resulting solution serves as our stock solution (SS) which is then subjected to specific incubation conditions outlined for each strain (Table 3) which are reference strains cataloged by the ATCC (American Type Culture Collection) [30].

Table 3- Reference strains by ATCC, media and incubation conditions.

Microorganism	Liquid culture medium	Solid culture medium	Incubation conditions
Staphylococcus aureus, ATCC 6538	TSB	TSA	30-35 ºC, 18-24 h
Pseudomonas aeruginosa, ATCC 9027	TSB	TSA	30-35 ºC, 18-24 h
Bacillus subtilis, ATCC 6633	TSB	TSA	30-35 ºC, 18-24 h
Candida albicans, ATCC 10231	TSB / SDB	TSA/SDA	30-35 ºC, 1-3 days
Aspergillus brasiliensis, ATCC 16404	TSB	TSA/SDA	30-35 ºC, 2-7 days
Escherichia coli, ATCC 8739	TSB	TSA	30-35 ºC, 18-24 h
Salmonella typhimurium, ATCC 14028	TSB	TSA	30-35 ºC, 18-24 h
Clostridium sporogenes, ATCC 11437	TSB / RCM	TSA	30-35º C, 18-48 h, anaerobic conditions
Listeria monocytogenes, ATCC 13932	TSB / HFB	TSA/ALOA/OA	30-35 ºC, 18-24 h
Listeria innocua, ATCC 33090	TSB / HFB	TSA/ALOA/OA	30-35 ºC, 18-24 h

Upon the conclusion of the prescribed incubation period, turbidity becomes evident within the growth medium, serving as an indicator of microbial proliferation under optimal growth conditions. After this observation various dilutions are meticulously prepared. Under strict aseptic conditions, 9 mL of buffered sodium chloride-peptone solution at a pH of 7.0 is added to multiple test tubes. Following, 1000 µL is transferred from the stock solution into the first test tube, representing a 10^{-1} dilution. Sequentially, 1000 µL is withdrawn from each dilution and successively added to the next (from 10^{-1} to 10^{-10}). Following the preparation of dilutions, 100 µL of each dilution is inoculated onto the designated solid growth medium, specified on Table 3, using an inoculation loop for even distribution. The dilutions aim is to find the dilution where the CFU/plate count is at a concentration between 10 and 100 to be subsequently used in the growth promotion phase.

The original stock solution is then stored under refrigeration conditions and employed to generate a fresh stock solution the subsequent week , comprising 9

mL of TSB supplemented with 100 µL of the original stock solution. The following dilutions are prepared from this newly obtained stock solution, and according to the Ph. Eur not more than five passages are conducted from the original master seed-lot [31].

When working with microorganism strains in a laminar airflow chamber, it is an important step to turn on the UV lights after the microorganism manipulation for at least 15 minutes. This step ensures effective sterilization since UV light has germicidal properties that eliminate bacteria, viruses, microorganisms present on the chamber, reducing the risk of contamination.

3. Results and Discussion

The aim is to identify the dilution level at which the CFU count is ranging from 10 to 100. If the count is too low (< 10 CFU), it might not provide a sufficiently reliable indication of microbial presence. On the other hand, if the count is too elevated (> 100 CFU) it makes accurate counting difficult due to a great number of colonies that could merge. This specific range is desired because it ensures that there are enough colonies to provide a reliable indication of microbial presence.

3.1 Strains under assessment

The tables (Tables 4 to 13) highlight the chosen dilution for each strain, that is between 10 and 100 CFU/ 100 µL.

3.1.1 Pseudomonas aeruginosa, ATCC 9027

Table 4- Pseudomonas aeruginosa (ATCC 9027) strain preparation results.

Dilution	SS	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	**10^{-6}**	10^{-7}	10^{-8}	10^{-9}	10^{-10}
CFU/ 100 µL	uncountable	>100	>100	>100	>100	140	**12**	1	0	0	0

3.1.2 *Escherichia coli, ATCC 8793*

Table 5-Escherichia coli (ATCC 8793) strain preparation results.

Dilution	SS	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	**10^{-6}**	10^{-7}	10^{-8}	10^{-9}	10^{-10}
CFU/ 100 µL	uncountable	>100	>100	>100	>100	150	**43**	7	0	0	0

3.1.3 *Bacillus subtilis, ATCC 6633*

Table 6-Bacillus subtilis (ATCC 6633) strain preparation results.

Dilution	SS	10^{-1}	10^{-2}	10^{-3}	10^{-4}	**10^{-5}**	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
CFU/ 100 µL	uncountable	>100	>100	>100	180	**71**	3	0	0	0	0

3.1.4 *Staphylococcus aureus, ATCC 6538*

Table 7- Staphylococcus aureus (ATCC 6538) strain preparation results.

Dilution	SS	10^{-1}	10^{-2}	**10^{-3}**	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
CFU/ 100 µL	uncountable	>100	>100	**48**	9	1	0	0	0	0	0

3.1.5 *Salmonella thyphimurium, ATCC 14028*

Table 8-Salmonella thyphimurium (ATCC 14028) strain preparation results.

Dilution	SS	10^{-1}	**10^{-2}**	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
CFU/ 100 µL	uncountable	>100	**34**	9	0	0	0	0	0	0	0

3.1.6 *Candida albicans, ATCC 10231*

Table 9- Candida albicans (ATCC 10231) strain preparation results.

Dilution	SS	10^{-1}	10^{-2}	10^{-3}	**10^{-4}**	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
CFU/ 100 µL	uncountable	>100	>100	120	**62**	6	0	0	0	0	0

3.1.7 *Aspergillus brasiliensis, ATCC 16404*

Table 10- Aspergillus brasiliensis (ATCC 16404) strain preparation results.

Dilution	SS	10^{-1}	**10^{-2}**	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
CFU/ 100 µL	uncountable	>100	**22**	2	0	0	0	0	0	0	0

3.1.8 *Listeria monocytogenes, ATCC 13932*

Table 11- Listeria monocytogenes (ATCC 13932) strain preparation results.

Dilution	SS	10^{-1}	10^{-2}	10^{-3}	**10^{-4}**	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
CFU/ 100 µL	uncountable	>100	>100	>100	**52**	10	5	0	0	0	0

3.1.9 Listeria innocua, ATCC 33090

Table 12- Listeria innocua (ATCC 33090) strain preparation results.

Dilution	SS	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	**10^{-6}**	10^{-7}	10^{-8}	10^{-9}	10^{-10}
CFU/ 100 µL	uncountable	>100	>100	>100	>100	100	**42**	4	1	0	0

1.1.10 Clostridium sporogenes, ATCC 11437

Table 13- Clostridium sporogenes (ATCC 11437) strain preparation results.

Dilution	SS	10^{-1}	10^{-2}	10^{-3}	**10^{-4}**	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
CFU/ 100 µL	uncountable	>100	>100	>100	**52**	5	0	0	0	0	0

Following the enumeration of CFU/plate for every dilution of each strain, a crucial step succeeds : the identification and selection of the dilution that meets the criteria, that will be used as positive control in culture media. When conducting growth promotion tests, the appropriate dilution for each strain is now determined with precision.

Chapter IV – Growth Promotion

1. Introduction

In microbiology, positive control for culture medium plays a crucial role ensuring that the growth conditions provided by a particular media are appropriate for the growth of the intended microorganism. According to the World Health Organization (WHO), growth promotion should be done on all media and on every batch [32].

The preparation of microorganism strains is essential in serving as a positive control for culture media. By inoculating a known, well-characterized strain of the target microorganism onto culture media under standardized conditions it is possible to validate the efficacy of the culture medium, ensure its suitability for the intended microorganism's growth, and detect any deviations in the medium's growth-promoting properties. To this end, all batches of prepared culture media undergo rigorous testing, encompassing growth promoting (GP), growth inhibition (GI), and indicative properties (IP) assessments, conducted according to prescribed methodologies.

2. Materials and Methods

2.1 Microorganisms strains

The inoculations are meticulously prepared for each microorganism, employing strains with previous known concentrations (between 10-100 CFU/ 100 µL) to maintain consistency. Duplicate tests are executed for a comparison between the new and a previously approved batch. Inoculation protocols entail the application of 100 µL per agar plate for solid media, or tube for liquid media. The concentration criterion is less than 100 CFU/ 100 µL for growth inhibition and indicative properties and greater than 100 CFU/ 100 µL for growth promotion. Temperature and incubation periods are outlined in Table 14 and aligned with the Ph. Eur guidelines to ensure standardized conditions.

Table 14- Growth promotion, inhibitory and indicative properties for culture media.

Assay/ Culture Medium		**Property**	**Strains to test**	**Incubation conditions**
TAMC	TSA	GP	*Staphylococcus aureus, ATCC 6538* *Pseudomonas aeruginosa, ATCC 9027* *Candida albicans,* ATCC 10231 *Aspergillus brasiliensis*, ATCC 16404	30-35 °C, < 3 days 30-35 °C, < 3 days 30-35 °C, < 5 days 30-35 °C, < 5 days
TYMC	SDA	GP	*Candida albicans,* ATCC 10231 *Aspergillus brasiliensis*, ATCC 16404	20-25 °C, < 5 days 20-25 °C, < 5 days
Gram-negative bacteria	MB	GP	*Escherichia coli*, ATCC 8739 *Pseudomonas aeruginosa, ATCC 9027*	30-35 °C, < 24 hours
		GI	*Staphylococcus aureus, ATCC 6538*	30-35 °C, > 48 hours
	VRBD	GP + IP	*Escherichia coli*, ATCC 8739 *Pseudomonas aeruginosa, ATCC 9027*	30-35 °C, < 18 hours
Escherichia coli	MCB	GP	*Escherichia coli*, ATCC 8739	42-44 °C, < 24 hours
		GI	*Staphylococcus aureus, ATCC 6538*	42-44 °C, > 48 hours
	MCA	GP + IP	*Escherichia coli*, ATCC 8739	30-35 °C, < 18 hours
Salmonella spp.	RVB	GP	*Salmonella typhimurium*, ATCC 14028	30-35 °C, < 18 hours
		GI	*Staphylococcus aureus, ATCC 6538*	30-35 °C, > 24 hours
	XLD	GP + IP	*Salmonella typhimurium*, ATCC 14028	30-35 °C, < 18 hours
Staphylococcus aureus	MSA	GP	*Staphylococcus aureus, ATCC 6538*	30-35 °C, < 18 hours
		GI	*Escherichia coli*, ATCC 8739	30-35 °C, > 72 hours
Pseudomonas aeruginosa	CTA	GP	*Pseudomonas aeruginosa, ATCC 9027*	30-35 °C, < 18 hours
		GI	*Escherichia coli*, ATCC 8739	30-35 °C, > 72 hours
Clostridia	RCM	GP	*Clostridium sporogenes*, ATCC 11437	30-35 °C, < 48 hours
	CA			
Candida albicans	SDB	GP	*Candida albicans,* ATCC 10231	30-35 °C, < 3 days
	SDA	GP + IP	*Candida albicans,* ATCC 10231	30-35 °C, > 48 hours
Listeria monocytogenes	HFB	GP	*Listeria monocytogenes*, ATCC 13932	29-30 °C, < 24 hours
		GI	*Escherichia coli*, ATCC 8739	29-30 °C, > 26 hours
	FB	GP	*Listeria monocytogenes*, ATCC 13932	36-38 °C, < 22 hours
		GI	*Escherichia coli*, ATCC 8739	36-38 °C, > 26 hours
	ALOA	GP + IP	*Listeria monocytogenes*, ATCC 13932	36-38 °C, < 46 hours
			Listeria innocua, ATCC 30090	36-38 °C, < 46 hours
		GI	*Escherichia coli*, ATCC 8739	36-38 °C, > 50 hours

	OA	GP + IP	*Listeria monocytogenes*, ATCC 13932	36-38 ^{o}C, < 46 hours
			Listeria innocua, ATCC 30090	36-38 ^{o}C, < 46 hours
		GI	*Escherichia coli*, ATCC 8739	36-38 ^{o}C, > 50 hours

For solid culture media, the recovery rate of colonies in the new batch is evaluate relative to the previously approved batch, aiming for a recovery rate between 50 to 200%. This means that the average number of colonies on the plates of the new batch should be within a factor of two when compared to the average number of colonies from the previous approved batch. The recovery rate can be calculated by the following equation :

$$\text{Recovery rate } (\%) = \frac{No.\,of\ CFU\ \text{recovered in the new batch}}{No.\,of\ CFU\ \text{recovered in the previous batch}} \times 100$$

In the case of liquid media, while no quantitative definition is provided, similarity in turbidity serves as a qualitative indicator of consistency.

Overall, the growth promotion of microorganism strains in positive control plays an essential role ensuring the accuracy of microbiological assays. If the microorganisms demonstrate robust growth consistent with expectations, it confirms the suitability and efficacy of the medium for supporting microbial growth under the specified conditions [33].

3. Results and Discussion

Given that the ideal dilution for each strain is now established, it is possible to proceed with testing the culture media.

In the context of growth-promoting assessments on solid media, it is crucial to achieve a recovery rate between 50 and 200 %. The table below (Table 15) provides the outcomes of the growth promotion assays on solid media, comparing the media under evaluation with the previously approved media along with the corresponding recovery rates. It is important to highlight that the comparison with the previously approved media can be executed either using the same media or employing TSA. This approach aims to ensure that the strains

failure to grow is not due to inherent quality issues. By testing with TSA, it can be concluded that the strain can grow but failed to do so in the media that it is not supposed to.

Table 15- Solid media growth promotion results.

Culture Medium	Strains to test	Previously approved media	Media to test	Recovery rate (%)
TSA	*Staphylococcus aureus, ATCC 6538*	82	80	98
	Pseudomonas aeruginosa, ATCC 9027	84	90	107
	Bacillus subtilis, ATCC 6633	30	39	130
	Candida albicans, ATCC 10231	31	31	100
	Aspergillus brasiliensis, ATCC 16404	22	23	105
SDA	*Candida albicans,* ATCC 10231	30	34	113
	Aspergillus brasiliensis, ATCC 16404	22	26	118
VRBD	*Pseudomonas aeruginosa, ATCC 9027*	84	80	95
	Escherichia coli, ATCC 8739	88	92	105
MCA	*Escherichia coli*, ATCC 8739	88	94	107
XLD	*Salmonella typhimurium*, ATCC 14028	50	60	112
MSA	*Staphylococcus aureus, ATCC 6538*	82	88	107
	Escherichia coli, ATCC 8739	103	0	0
VRB	*Pseudomonas aeruginosa, ATCC 9027*	84	78	93
	Escherichia coli, ATCC 8739	88	90	102
CTA	*Pseudomonas aeruginosa, ATCC 9027*	32	40	125
	Escherichia coli, ATCC 8739	109	0	0
CA	*Clostridium sporogenes*, ATCC 11437	39	42	108
OA	*Escherichia coli*, ATCC 8739	100	0	0
	Listeria monocytogenes, ATCC 13932	59	69	103
	Listeria innocua, ATCC 33090	39	37	95
ALOA	*Escherichia coli*, ATCC 8739	100	0	0
	Listeria monocytogenes, ATCC 13932	59	60	102
	Listeria innocua, ATCC 33090	39	40	103

If considered the examination of MSA media: the *S. aureus, ATCC 6538* strain characterized by growth-promoting traits, exhibited growth, manifesting a 107% recovery rate. On the other hand, the *E. coli*, ATCC 8739 strain, possessing growth inhibition properties, did not exhibit growth as expected (Table 15).

As previously stated, the assessment of bacterial growth in liquid media involves the examination of turbidity, or lack, within the medium. A positive result (+) indicates that the media has supported the growth of the bacterial strain with growth-promoting properties while growth-inhibiting strains fail to proliferate as expected (-) (Table 16).

Table 16- Liquid media growth promotion results.

Culture Medium	Strains to test	Previously approved media	Media to test	Recovery rate (%)
TSB	*Staphylococcus aureus, ATCC 6538*	+	+	OK
	Pseudomonas aeruginosa, ATCC 9027	+	+	
	Bacillus subtilis, ATCC 6633	+	+	
MB	*Staphylococcus aureus, ATCC 6538*	-	-	OK
	Pseudomonas aeruginosa, ATCC 9027	+	+	
	Escherichia coli, ATCC 8739	+	+	
MCB	*Staphylococcus aureus, ATCC 6538*	-	-	OK
	Escherichia coli, ATCC 8739	+	+	
RVB	*Staphylococcus aureus, ATCC 6538*	-	-	OK
	Salmonella typhimurium, ATCC 14028	+	+	
RCM	*Clostridium sporogenes*, ATCC 11437	+	+	OK
SDB	*Candida albicans,* ATCC 10231	+	+	OK
HFB	*Escherichia coli*, ATCC 8739	-	-	OK
	Listeria monocytogenes, ATCC 13932	+	+	
FB	*Escherichia coli*, ATCC 8739	-	-	OK
	Listeria monocytogenes, ATCC 13932	+	+	

By the analysis of Table 16, consider the strain *S.aureus, ATCC 6538,* which has growth inhibition properties when testing MB media. As expected, the absence of turbidity serves as an indication of successful growth inhibition. On the contrary, strains such as *P. aeruginosa, ATCC 9027, and E.coli*, ATCC 8739 demonstrate growth promotion characteristics on the same media. In accordance with this trait, the presence of turbidity following inoculation represents a successful growth in this media.

It is possible to make meaningful conclusions regarding the efficacy of the solid and liquid media, considering the highly favorable results that have been presented.

Chapter V – Analysis and Samples

1. Introduction

1.1 Standard Specifications

The products are analyzed according to the company's internal regulations that are based on Ph. Eur. As mentioned previously, the aim of the Ph. Eur is to establish and provide standards for the quality, safety, and efficacy of pharmaceutical substances, medical products, and related materials [34].

According to the Ph. Eur, the following table (Table 17) specifies the standard specifications for drugs, nutritional supplements, raw materials, and alcoholic extracts, as well as the required microorganism analysis for each category.

Table 17-Standard specifications for samples of drugs, nutritional supplements, and raw materials.

		Drugs	Nutritional supplements	Raw Materials	Alcoholic extracts
TAMC (CFU/ g or mL)		10^3	10^5	10^4	10^5
TYMC (CFU/g or mL)		10^2	10^4	10^2	10^4
Gram-Negative Bacteria		—	10^4	10^2	10^4
Specific microorganisms	***Escherichia coli***	Absence of *E.Coli* (1 g/mL)	Absence of *E.Coli* (1 g/mL)	Absence of *E.Coli* (1 g/mL)	Absence of *E.Coli* (1 mL)
	Salmonella spp.	—	Absence of *Salmonella* (10 g/mL)	Absence of *Salmonella* (25 g/mL)	Absence of *Salmonella* (25 mL)
	Staphylococcus aureus	—	Absence of *S. Aureus* (1 g/mL)	—	
	Listeria monocytogenes	—	Absence of *Listeria* (25 g/mL)	—	

2. Materials and Methods

2.1 Specific Analysis for Each microorganism

The following tables (Tables 18 to 26) specify all the analyses conducted for each microorganism, the culture media transfer along with the corresponding incubation conditions to which they are subjected.

2.1.1 TAMC (Total Aerobic Microbial Count) and TYMC (Yeast and Mold Count)

For the analysis of Total Aerobic Microbial Count (TAMC) and Yeast and Mold Count (TYMC), the initial step involves measuring 1 g/mL of the sample. Adhering to the specifications outlined by the Ph. Eur, the number of dilutions (10^x) is determined. Dilutions are prepared as follows: 1 g/mL of the sample is measured, and 9 mL of peptone water (PEP) is added, that represents the first dilution 10^{-1}. Subsequently, 1 mL of the previous solution is added to a test tube containing 9 mL of PEP, representing the 10^{-2} dilution, and so forth. This systematic dilution process is integral to achieving accurate microbial counts, aligning with established standards, and ensuring the reliability of the results (Figure 1).

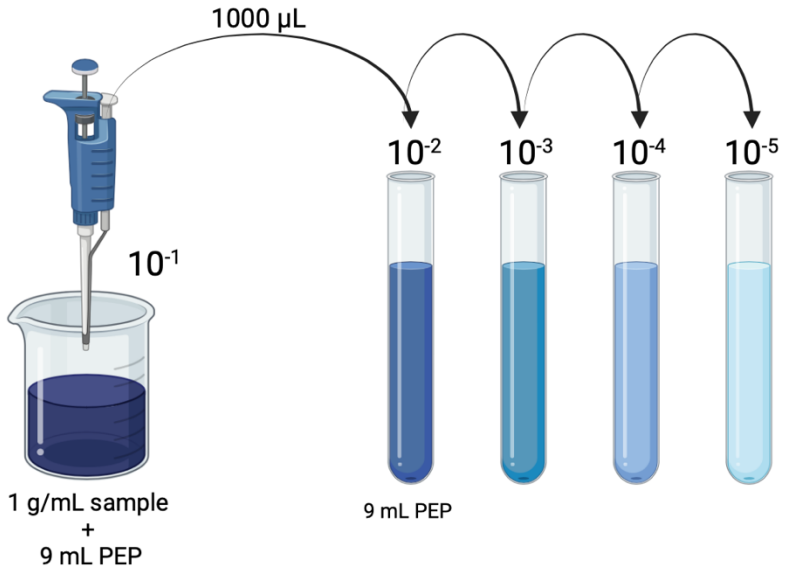


Figure 1- Schematic representation of the dilution process. Created with BioRender.com.

Consequently, 1 mL of each dilution is added to an empty Petri dish to add the appropriate media. For the TAMC analysis , TSA media is added, while in the case of TYMC the appropriate media is SDA, as illustrated in Figure 2. Both media are previously melted on a water bath method at 100 ºC but will solidify in a few minutes once they are poured onto the petri dishes.

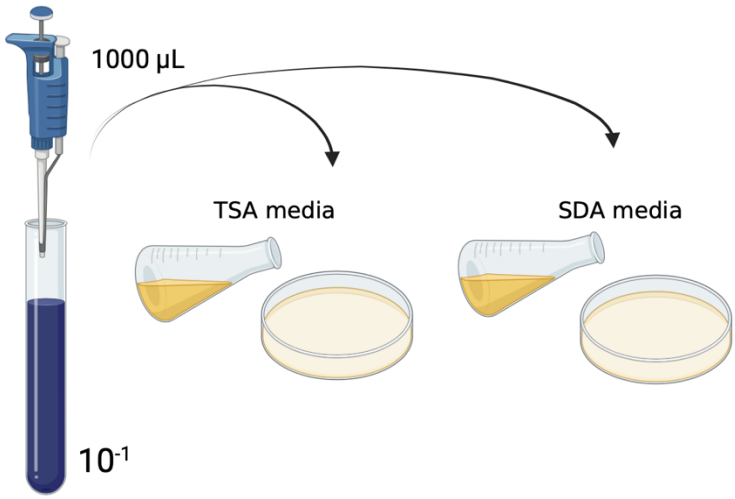


Figure 2- Schematic representation of the pour plate method. Created with BioRender.com.

Once the media is solidified, the petri dishes will be subjected to different incubation conditions (Table 18).

The results regarding this analysis are assessed quantitatively, therefore the results will be analyzed by counting of colony-forming units per g or mL (CFU/g or mL).

Table 18- Media and incubation conditions for TAMC and TYMC analysis.

			Media	
	Sample	PEP	TSA	SDA
TAMC	10^x corresponding to 1 g/mL	Add 9mL	T (ºC): 30-35 ºC for 3-5 days	--------
TYMC	10^x corresponding to 1 g/mL	Add 9mL	--------	T (ºC): 20-25 ºC for 5-7 days

2.1.2 Gram-Negative Bacteria

For the analysis of Gram-negative bacteria, the process begins with a short initial incubation in TSB media, followed by a transfer to MB liquid media and a final incubation in VRBD solid media (Table 19).

The result of this analysis is assessed quantitively by CFU/ g or mL.

Table 19- Media and incubation conditions for Gram-Negative Bacteria analysis.

		Media		
	Sample	TSB	MB	VRBD
Gram-negative bacteria	x mL/g	T (ºC): 20-25 ºC for 2-5h	T (ºC): 30-35 ºC for 24-48h	T (ºC): 30-35 ºC for 18-24h

2.1.3 Escherichia Coli

In regards to the analysis of the presence of *E. coli* , the medium that starts the incubation is TSB, followed by a transition to MCB liquid media, and ultimately a solid MCA media. The results are evaluated qualitatively, consequently they will be presented based on the presence or absence of colony-forming units per gram or milliliter (CFU/g or mL) (Table 20).

Table 20- Media and incubation conditions for E.Coli analysis.

		Media		
	Sample	TSB	MCB	MCA
Escherichia coli	x mL/g	T (ºC): 30-35 ºC for 18-24h	T (ºC): 42-44 ºC for 24-48h	T (ºC): 30-35 ºC for 18-72h

2.1.3.1 Indole Test

By detecting the bacteria's ability to metabolize tryptophan and produce indole, the indole test remains a fundamental procedure to identify *E. coli.*

To perform the test, using a sterile inoculating loop transfer a small amount of the bacterial culture to a tube with peptone water. Inoculate that solution at 30-35 ºC during 24-48 hours. After incubation add 2-3 drops of Kovac's reagent, stored at room temperature, to the culture broth. Gently shake the tube and observe the top layer of the liquid change color. The appearance of a pink layer indicates a positive test, confirming the presence of indole and suggesting the presence of *E. coli.* On the other hand, the appearance of a green layer indicates a negative test [35].

2.1.4 Salmonella spp.

In the case of *Salmonella* analysis, the choice of the initial incubation media varies based on the product and the Ph. Eur specification. In cases where the specification indicates the absence of Salmonella in 25 g/mL the first medium used is PEP ISO. Conversely, if the specification specifies a value under 25 g/mL then the initial medium is TSB. The following media is common in both cases, RVB, and the final one is XLD. (Table 21).

The outcomes are evaluated qualitatively and presented based on the presence or absence of CFU/ g or mL.

Table 21- Media and incubation condition for Salmonella spp. analysis.

				Media	
Sample				RVB	XLD
Salmonella spp.	x mL/g	25 g/mL	PEP ISO T (ºC): 30-35 ºC for 18-24h	T (ºC): 30-35 ºC for 18-48h	T (ºC): 30-35 ºC for 18-48h
		<25 g/mL	TSB T (ºC): 30-35 ºC for 18-24h		

2.1.5 Staphylococcus aureus and Pseudomonas aeruginosa

For the analysis of *S. aureus* and *P. aeruginosa*, both undergo a two-step process involving a transition from a liquid media to solid media. In both cases, the initial incubation is done by using TSB media. Subsequently, for *S. aureus*, the solid media used is MSA, while for *P. aeruginosa*, the solid media is CTA. This sequential approach ensures a comprehensive analysis of these bacterial strains, contributing to the qualitative evaluation of their presence or absence in tested samples (Tables 22 and 23).

Table 22- Media and incubation conditions for Staphylococcus aureus analysis.

		Media	
	Sample	TSB	MSA
Staphylococcus aureus	x mL/g	T (ºC): 30-35 ºC for 18-24h	T (ºC): 30-35ºC for 18-72h

Table 23- Media and incubation conditions for Pseudomanas Aeruginosa analysis.

		Media	
	Sample	TSB	CTA
Pseudomonas aeruginosa	x mL/g	T (ºC): 30-35 ºC for 18-24h	T (ºC): 30-35 ºC for 18-72h

2.1.5.1 Coagulase Test

S. aureus is a significant human pathogen responsible for a range of infections, from minimal skin infection to life-threatening diseases as mentioned previously. Coagulase is an enzyme produced by *S. aureus* that causes blood plasma to coagulate by converting fibrinogen to fibrin [36].

The coagulase test is the most reliable method to differentiate *S. aureus,* which produces the enzyme coagulase, from other *Staphylococcus* species that do not produce coagulase [37].

The test contains latex particles coated with fibrinogen, IgG (immunoglobulin G), monoclonal antibodies (mAb), bovine albumin solution and a bovine albumin solution as a negative control, to ensure specificity and rule out non-specific agglutination. It is also provided, disposable agglutination cards and disposable mixing sticks. Proper storage of the reagents at 2-6 ºC is important to maintain test accuracy and reliability.

On the agglutination cards, two circular areas are displayed. Place a drop of the latex reagent on one circle and one drop of the negative control on the other. Using a mixing stick, add a colony of the bacterial sample to both reagents. Gently rock the card back and forth and observe agglutination within a few seconds. A positive result is observed when there is agglutination (clumping), suggesting the presence of *S. aureus*. No agglutination indicates the absence of this microorganism. The absence of agglutination in the negative control confirms the specificity of the test.

2.1.6 Candida Albicans

For the analysis of *C. albicans*, the microorganism has a two-step process involving a transition from a liquid media, SDB, to a solid media, SDA. The results are expressed qualitatively; therefore, the results will be analyzed by the presence or absence of colony-forming units per g or mL (CFU/g or mL) (Table 24).

Table 24- Media and incubation conditions for Candida Albicans analysis.

		Media	
	Sample	SDB	SDA
Candida albicans	x mL/g	T (ºC): 30-35 ºC for 3-5 days	T (ºC): 30-35 ºC for 24-48h

2.1.7 Clostridium

For the analysis of *Clostridium,* the incubation process initiates by measuring 1g or mL of the sample and adding 9 mL of PEP media. In this particular case , the samples are measured in duplicate, with one undergoing condition of 80ºC and the other kept at room temperature for 10 minutes. Subsequently, both samples are transferred to RCM media and finally to CA, with both stages conducted under anaerobic conditions. Clostridia are anaerobic bacteria , meaning they are unable to survive in the presence of oxygen. When subjected to aerobic conditions during incubation, clostridia will not grow, and the analysis may yield false negatives. To achieve an oxygen-free environment it is used anaerobic jars supplemented with chemical packages. These chemicals react with the available oxygen inside the jar, effectively removing it.

The results are evaluated qualitatively (CFU/g or mL) (Table 25).

Table 25- Media and incubation conditions for Clotridia analysis.

		Media		**Media**	
	Sample	PEP		RCM	CA
Clostridia	x mL/g	1:10 dilution	- 80ºC - Room temperature	T (ºC): 30-35 ºC for 48h	T (ºC): 30-35 ºC for 48-72h
				Under anaerobic condition	

2.1.8 Listeria monocytogenes

For the analysis of *Listeria* , the process begins with an initial incubation in HFB media. Subsequently, the samples progress to both a liquid media (FB) and solid media (ALOA and OA). Those in FB will undergo further incubation in ALOA and OA as well. The results are assessed qualitatively and therefore analyzed by the presence or absence of CFU per g or mL (CFU/ g or mL) (Table 26).

Table 26- Media and incubation conditions for Listeria Monocytogenes analysis.

	Media			
	Sample	HFB		
Listeria monocytogenes	x mL/g	T (°C): 29-31 °C for 24-26h	FB T (°C): 36-38°C for 22-26h ALOA/OA T (°C): 36-38°C for 46-50h	ALOA/OA T (°C): 36-38°C for 46-50h —

2.2 Specific Sample Specifications

To achieve more consistent and reliable results, it is going to be sampled multiple samples of each category: Drugs, Nutritional Supplements, Raw materials, Alcoholic extracts, and Stability tests, in various forms (capsules, pills, syrups, or powder).

The following tables (Tables 27 to 31) , detail the specifications associated with each sample, providing guidance on the necessary analysis to be performed. To ensure the security and privacy of the company, none of the product names will be disclosed.

2.2.1 Drugs

Table 27- Drug sample specification.

		Drug sample 1	**Drug sample 2**	**Drug sample 3**
TAMC (CFU/ g or mL)		10^3	10^4	10^3
TYMC (CFU/g or mL)		10^2	10^2	10^2
Gram-Negative Bacteria		—	10^2	—
Specific microorganisms	*Escherichia coli*	Absence of *E.coli* (1g)	Absence of *E.coli* (1g)	Absence of *E.coli* (1g)
	Salmonella spp.	—	Absence of *Salmonella* (1g)	—
	Staphylococcus aureus	—	Absence of *S. aureus* (1g)	—
	Listeria monocytogenes	—	—	—

2.2.2 Nutritional Supplements

Table 28- Nutritional Supplements specification.

		Nutritional Supplement 1	Nutritional Supplement 2	Nutritional Supplement 3
TAMC (CFU/ g or mL)		10^4	10^4	10^4
TYMC (CFU/g or mL)		10^2	10^2	10^2
Gram-Negative Bacteria		10^2	10^2	10^2
Specific microorganisms	*Escherichia coli*	Absence of *E. coli* (1mL)	Absence of *E. coli* (1g)	Absence of *E. coli* (1mL)
	Salmonella spp.	Absence of *Salmonella* (10mL)	Absence of *Salmonella* (10g)	Absence of *Salmonella* (10mL)
	Staphylococcus aureus	Absence of *S. aureus* (1mL)	Absence of *S. aureus* (1g)	Absence of *S. aureus* (1mL)
	Listeria monocytogenes	—	Absence of *Listeria* (25g)	—
	Clostridia	—	—	Absence of *Clostridia* (1mL)

2.2.3 Raw Materials

Raw materials are often and easily contaminated with a range of microorganisms including bacteria and fungi, which can compromise the safety of the final product [38].

Analysis of raw materials is conducted both before and after decontamination. An external laboratory is responsible for the decontamination using a UV light and, by performing analysis on raw materials before decontamination, the cost can be avoided. It is expected that raw materials prior to decontamination will exhibit higher microbial counts, but the results may still meet the allowed specification (Table 29). Four different raw material will be analyzed, two before decontamination and two after decontamination.

Table 29-Raw Materials specification.

		Before decontamination		After decontamination	
		Raw material 1	**Raw material 2**	**Raw material 3**	**Raw material 4**
TAMC (CFU/ g or mL)		10^4	10^5	10^4	10^4
TYMC (CFU/g or mL)		10^2	10^4	10^2	10^2
Gram-Negative Bacteria		__	10^4	10^2	10^2
Specific microorganisms	*Escherichia coli*	Absence of *E. coli* (1g)	Absence of *E. coli* (1g)	Absence of *E. coli* (1g)	Absence of *E. coli* (1g)
	Salmonella spp.	Absence of *Salmonella* (1 g)	Absence of *Salmonella* (25 g)	Absence of *Salmonella* (25 g)	Absence of *Salmonella* (25 g)
	Staphylococcus aureus	Absence of *S. aureus* (1g)	—	—	—
	Listeria monocytogenes	—	—	—	—

2.2.4 Alcoholic Extracts

Alcoholic extracts play an important role in the manufacturing of pharmaceutical products due to their ability to extract bioactive compounds from plant materials efficiently. Alcoholic extracts are invaluable in pharmaceutical product manufacturing due to the efficient extraction, preservation of active ingredients, and ability to enhance bioavailability.

Table 30-Alcoholic extracts specifications.

		Alcoholic Extract 1	**Alcoholic Extract 2**	**Alcoholic Extract 3**
TAMC (CFU/ g or mL)		10^5	10^5	10^5
TYMC (CFU/g or mL)		10^4	10^4	10^4
Gram-Negative Bacteria		10^4	10^4	10^4
Specific microorganisms	*Escherichia coli*	Absence of *E. coli* (1mL)	Absence of *E. coli* (1mL)	Absence of *E. coli* (1mL)

	Salmonella spp.	Absence of *Salmonella* (25mL)	Absence of *Salmonella* (25mL)	Absence of *Salmonella* (25mL)
	Staphylococcus aureus	—	—	—
	Listeria monocytogenes	—	—	—

2.2.5 Stability Tests

Table 31- Stability test specifications.

		Stability test 1	**Stability test 2**	**Stability test 3**
TAMC (CFU/ g or mL)		10^3	10^5	10^4
TYMC (CFU/g or mL)		—	10^4	10^2
Gram-Negative Bacteria		—	10^4	10^2
Specific microorganisms	*Escherichia coli*	Absence of *E. coli* (1g)	Absence of *E. coli* (1mL)	Absence of *E. coli* (1mL)
	Salmonella spp.	—	Absence of *Salmonella* (1 mL)	Absence of *Salmonella* (10 mL)
	Staphylococcus aureus	Absence of *S. aureus* (1g)	—	Absence of *S. aureus* (1mL)
	Pseudomonas aeruginosa	Absence of P. aeruginosa (1g)	—	—
	Candida Albicans	Absence of *C. albicans* (1g)	—	—
	Listeria monocytogenes	—	—	—

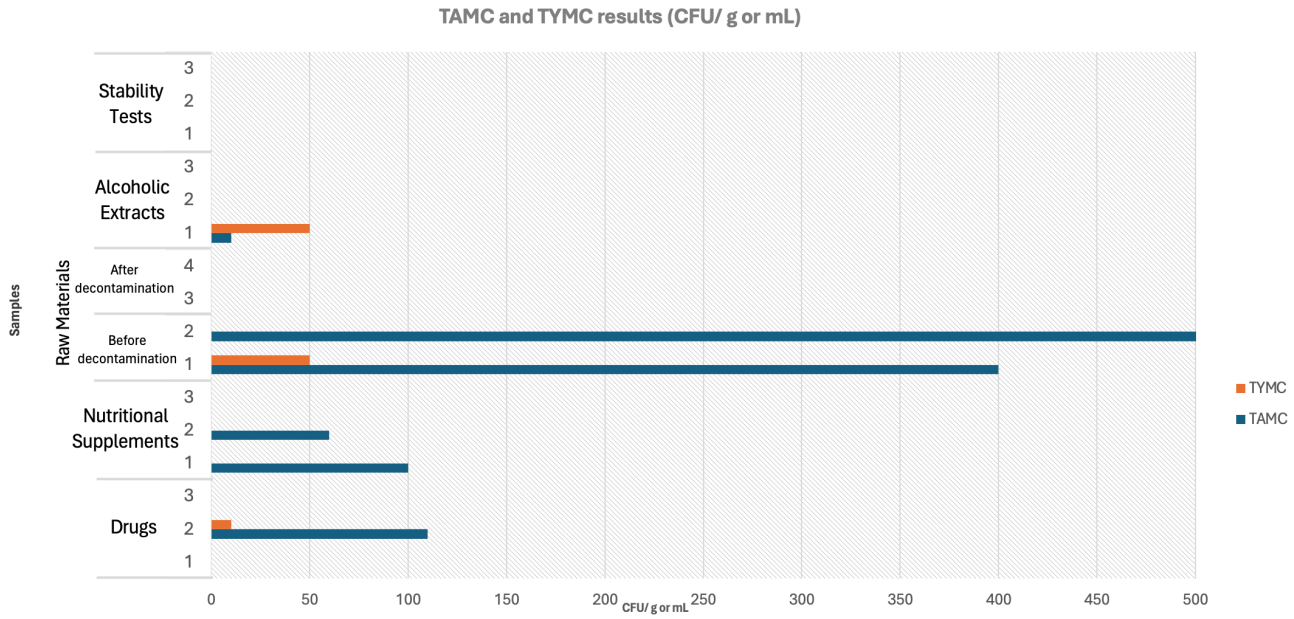
3. Results and Discussion

3.1.1 TAMC and TYMC

To assess the TAMC and TYMC results, dilutions are prepared according to the specifications outlined in the Ph. Eur, as mentioned previously. Following the dilutions, the number of colony-forming units (CFU) is counted for each dilution on their respective plate.

Each dilution reduces the concentration of microorganisms in the sample by a specific factor. For example, a 10^{-1} dilution means that the sample has been diluted to one-tenth of its original concentration. For that reason, in a 10^{-1} dilution, the number of CFU/plate must be multiplied by 10 to estimate the number in the sample. Similarly, in a 10^{-2} dilution, the CFU count must be multiplied by 100, in a 10^{-3} for 1000 and so forth.

The following figure (Figure 3) represents the results for TAMC and TYMC, for each sample within every category.



	Drugs			Nutritional Supplements			Raw materials				Alcoholic Extract			Stability tests		
							before decontamination		after decontamination							
	1	2	3	1	2	3	1	2	3	4	1	2	3	1	2	3
TYMC	0	10	0	0	0	0	50	0	0	0	50	0	0	0	0	0
TAMC	0	110	0	100	60	0	400	50000	0	0	10	0	0	0	0	0

Figure 3- TAMC and TYMC results by count of CFU/ g or mL.

To present the results, we focus on the worst-case scenario. For instance, consider raw material sample number one. The TAMC dilution results are as follows: 10^{-1}: 30 CFU/ g; 10^{-2}: 4 CFU/ g; 10^{-3}: 0 CFU/ g; 10^{-4}: 0 CFU/ g; 10^{-5}: 0 CFU/ g. After applying the respective multiplication factors, the highest count is obtained from the 10^{-2} dilution : 4 CFUs x 100= 400 CFUs in the original sample.

It is notorious that there is a significant difference between the microbial load in raw materials before and after decontamination. As anticipated, the raw materials prior to decontamination exhibit a substantially higher value of CFUs. As it is demonstrated in Figure 3 , the sample number 2 exceeds the specification

limit for TAMC. In this case, it is recommended to decontaminate the raw material and reanalyze it. As expected, when decontaminated, the TAMC count was within the specification limit.

Overall, the results were positive, demonstrating values that fall within the specifications outlined in the Ph. Eur. The majority of the samples revealed absence or low TAMC and TYMC counts, indicating a highly adequate result in this assessment.

3.1.2 Gram-Negative Bacteria

As mentioned previously, the assessment of gram-negative bacteria is analyzed quantitatively. In regards to the results, it is noteworthy that none of the analyzed samples exhibited microbial count (Table 32).

Table 32- Gram-Negative Bacteria results.

	Drugs			**Nutritional Supplements**			**Raw Materials**				**Alcoholic Extracts**			**Stability Tests**		
	1	2	3	1	2	3	1	2	3	4	1	2	3	1	2	3
Gram-Negative Bacteria	<10 CFU/ g or mL															

Enterobacteriaceae, a family of Gram-Negative bacteria, exhibits a morphology characterized by purple colonies surrounded by a red halo when cultured on VRBD agar [39]. If the presence of Gram-negative bacteria was detected in any of the samples, Figure 4 illustrates the appearance of the colonies.

When inoculating on solid media, the streak plate method is employed to isolate individual colonies of microorganisms. This process involves using an inoculation loop to systematically streak the sample across the agar plate. Each streak

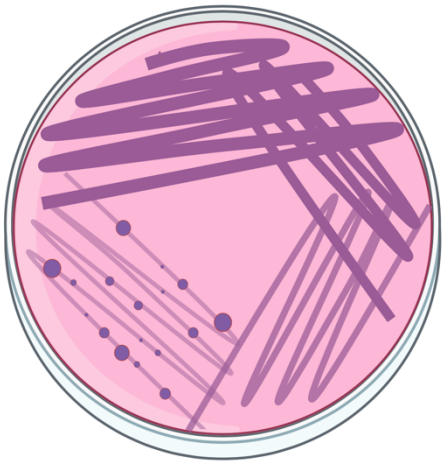


Figure 4- Gram-Negative bacteria colony morphology on VRBD agar. Created with BioRender.com.

reduces the number of microorganisms transferred to the next section of the plate, diluting the sample. By the final streak the density is low enough so that individual colonies can be isolated, as illustrated in Figure 4.

3.1.3 Specific Microorganisms

The following table (Table 33) presents the qualitative results of the specific microorganism analysis based on the following criteria: Presence of CFU, Absence of CFU, or Absence of characteristic CFU . The presence of Colony-Forming units refers to CFUs indicative of particular and common microorganisms, recognized as known contaminants or pathogens. The absence of characteristic CFUs ensures that the product does not contain these common microorganisms. In some cases, this is a result of failure to maintain aseptic conditions or improper handling techniques within the laminar flow environment.

Table 33- Specific microorganism results.

Samples		*Escherichia coli*	*Salmonella spp.*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Candida Albicans*	*Clostridia*	*Listeria monocytogenes*
Drugs	1	Absence/ g	—	—	—	—	—	—
	2	Absence/ g	Absence/ g	Absence of characteristic CFU	—	—	—	—
	3	Absence/ g	—	—	—	—	—	—
Nutritional Supplements	1	Absence/ mL	Absence/ 10mL	Absence of characteristic CFU	—	—	—	—
	2	Absence/ g	Absence/ 10g	Absence of characteristic CFU	—	—	—	Absence/ 25g
	3	Absence/ mL	Absence/ 10mL	Absence/ mL	—	—	Absence/ mL	—
Raw Materials	1	Absence/ g	Absence/ g	Absence of characteristic CFU	—	—	—	—
	2	Absence/ g	Absence of characteristic CFU	—	—	—	—	—
	3	Absence of characteristic CFU	Absence of characteristic CFU	—	—	—	—	—
	4	Absence/ g	Absence/ 25g	—	—	—	—	—

Alcoholic Extracts	1	Absence/ mL	Absence/ 25mL	—	—	—	—	—
	2	Absence/ mL	Absence/ 25mL	—	—	—	—	—
	3	Absence/ mL	Absence/ 25mL	—	—	—	—	—
Stability Tests	1	Absence/ g	—	Absence/ g	Absence/ g	Absence/ g	—	—
	2	Absence/ mL	Absence/ mL	—	—	—	—	—
	3	Absence/ mL	Absence/ 10mL	Absence/ mL	—	—	—	—

To verify the presence or absence of these specific microorganisms, it is important to know and differentiate the colony morphology of each one.

As explained previously, *E. coli* is incubated on MCA agar. MCA includes a pH indicator that changes to pink in acidic environments. As a result of this reaction, lactose-fermenting gram-negative bacteria will produce pink colonies. *E. coli,* a lactose-fermenting bacterium, breaks down the lactose in the medium resulting in acid production. Therefore, *E. coli* colonies on MCA agar are characteristically pink (Figure 5A) [35], [40].

When *S. aureus* is cultured on MSA media, the colonies exhibit distinctive morphological features. The colonies appear yellow and may have a yellow halo around them. This coloration occurs because *S. aureus* ferments mannitol, which lowers the pH of the medium. The acid production from mannitol fermentation causes the pH indicator (phenol red) in the medium to turn yellow (Figure 5B) [36].

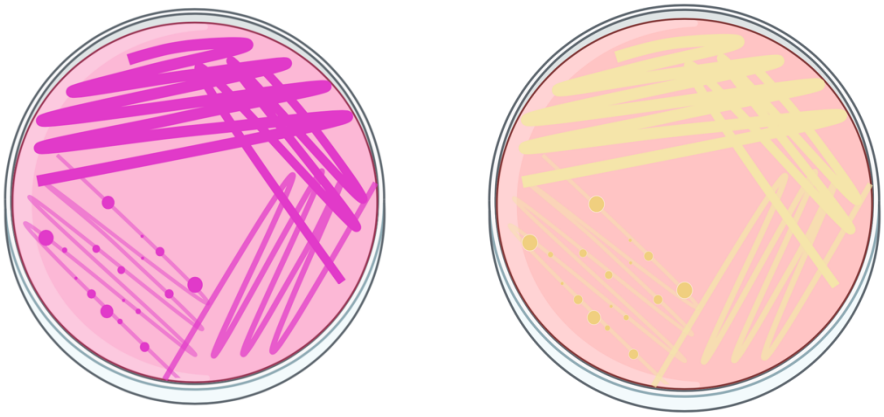


Figure 5- (A) E. coli colony morphology on MCA agar. (B) S. aureus colony morphology on MSA agar. Created with BioRender.com.

In the analysis of *E. coli and S. aureus,* should CFUs be present, the confirmation of the presence of these two microorganisms is achieved through the indole and coagulase tests. If the morphology of the colonies resembles the typical colony characteristics of *E. coli* and *S. aureus*, these tests clarify the presence or absence of these two common microorganisms.

When evaluating the results of these tests, by interpretation of the Table 18, the CFUs detected did not originate from *E. coli* and *S. aureus,* and the result is expressed as “absence of characteristic CFUs”.

On the other hand, *Salmonella, P. aeruginosa, C. albicans, Clostridia* and *Listeria monocytogenes* colonies possess distinct characteristics, facilitating the identification of this microorganism without the need for additional identification tests.

On XLD agar inoculated with *Salmonella spp.* , the colonies are characterized by a red color with black centers [41]. In an initial phase, the colonies appear yellow due to the fermentation of xylose, creating an acidic environment. Subsequently, the colonies turn red as a result of lysine decarboxylation, restoring the pH to alkaline. The production of hydrogen sulfide (H_2S) by *Salmonella* leads to the formation of black centers [42].

P. aeruginosa produces a distinct green pigment that diffuses into the agar aiding in its identification. Under UV light, the colonies may display a yellow-green fluorescence due to the production of fluorescein [43].

Candida albicans presents growth on SDA medium after 24-48h of incubation at 30-35 ^{o}C. It appears in the form of medium/large and white to cream-colored colonies.

Listeria presents different colony morphology depending on the media, ALOA, or OA. On ALOA the colonies typically appear blue with a clear/greyish halo surrounding them. This halo is crucial to differentiate *Listeria Monocytogenes* from other *Listeria* species [44]. On OA, due to the hydrolysis of esculin in the medium that produces a black precipitate, the colonies appear to be black.

Overall, every sample yielded positive results, with no detected presence of any specific microorganism. This represents a positive outcome of the analysis, where the results indicate either the absence of characteristic CFU or the absence of CFU.

Upon analyzing the TAMC, TYMC, Gram-negative bacteria, and specific microorganism analysis results, it is concluded that all drugs, nutritional supplements, raw materials, alcoholic extracts, and stability tests have positive outcomes and, therefore, were all approved. Each product tested met the strict criteria set forth for microbial safety, affirming their quality and compliance with standards.

Chapter VI – Environmental Control

1. Introduction

Assessing and monitoring the environmental conditions of the manufacturing area is crucial to ensure the quality and safety of the product. It is important to guarantee the microbiological quality because it can have a direct impact on the final pharmaceutical product. It involves the analysis and data collection of the microorganisms present in the air and on the surfaces of pharmaceutical production environments, as well as sanitation and packaging rooms [45].

2. Materials and Methods

Every room within the company undergoes a semestral, trimestral, or annual environmental control assessment depending on the International Organization for Standardization (ISO) assigned class (5, 8, or 9) (Table 34) [46].

Table 34-Periodicity according to each ISO class.

ISO	Frequency
5	Semestral
8	Semestral
9	Annual

Areas classified as ISO 5 and ISO 8 are involved in the manufacturing of drugs, and thus subjected to more frequent monitoring. On the other hand, rooms designated as ISO 9, which are not in direct contact with the product, are only monitored on an annual basis.

2.1 Air Control

The air control monitoring aims to maintain optimal air quality and hygiene standards throughout the manufacturing process. Monitoring air quality regularly enables early detection of deviations that could compromise product quality [47].

According to ISO 14644-1:2015 the number of air samples required is contingent upon the square meters of the room under analysis (Table 35).

Table 35- Number of samples required per room area.

Area (m^2)	Minimum number of sample points
8	4
10	5
24	6
28	7
32	8
36	9
52	10
56	11
64	12
68	13
72	14
76	15
>1000	See formula below

For rooms with an area over 1000 m^2, the minimum number of sampling points (NL) is calculated with the following equation, where A represents the room area in m^2.

$$NL = 27 \times (\frac{A}{1000})$$

The air control monitoring can be assessed using two methods:

a. Air sampling method (Centrifugation)

Two air samplers will be employed, one to place plates of TSA, to analyze aerobic bacteria, and the other containing plates of SDA, to analyze molds and yeast. Each air sampler will be strategically positioned in different locations within the room, according to the number of samples required. The air samplers used have a perforated lid, a vacuum, and a rotation system ensuring exposure to approximately 1 m^3 of air. Prior to each use, the lid of the air samplers are meticulously disinfected with 70% ethanol. TSA plates will be incubated at temperatures ranging between 30-35 ^oC for a duration of $\geq$ 5 days, and SDA plates will undergo incubation at temperatures between 20-25 ^oC for a period of 5-7 days [48].

The results will then be analyzed and expressed in CFU/m^3.

b. Plate Sedimentation method (Settle plates)

The plate sedimentation method is only applicable to areas classified as ISO 5 with laminar flow. To perform this test, TSA and SDA plates are exposed at specific points in the room, usually inside the laminar flow hood, with the lids removed for a defined period of time (4 hours) according to a specific monitoring plan. The microorganisms suspended in the air will eventually settle onto the surface of the agar medium.

Subsequently, the plates will undergo incubation at the conditions mentioned previously, followed by the enumeration of CFU/plate.

According to the ISO and the Ph. Eur there are two levels to evaluate the results: Alert and Action levels. Alert levels indicate that the system is surpassing normal operating values, signaling potential overload. These alert values serve exclusively as a warning regarding the systems deviation from its regular operational parameters and do not require immediate action. Upon reaching the action threshold, it becomes imperative to initiate a corrective intervention to the restore the system to its normal operating conditions [48].

The table below (Table 36) expresses the action and alert levels for the air sampling and plate sedimentation methods [31].

Table 36- Alert and action levels for air sampling and plate sedimentation methods

ISO	Air sampling CFU/m³		Settle plates (CFU/ plate)	
	Action	Alert	Action	Alert
5	8	5	4	3
8	150	100	75	50

As mentioned previously, rooms designated with ISO 9 are monitored annually. The reason behind this classification and periodicity of sampling is attributed due to the rooms function as a secondary packaging area for the products. Consequently, the ISO 9 classification has no action and alert limits established.

2.2 Surface Control

Surface monitoring is conducted to perform microbiological analysis aimed at overseeing the sanitization efficacy of surfaces within manufacturing facilities. During surface control sampling, the selection of sampling points is independent of the room's size. Five distinct sampling points will be designated : the wall, ceiling, floor, and door, along with the support table. This comprehensive approach ensures a thorough assessment of the microbial contamination across various surfaces critical to the manufacturing process. To perform this analysis, it is required contact plates, which are petri dishes that contain sterile growth medium, prepared in such a way that the surface of the medium is above the edges of the plate. Each designated area will be subjected to a TSA and SDA contact plate application for a duration of 10 ± 1 seconds, facilitating the efficient transfer of any existing microorganisms from the surface to the contact plate.

Following sampling, the contact plates undergo incubation conditions identical to those mentioned previously for TSA and SDA plates. The results are expressed in CFU/plate, which is equivalent to CFU/ 25 cm^2, and analyzed according to the Alert and Action levels established (Table 37).

Table 37- Alert and action levels for surface analysis

ISO	Surface analysis CFU/25cm²	
	Action	Alert
5	4	3
8	38	25

3. Results and Discussion

As previously stated , analyses are systematically carried out within every room across all sectors of the factory, occurring on an annual, semi-annual, or quarterly basis, contingent upon the specific room in question. Additionally, ISO 5, 8, and 9 classifications is assigned to each room implying that the alert and action levels differ according to each classification.

For a comprehensive overview, the following graphics present the mean values of air sampling and surface analysis with ISO 5 and 8 classifications, concerning the rooms on the first floor of the facility since the month of November of 2023.

3.1 Air control

In the following graphic, the average values of TAMC and TYMC for the air control in the first-floor rooms with ISO 8 classification analyzed up to the present moment are illustrated (Figure 6). For a better understanding, the Alert and Action level values for the respective ISO classification are highlighted.

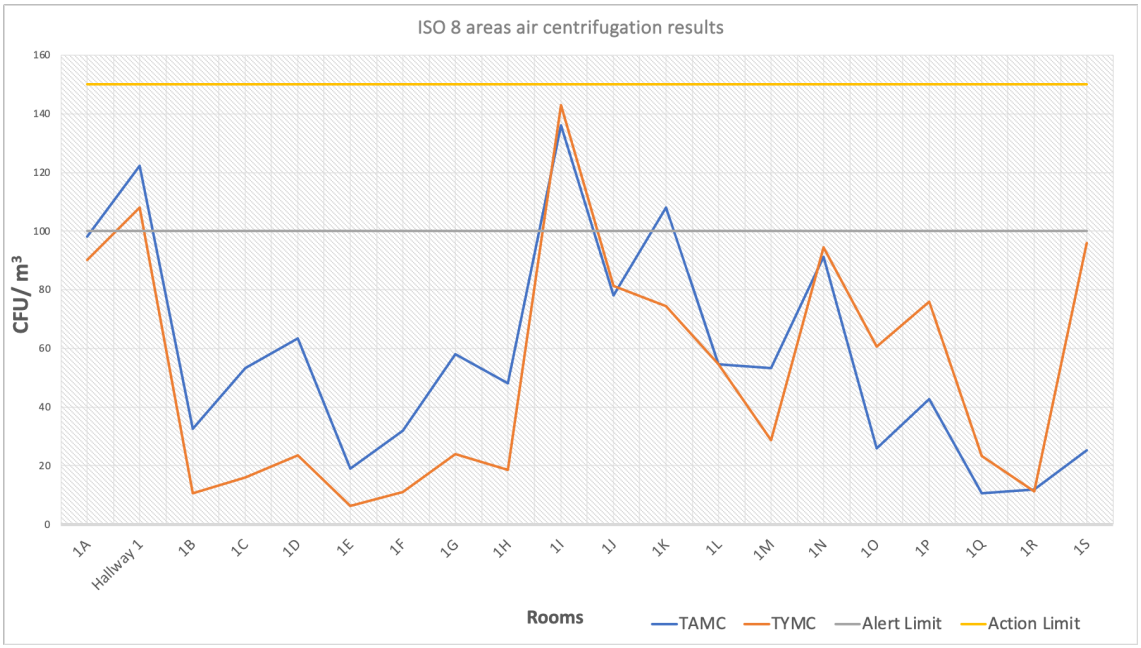


Figure 6- Mean TAMC and TYMC value of each sampling point regarding the air sampling analysis of every first floor ISO 8 rooms.

According to the graphic, the areas exposed to a large amount of movement of people tend to have a higher TAMC and TYMC value (Hallway 1). It is important to clarify that humidity and ventilation are crucial factors in bacterial growth [49]. The lack of air circulation in a small, closed material drying room (1I) has resulted in a higher level of humidity and dust which lead to high values of TAMC and TYMC.

As the graphic validates, the rooms with ISO 8 classification on the first floor have values that are below the set limits, which indicates good air quality which will have no impact on the process or the outcome of the final product.

The following graphic demonstrates the outcome through the air sampling and plate sedimentation method within ISO 5 rooms situated on the first floor (Figure 7). As mentioned previously, the Alert and Action limits diverge across different ISO classifications, and in this context, they also vary according to the method employed (air centrifugation or plate sedimentation method). Specifically, the plate sedimentation analysis has more stringent limit values, a distinction attributed to its correlation with rooms featuring laminar flow.

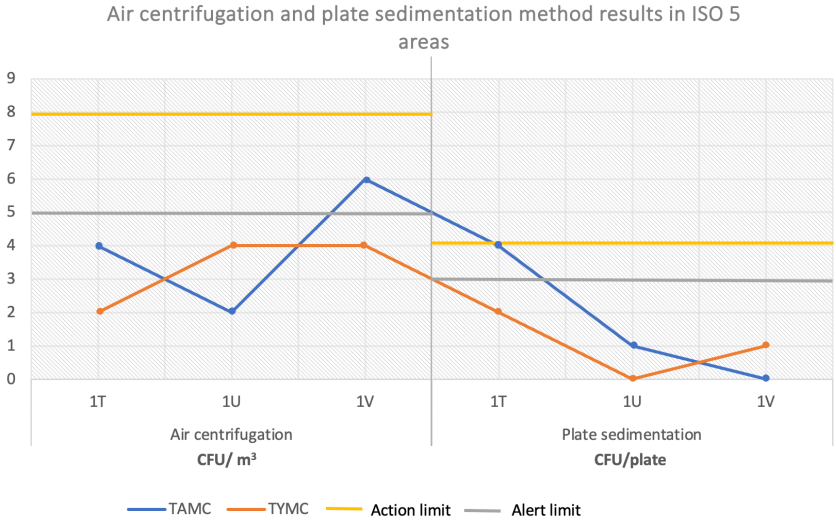


Figure 7- Mean TAMC and TYMC value of each sampling point regarding the air centrifugation and plate sedimentation analysis of every first floor ISO 5 rooms.

By the interpretation of the graphic, it can be concluded that the rooms 1T, 1U and 1V exhibit values below the limits in both air analyses. As these rooms are classified as ISO 5, it is crucial to ensure thorough and consistent cleaning to maintain hygienic standards. Consequently, it can be concluded that these rooms meet the standards, resulting in low TAMC and TYMC levels as expected.

3.2 Surface control

In regards to surface control, the outcomes from rooms classified as ISO 5 and ISO 8 will be presented in the following graphics. In this case the sampling is carried out using the same methodology, as explained previously, and at identical sampling points. It is presented in the following graphics (Figures 8 and 9) the mean value of TAMC and TYMC of every room specified in the air control graphic.

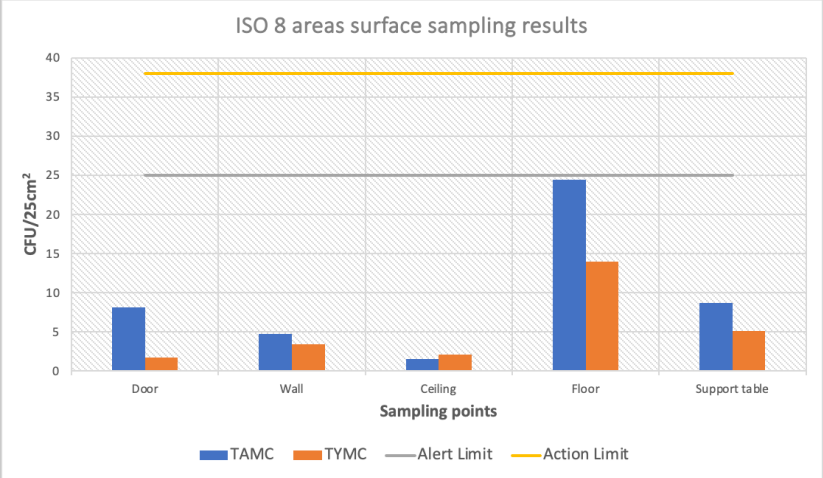


Figure 8- Mean TAMC and TYMC value of each sampling point regarding the surface sampling analysis of every first floor ISO 8 rooms.

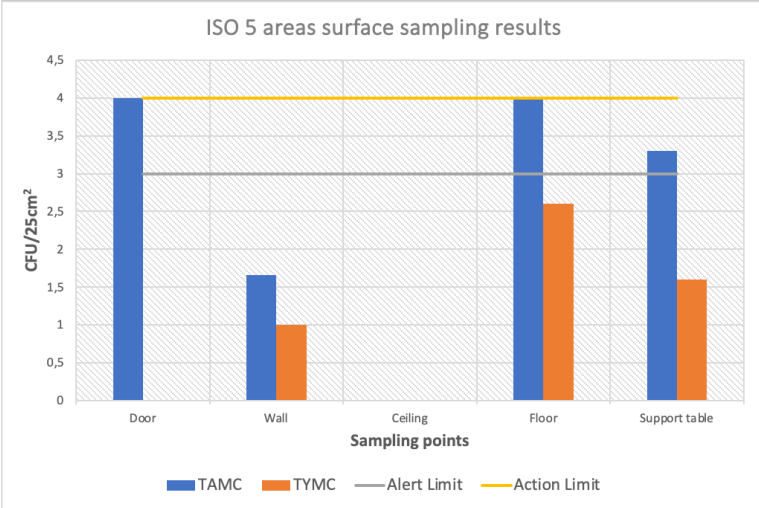


Figure 9- Mean TAMC and TYMC value of each sampling point regarding the surface sampling analysis of every first floor ISO 5 rooms.

As expected , the surface with higher levels of aerobic microorganisms and yeast and mold presence is the floor in both cases. On the other hand, the surface presenting the least contamination is the ceiling (Figures 8 and 9). It is possible to conclude that human activity significantly contributes to this differential distribution of microbial contaminants.

In accordance with the Action levels delineated by the Ph. Eur, it is noteworthy that the designated rooms do not surpass any predefined thresholds. Without exceeding this limit in any instance, it is possible to conclude the appropriate sanitization and efficacy of the surface decontamination within the rooms.

For a more detailed and specific analysis, four rooms have been randomly selected from various floors and sectors of the factory with different ISO classifications (Table 38).

Table 38- ISO classification for rooms 1T, 2D, 1B and 1X.

Room	ISO
1T	5
2D	8
1B	8
1X	9

Room 1T

Room 1T is classified as ISO 5, serving as a drug weighing area. With a total area of 7.72 m^2, only two sampling points are required to analyze this room.

The following figure (Figure 10) represents the position of the sampling points within this room, for a better understanding of the results. The surface sampling points are represented by the following legend: D- Door; W- Wall; F- Floor; C- Ceiling; T- support Table.

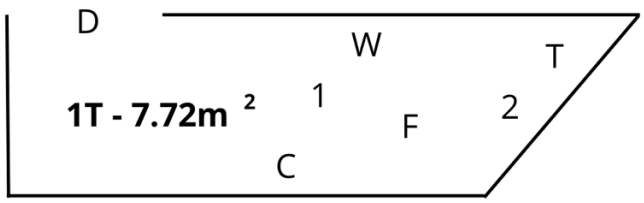


Figure 10- Sampling points position in room 1T.

Given the presence of a laminar flow chamber, air sampling analysis must be conducted using both methods: air centrifugation and plate sedimentation. The following graphic (Figure 11) illustrates the results obtained from the two sampling points using both methods.

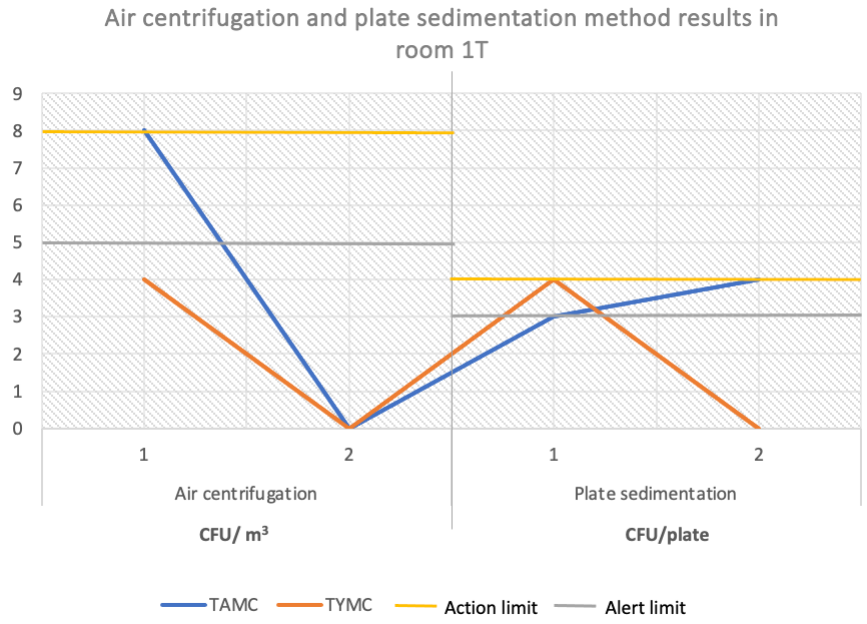


Figure 11- Room 1T air sampling results.

In both analyses the results are below the established limits. For that reason, it is possible to conclude that the laminar flow is working properly, with no microbial contamination. In a drug weighing room, it is imperative that this condition remains as it can influence the entirety of the product manufacturing.

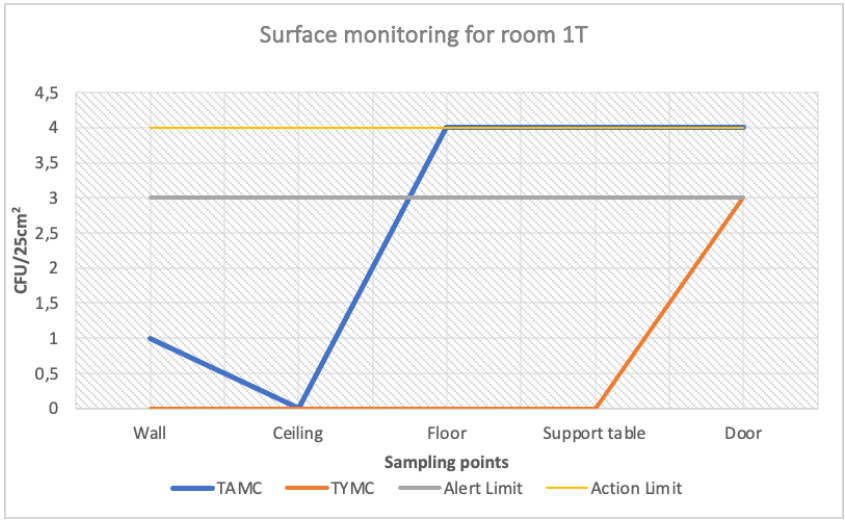


Figure 12- Room 1T surface sampling results.

In regards to the surface monitoring in this room, it is possible to conclude that the surfaces are properly cleaned, presenting values under the action limit

(Figure 12). It is possible to conclude that the environmental conditions of room 1T will not negatively affect the manufacturing process.

Room 2D

Room 2D is classified as ISO 8 and it is not directly involved in the manufacturing process of the products. This room functions as a quarantine of liquid, semi-solid, and finished product intermediates so it can be expected higher values. This room requires 6 sampling points to be analyzed, since it has an area of 25.96 m^2.

For a better understanding of the results, the following figure (Figure 13) illustrates the location of each air sampling point alongside the areas within the room in which surface sampling points were collected.

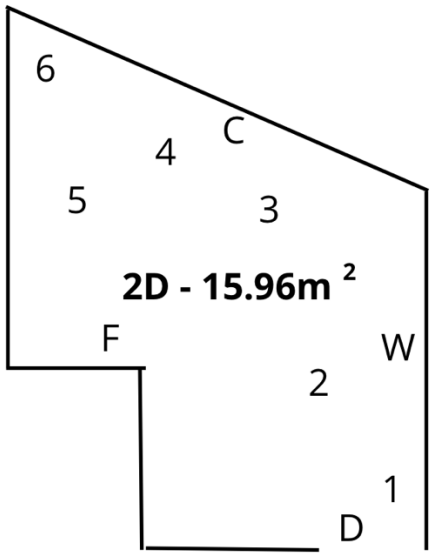


Figure 13- Sampling points position in room 2D.

As illustrated in Figure 13, each sampling point, whether for air or surface sampling, is strategically distributed throughout the room, ensuring a comprehensive evaluation of all areas. The results of that evaluation are expressed in the figure below (Figure 14).

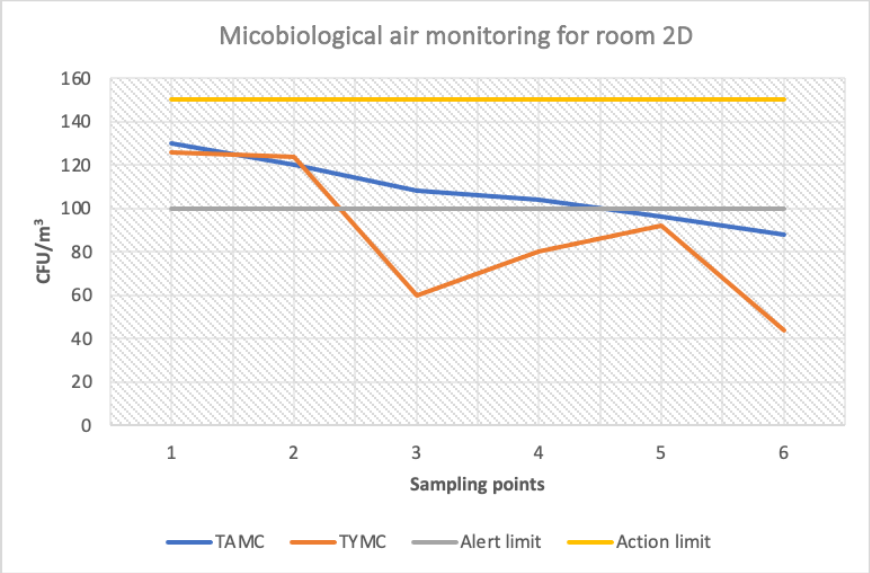


Figure 14- Room 2D air sampling results.

Notably, sampling points in close proximity to the door (1 and 2) exhibit elevated levels of TAMC and TYMC where there is increased movement and higher potential for contamination. The frequent traffic associated with doors can introduce external contaminants into the room, raising microbial load in the air.

Despite the anticipated high values corresponding with the rooms function, the overall results are positive, without surpassing the Action limit.

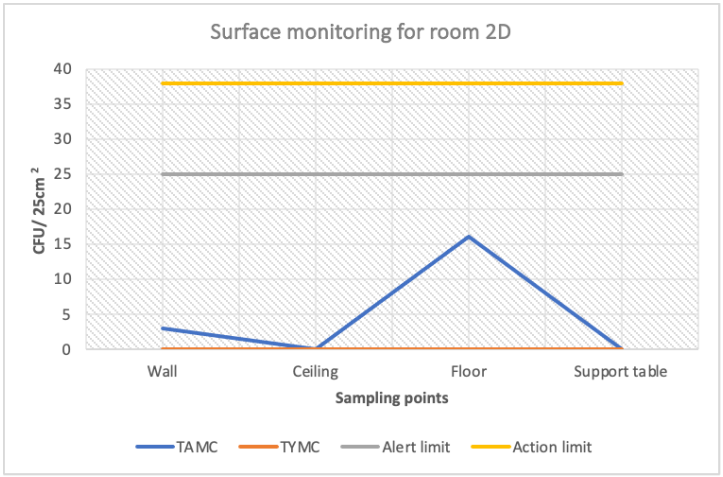


Figure 15- Room 2D surface sampling results.

Concerning the surface control (Figure 15), the room presents adequately sanitized surfaces. It is noteworthy that typically, the floor area registers the highest TAMC count but does not reach the alert limit.

Room 1B

Room 1B serves as a bulk preparation room and it is classified as ISO 8. Six sampling points were uniformly distributed across the room, covering a total area of 15.12m^2. This strategic distribution aims to provide comprehensive and representative data for a more accurate evaluation (Figure 16).

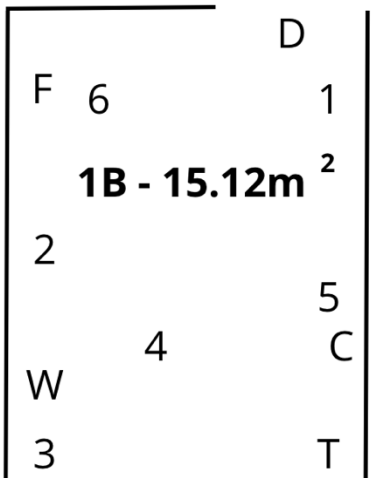


Figure 16- Sampling points position in room 1B.

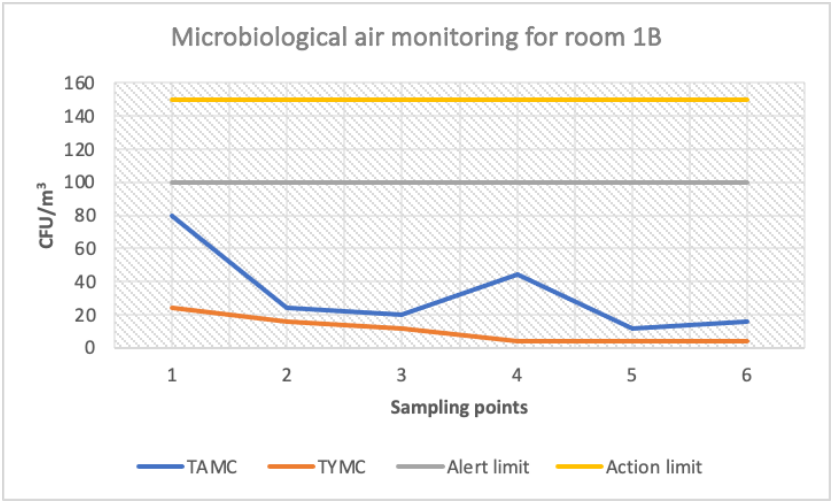


Figure 17- Room 1B air sampling results.

As expected, the air quality results obtained from both the TAMC and TYMC assessments reveal the highest concentrations at the sampling point 1, proximate to the entrance door (Figure 17).

The results obtained from the air monitoring for room 1B were positive, with low TAMC and TYMC counts below the alert limit.

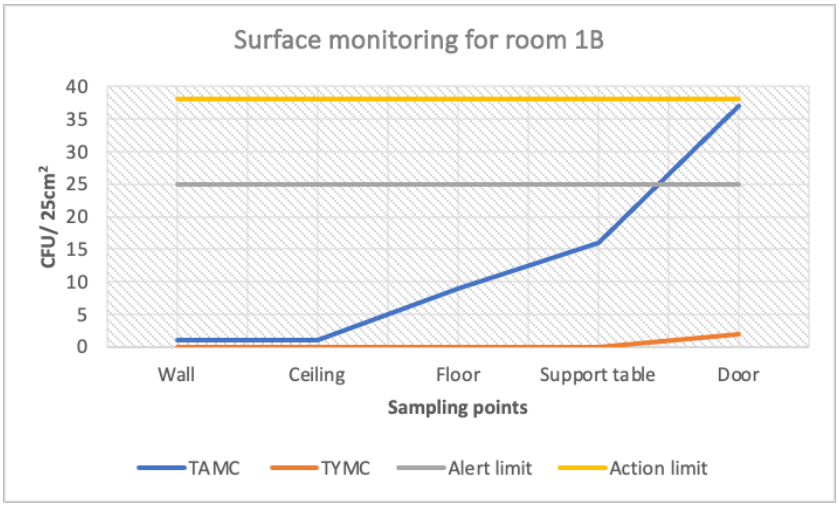


Figure 18- Room 1B surface sampling results.

In the surface assessment, it was observed that the door exhibited the highest TAMC and TYMC values (Figure 18). The door represents an area where greater microbial contamination is expected as it is subjected to frequent handling and interaction throughout the day.

Overall, the surface results were positive, with only one sampling point surpassing the alert limit.

Room 1X

Room 1X is a secondary packaging area and is analyzed annually due to its ISO 9 classification. Unlike primary packaging areas where products are directly handled and processed, secondary packaging involves tasks such as labeling, and boxing, which are focused on the outer packaging of the product. For that reason, the products quality and safety cannot be compromised in this room, thus the ISO 9 classification and only annual analysis are required.

Having a total area of 25.58 m^2, seven air sampling points and five surface sampling points are analyzed throughout the room (Figure 19).

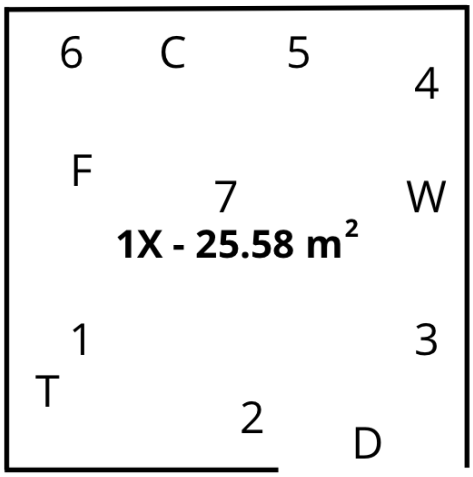


Figure 19- Sampling points position in room 1X.

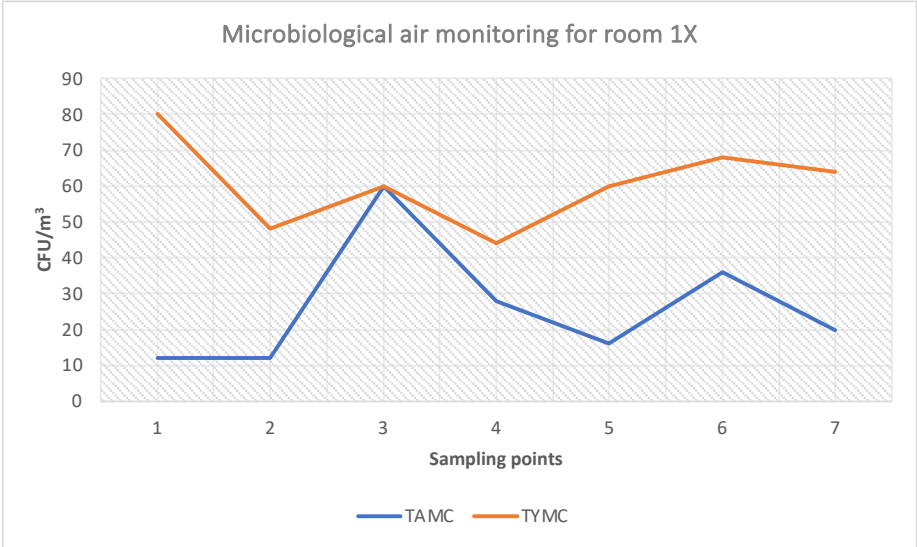


Figure 20- Room 1X air sampling results.

Due to its low humidity, and low aerial dust from packaging materials, this room exhibits low levels of TAMC and TYMC at every sampling point (Figure 20). Sampling point number 1, near the support table, can have a higher human presence, resulting in a higher TYMC value. Sampling point number 3, positioned directly in front of the door, registers a higher microbial count due to its proximity

to a frequent point of entry and the potential for increased environmental contamination.

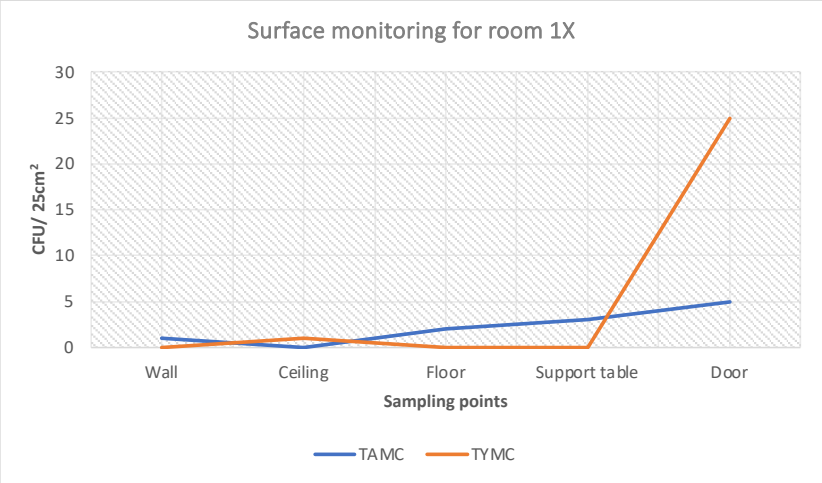


Figure 21- Room 1X surface sampling results.

As well as the air sampling results, the surface monitoring outcomes are also very promising (Figure 21). As mentioned previously, the ISO 9 classification has no action and alert limits established but the microbial count does not reach high values on the air and surface monitoring.

Chapter VII – Purified Water Control

1. Introduction

Water stands as a fundamental commodity extensively used within the Pharmaceutical sector. It serves as a key component in the production, formulation, and manufacturing processes of pharmaceutical products, functioning both as a raw material and a solvent. Additionally, purified water is also used in the cleaning process of equipment and production rooms.

Given its indispensable role in this industry, ensuring stringent microbiological control over the purified water system becomes crucial [50].

2. Materials and Methods

2.1 Sample collection

Water samples are collected from various points within the facility's water system, daily. Regular water sampling ensures the quality and safety, and it allows for the identification of potential contaminants that could compromise the integrity of the water supply.

Thoroughly disinfect the collection valve using a 70% ethanol solution, ensuring both the interior and exterior surfaces are treated. Subsequently, open the tap and allow water to flow for approximately one minute. Following this, gather the sample into a sterile vial, with each sample comprising a different volume. The periodicity and required analysis for each water source are expressed in Table 39 [51].

Table 39- Periodicity and required analysis for each water source.

Water sampling points	Periodicity	Analysis	Media
Purified water	Every two weeks	TAMC (100mL)	R2A
Net water (NW)	Weekly	TAMC (100mL)	R2A
		E. coli (100mL)	MCA
		Coliforms (100mL)	VRB
Net water (AF)	Semesterly	TAMC (10 mL and 1mL)	R2A
		E. coli (100mL)	MCA
		Coliforms (100mL)	VRB

In the analysis of the net water (NW and AF) it is required to conduct the analysis to determine TAMC, *E. coli,* and coliforms presence. The principal disparity between NW and AF lies in the frequency of sampling and the designated sampling sites. In the AF system, numerous sampling points are distributed throughout the rooms, whereas in the NW system, there is only a single sampling site.

2.2 Bioburden Testing

Bioburden refers to the presence of viable microorganisms on a material, within the environment, inside a device, etc. In the present study, bioburden testing is used to detect and quantify the number of viable microorganisms present in a water sample. There are three most common methods : membrane filtration, direct plate, and most probable number (MPN) for bioburden testing [52]

In this case, it is employed the membrane filtration method for bioburden testing. Following collection of the water, in a laminar flow hood, a membrane filter with a pore size of 0.45 μm is carefully positioned with the gridded side facing upward. The filtration unit is equipped with a funnel attached to its base, ensuring that the membrane filter is positioned between the funnel and the base. The precise volume specified in the previous table is poured into the funnel, and then the vacuum is activated to absorb the water into the filter.

Using forceps, the membrane filter is delicately lifted by its edge and placed either on an R2A agar plate, MCA or VRB. For the TAMC analysis, the

R2A agar petri dish is then subjected to an incubation period of five days within a temperature-controlled environment set at 30-35 ºC. For the *E. coli* presence analysis, the filter is placed on an MCA plate and incubated at 30-35 ºC for 18-72 hours. Finally, for the coliform analysis, the filter is positioned on a VRB plate and incubated at 30-35 ºC for 18-24 hours [53]. A schematic representation of this process is illustrated on Figure 22.

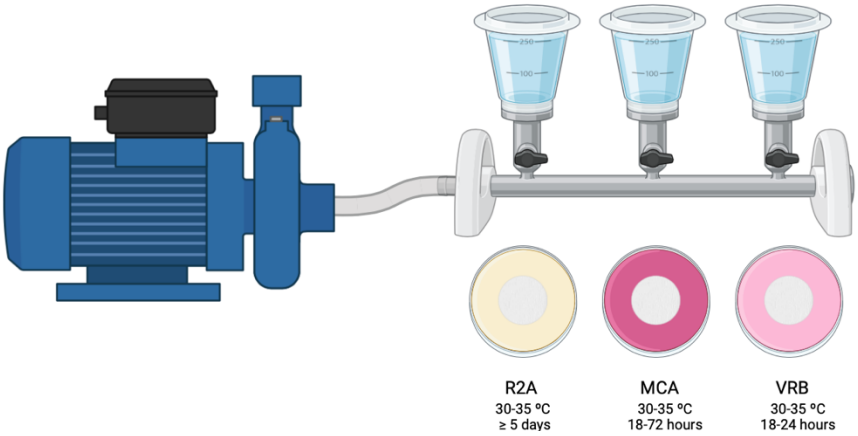


Figure 22- Schematic representation of the purified water analysis. Created with BioRender.com.

The results are analyzed by counting of CFU/mL and evaluated according to action and alert levels (Table 40).

Table 40- Alert and Action levels for water control.

ISO	Water samples CFU/mL	
	Action	Alert
5	80	50

3. Results and Discussion

For a clearer understanding of the purified water result, it is going to be presented the mean TAMC values for each sampling point since the beginning of the present year.

It is important to notice that the purified water sampling points are strategically distributed across the facility, with many situated within production rooms directly involved in the manufacturing of pharmaceutical and nutraceutical products. Consequently, the positivity of these results holds great importance as they directly impact production safety.

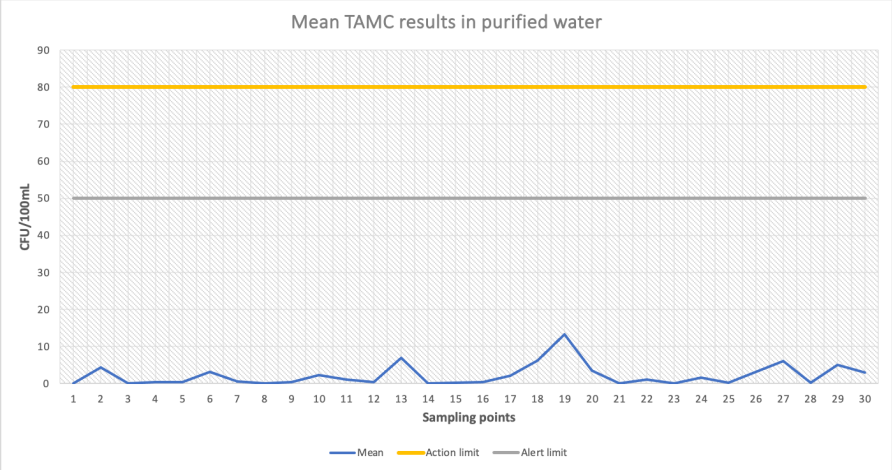


Figure 23- Mean TAMC results in purified water analysis.

It was expected that higher values would be observed at sampling points with less frequent use, such as points 2, 13, 19, and 27. Stagnant conditions in these sampling points create a more favorable environment for bacterial growth. Despite this and based on the interpretation of the graphic (Figure 23), it can be deduced that each sampling point exhibits a TAMC mean value below the alert limit indicating a positive trend.

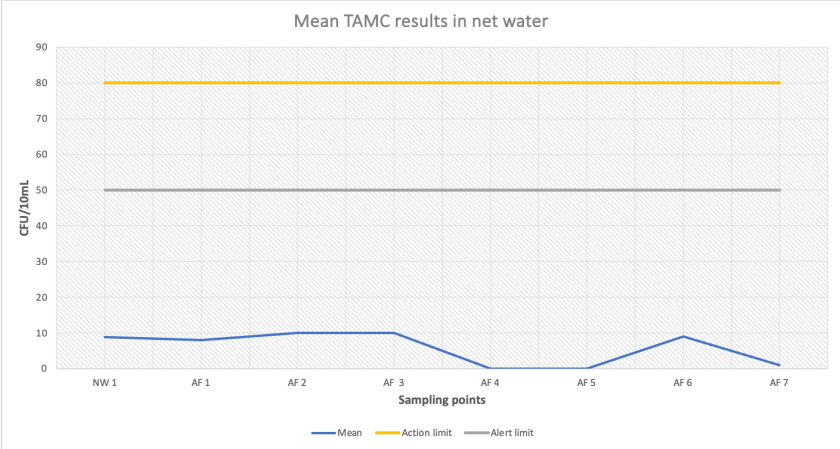


Figure 24- Mean TAMC results in net water analysis.

In regards to the TAMC results (Figure 24), it is notable that every net water sampling point demonstrates a low count of CFUs.

In the analysis conducted on the net water sampling points, it is noteworthy that no presence of *E.coli* and coliforms was detected. Consequently, there are no results to be presented in this regard.

Based on the purified water results, it can be established that the quality and safety of the water system are satisfactory and will not adversely affect the manufacturing process.

Chapter VIII – Operator Control

1. Introduction

In controlled environments, most microbial contamination is predominantly of human origin [54]. This approach acknowledges the human factor as a key contributor to potential microbial contaminations and underscores the importance of implementing measures to enhance control and maintain the integrity of the controlled environment. This control is conducted every three months across every sector of the company, mainly targeting the operators involved in the manufacturing phase of the products.

2. Materials and Methods

2.1 Sample collection

TSA and SDA contact plates are used to assess microbial contamination on two sampling points : the gloves and forearms of operators. When conducting glove testing, each finger of both the right and left hand should be pressed onto the contact plate. As for the forearms, the plate should be pressed against both the right and left forearms for a duration of 10 ± 1 seconds. Subsequently, the TSA plates are incubated at 30-35 ºC for 3-5 days, while the SDA plates undergo incubation at 20-25 ºC for 5-7 days. The results are analyzed through the enumeration of CFU/ plate or CFU/25 cm^2 (Table 41) [55].

Table 41- Alert and Action levels for operator control monitoring.

	Operator samples CFU/25cm²	
	Action	**Alert**
Gloves	50	25
Forearms	50	25

3. Results and Discussion

As mentioned previously, the operator analysis is conducted every three months throughout all sectors of the factory. Subsequently, it will be presented the results of the two sampling moments this year, 2024 (Figure 25).

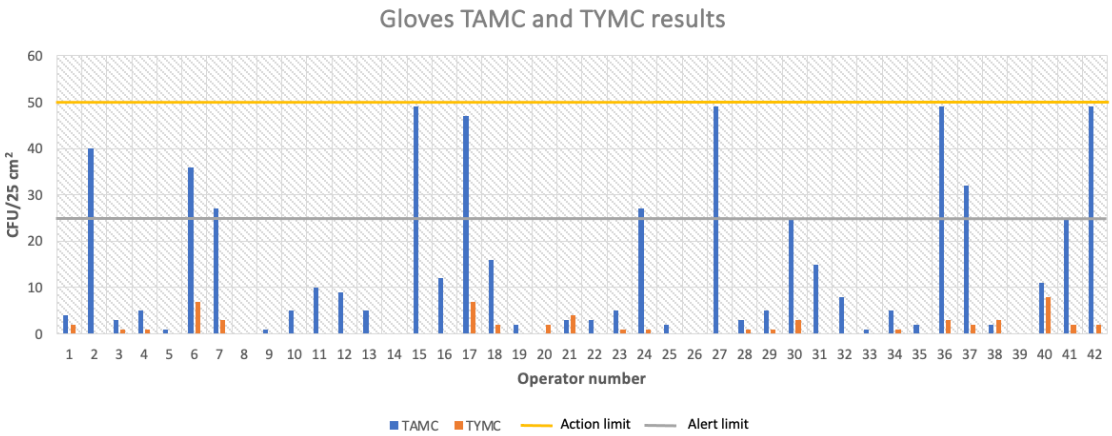


Figure 25- TAMC and TYMC gloves results for each operator where half of the results represent the first sampling time of the year 2024, and the other half represents the second.

As expected , the gloves exhibit low TAMC and TYMC attributed to their frequent disinfection and regular replacement (Figure 25). The elevated TAMC values observed in some specific operators (15, 17, 27, 36 and 42) may be attributed to the handling of certain materials without replacing the gloves at the time of sampling.

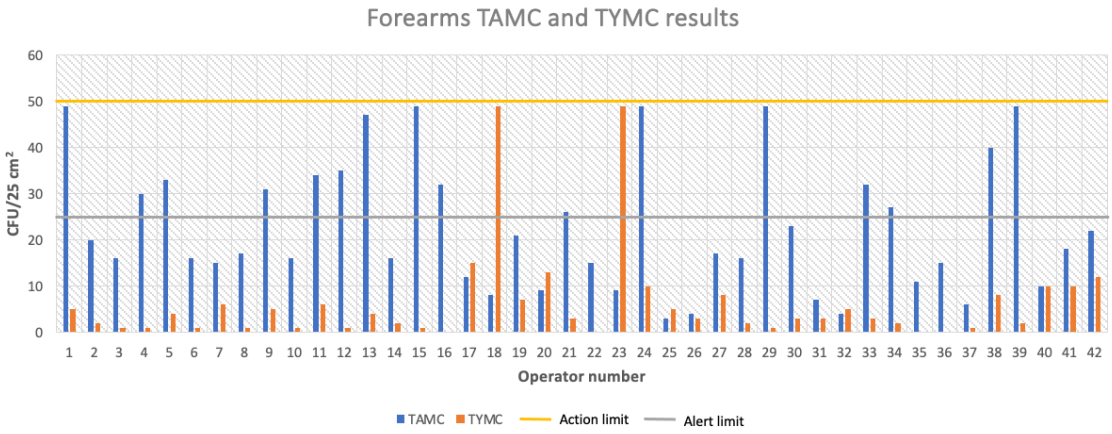


Figure 26- TAMC and TYMC forearms results for each operator where half of the results represent the first sampling time of the year 2024, and the other half represents the second.

Typically, the uniforms are worn one or two days before their replacement. The higher microbial count on some operators may be due to their function , when working on a production area, being in contact with raw materials, reagents, and dust it is common that there is a higher level of contamination. On the other hand, operators working in a packaging area typically have lower microbial count due to the lack of direct contact with the substances mentioned previously.

Despite this, none of the results indicate a count higher than the established action level (Figure 26).

As it can be concluded by the interpretation of all the analysis, purified water control and environmental control results, there is generally a higher count of TAMC compared to TYMC. This difference can be caused by multiple factors. Firstly, aerobic bacteria are more common and have a superior capacity to grow in various environmental conditions, they are more adaptable and can proliferate rapidly. In contrast, typically yeasts and molds require specific growth conditions, such as high humidity and specific substrates. Additionally, the decontamination agents used in these controlled environments are often more effective against fungi, which contributes to a lower TYMC count.

Chapter IX – Conclusion

All aspects that could compromise the quality and safety of the pharmaceutical products have been subjected to meticulous analysis. The results collected from this comprehensive analysis have been presented for thorough examination and interpretation.

Through a careful examination of each individual result, it becomes evident that every stage of the production process operates within the parameters of good conditions. Notably, neither the purified water system, crucial for the formulation of pharmaceutical compounds and responsible for the sanitization of the reactors, nor the environment where the products are manufactured at, maintained to adhere the standards of cleanliness and sterility, exhibit any indicators that could compromise or impact the quality or efficacy of the final product. Furthermore, an assessment of the human element within the production process reveals a commitment to established protocols and best practices. Each stage and factor, be it the purified water, the manufacturing environment, or the operators, function well to uphold the highest standards.

The strain preparation and the growth promotion protocols represent indispensable steps in this process. Through these precise procedures we confirm the efficacy and reliability of the methodologies used.

All of the analyzed final products, raw materials, alcoholic extracts and stability tests were approved with the results meeting the proper specification. This ensures that the pharmaceutical products emerging from Labialfarma's facility do not present a microorganism contamination that could lead to adverse health risks for the customers, reaffirming the company's commitment to the well-being of the consumers.

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Appendix I

The following figures (Figure 27 to 31) specify the obtained values of each analysis, in every sampling point.

Room	Sampling points	Total Aerobic Microbial Count (TAMC)												Mean TAMC	Total Yeast and Mold Count (TYMC)												Mean TYMC	Alert Limit	Action Limit
1A	9	80	64	100	124	108	100	92	104	112				98,2	124	64	56	48	100	108	98	102	112				90,22	100	150
Hallway 1	10	139	100	140	100	110	100	145	143	125	120			122,4	119	97	149	80	88	96	104	98	145	105			108,44	100	150
1B	6	80	24	20	44	12	16							32,7	24	16	12	4	4	4							10,67	100	150
1C	6	128	52	36	52	40	12							53,3	24	40	4	20	8	0							16,00	100	150
1D	6	68	32	104	20	36	120							63,3	0	12	44	1	32	52							23,50	100	150
1E	12	68	2	12	30	28	16	12	0	44	4	12	0	19,0	20	8	0	0	16	4	8	0	16	0	0	4	8,00	100	150
1F	7	20	48	64	36	24	16	16						32,0	16	12	0	25	20	0	4						11,00	100	150
1G	6	76	64	32	44	52	80							58,0	32	36	40	12	4	20							24,00	100	150
1H	6	149	28	8	60	36	8							48,2	32	16	20	20	8	16							18,67	100	150
1I	2	140	132											136,0	138	148											143,00	100	150
1J	6	44	132	36	64	16	176							78,0	96	108	44	40	56	144							81,33	100	150
1K	4	40	120	140	132									108,0	40	104	52	102									74,50	100	150
1L	3	52	40	72										54,7	24	60	80										54,67	100	150
1M	6	148	48	32	8	40	44							53,3	104	20	20	0	12	16							28,67	100	150
1N	5	84	60	92	112	108								91,2	124	64	112	72	100								94,40	100	150
1O	6	44	16	4	48	32	12							26,0	64	28	72	112	32	56							60,67	100	150
1P	6	48	44	56	48	0	60							42,7	60	76	88	80	80	72							76,00	100	150
1Q	6	28	8	12	0	4	12							10,7	16	0	4	52	32	36							23,33	100	150
1R	5	4	8	20	12	16								12,0	16	12	8	12	8								11,20	100	150
1S	6	28	48	44	8	24	0							25,3	124	128	124	52	136	12							96,00	100	150

Figure 27- Specific values for ISO 8 air control monitorization.

Room	Sampling points	Air Centrifugation Total Aerobic Microbial Count (TAMC)		tal Yeast and Mold Count (TYM		Mean TAMC	Mean TYMC	Alert limit	Action limit
1T	2	8	0	4	0	4	2	5	8
1U	2	4	0	8	0	2	4	5	8
1V	2	8	4	8	0	6	4	5	8

Figure 28- Specific values for ISO 5 air control monitorization.

Room	Sampling points	Plate Sedimentation Total Aerobic Microbial Count (TAMC)		tal Yeast and Mold Count (TYM		Mean TAMC	Mean TYMC	Alert limit	Action limit
1T	2	3	4	4	0	3,5	2	5	8
1U	2	1	0	0	0	0,5	0	5	8
1V	2	0	0	1	0	0	0,5	5	8

Figure 29- Specific values for ISO 5 plate sedimentation monitorization.

	Surfaces											
	Door		Wall		Ceiling		Floor		Support table			
Room	TAMC	TYMC	TAMC	TYMC	TAMC	TYMC	TAMC	TYMC	TAMC	TYMC	Alert Limit	Action Limit
1A	0	13	12	0	5	11	1	24	23	0	25	38
Hallway 1	24	0	0	0	13	0	40	3	0	0	25	38
1B	9	0	1	0	1	0	37	2	16	0	25	38
1C	2	0	0	0	0	0	37	26	2	0	25	38
1D	1	0	0	0	0	0	9	0	5	1	25	38
1E	18	0	0	0	0	0	37	0	20	8	25	38
1F	24	0	25	24	0	0	11	0	0	0	25	38
1G	36	0	3	1	6	5	37	36	0	0	25	38
1H	8	0	32	12	0	0	37	37	4	12	25	38
1I	0	1	1	5	0	0	14	27	6	16	25	38
1J	3	0	2	4	0	2	9	22	4	30	25	38
1K	0	0	0	4	0	0	20	12	0	0	25	38
1L	0	4	0	0	0	0	36	30	35	1	25	38
1M	10	0	2	0	4	1	36	0	7	0	25	38
1N	0	1	0	0	0	0	23	17	8	15	25	38
1O	0	1	0	0	0	0	30	29	5	3	25	38
1P	16	0	1	1	0	0	24	4	1	2	25	38
1Q	3	5	8	8	1	22	26	2	25	5	25	38
1R	9	2	6	5	0	1	19	3	0	0	25	38
1S	0	7	2	4	1	1	6	5	14	10	25	38
Mean	8,15	1,7	4,75	3,4	1,55	2,15	24,45	13,95	8,75	5,15		

Figure 30- Specific values for ISO 8 surface monitorization.

	Surfaces											
	Door		Wall		Ceiling		Floor		Support table			
Room	TAMC	TYMC	TAMC	TYMC	TAMC	TYMC	TAMC	TYMC	TAMC	TYMC	Alert Limit	Action Limit
1T	4	0	1	0	0	0	4	3	4	0	3	4
1U	4	0	4	3	0	0	4	2	2	1	3	4
1V	4	0	0	0	0	0	4	3	4	4	3	4
Mean	4	0	1,66	1	0	0	4	2,66	3,33	1,66		

Figure 31- Specific values for ISO 5 surface monitorization.