MSc. THESIS

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2024

Hungarian University of Agriculture and Life Sciences
Buda Campus

Stipendium Hungaricum

Food Safety and Quality Engineering MSc

Identification of bell pepper (*Capsicum annum* L.) microbiota and assessment of antibiotic resistance of foodborne bacteria

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BUDAPEST

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1. Introduction

The prevalence of pathogenic microorganisms in meat products, dairy products, and vegetables depends on several factors. In primary produce, the causes can be topography, land-use interactions, and climate. Moreover, pathogens can contaminate produce directly in the field through several routes, such as atmospheric deposition, absorption from polluted soils and groundwater, use of untreated or poorly treated manure and compost, exposure to contaminated water from irrigation or flooding, transmission by insects, or fecal contamination from livestock or wildlife (Alegbeleye et al., 2018).

In the case of processed products such as meat and dairy products the transmission routes can be controlled better, but in case they are not controlled there can be a potential risk for contamination, among those causes are poor manufacturing practices, such as bad hygiene, cross contaminations being the main source of contamination the co-workers. Besides, as is known meat is a good source of protein, essential fatty acids, minerals, and vitamins, but easily perishable because it provides a suitable medium for the growth of various microorganisms (Bantawa et al., 2018). Similar to the case of dairy products, like raw milk, which is prized for its nutritional value, can serve as a reservoir for antibiotic-resistant bacteria, posing significant risks to consumers, particularly vulnerable populations such as infants, elderly individuals, and immunocompromised individuals, having in this case as reasons of pathogen microorganism contamination (Calahorrano-Moreno et al., 2022).

The microbiology risks in the food industry are part of a big concern regarding human health. The availability of nutrients along the food production chain creates a great environment for microbial growth, resulting most of the time in financial losses and even in human losses due to the uncontrolled pathogenic bacteria that over time are becoming more resistant to antibiotic treatments. Worldwide is reported, that annually the consumption of contaminated food products is responsible for causing illness in 600 million people and for economic losses exceeding USD 100 billion (Grudlewska-Buda et al., 2023).

The growing concern of antibiotic resistance is not just about the known microorganisms like *Campylobacter* spp*, Escherichia coli,* or *Listeria monocytogenes* but also is in microorganisms that before were harmful and nowadays are being considered as pathogens because of many reasons like the changes in the vulnerable population, change food-vector type or change of symptoms, becoming their resistance to antibiotic treatments. Microbial contaminants in food, including bacteria resistant to antibiotics, pose serious risks to consumers, potentially leading to foodborne illnesses and complicating treatment modalities

(Salam et al., 2023). Understanding the emergence and spread of antibiotic-resistant microbes in food sources is critical for mitigating these risks and preserving the efficacy of antimicrobial therapies. Foodborne pathogens have long been recognized as major contributors to illness and disease outbreaks worldwide (Elbehiry et al., 2023).

Mismanagement of antibiotics in animal husbandry, such as incorrect selection or overuse, is recognized as a primary factor contributing to the emergence and spread of antibiotic-resistant foodborne pathogens (Grudlewska-Buda et al., 2023). The rise of antibiotic-resistant bacteria presents a formidable challenge to global public health, with foodborne transmission serving as a significant possibility for the dissemination of resistant strains. World Health Organization (WHO) reported that in 2019 antimicrobial resistance (AMR) was responsible for the deaths of 700,000 people and the estimation for 2050 is that it will rise to 20 million, representing a cost of around 2.9 trillion dollars, which triggers a huge concern about the live and economy of the people around the world (Uddin et al., 2021).

The antibiotic sensitivity of these pathogens is crucial. The presence of antibiotic-resistant bacteria in food items such as raw pork meat, raw milk, fruit, and vegetables underscores the complex interaction between food production, microbial contamination, and human health (Elbehiry et al., 2023; Okaiyeto et al., 2024). With a lack of validated diagnostic methods and effective treatment options, the antibiotic resistance exhibited by emerging foodborne pathogens can present a significant epidemiological threat (Grudlewska-Buda et al., 2023). Considering that, this study aims to investigate the antibiotic resistance profile of some bacteria isolated from bell pepper phyllosphere, raw pork meat, and raw milk, employing microbiological methods to determine the minimum inhibitory concentration (MIC) of oxytetracycline-hydrochloride. Explaining the dynamics of antibiotic resistance in foodborne bacteria, this research endeavors to expose the potential microorganisms with antibiotic resistance that can become a threat to the public health.

2. Goals of the thesis

There is a pressing need to investigate the resistance patterns of microorganisms present in food samples to ensure the safety and quality of the food supply chain. The thesis work was carried out connecting to a PhD project aiming for an overview about the antimicrobial resistance of foodborne microorganisms. The goal of this research was to isolate and identify microorganisms from the phyllosphere of bell pepper (Capsicum annuum L.) and to analyze the antibiotic resistance patterns of microorganisms isolated from samples of raw pork meat, raw milk and bell pepper phyllosphere, specifically to Oxytetracycline-hydrochloride. By understanding the antibiotic resistance profiles of these microorganisms, we aim to contribute to the assessment of their potential risks the they pose to food safety and public health.

Objectives:

1) Isolation of Microbial Populations: The first objective is to isolate microorganisms present in the samples using various culture mediums, including general and selective agar. This step is crucial for identifying and studying the microorganisms responsible for potential contamination.
2) Characterization of colony types: The next objective is to characterize the different types of colonies through visual morphological characteristics and microscopic examination. This will provide valuable insights into the diversity and composition of microbial populations in the food samples.
3) Biochemical characterization: To perform biochemical methods for the characterization of isolated microorganisms. This step will help in determining the biochemical properties of the microorganisms, aiding in their classification and identification.
4) Genus and Species-Level Identification: Using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS), we aim to identify the isolated microorganisms at the genus and species levels.
5) Literature Review and Pathogenicity Assessment: To conduct a comprehensive literature review to assess the potential pathogenicity of the identified bacteria. This step is essential for understanding the health risks associated with foodborne microorganisms and informing risk management strategies.
6) Determination of Minimum Inhibitory Concentration (MIC) Values: Finally, to determine the MIC values of selected microorganisms using the Disk Diffusion Test method and Spectroscopy method (Multiskan Ascent). This will provide valuable information on the susceptibility of microorganisms to oxytetracycline-hydrochloride.

3. Literature overview.

3.1. The Grossum Group: An Overview of Bell Pepper (*Capsicum annuum* L.).

The Grossum Group, commonly known as Bell Pepper, encompasses several popular names such as Sweet Pepper, Green Pepper, and Red Pepper. Taxonomically, these plants belong to the Solanaceae family, also known as the Nightshade family. While the Grossum Group is inherently a tropical perennial, it is predominantly cultivated as an annual vegetable in agricultural practices (Meyer, n.d.).

3.1.1. Morphological Characteristics.

The Grossum Group exhibits an upright and bushy growth habit. The plant is characterized by its dark green lance-shaped leaves and produces small white flowers. The most distinctive feature of this group is its edible fruit, which is botanically classified as a berry. These fruits present a diverse colour palette, transitioning from green during the immature stage to shades of yellow, orange, red, purple, black, and even white as they mature. Furthermore, the taste profile of the fruit evolves from a mildly bitter to a sweeter flavour as it ripens (Meyer, n.d.)

3.1.2. Biochemical compounds present in Bell peppers.

Capsicum annuum L. is a good source of phytochemicals including phenolic, flavonoids, and carotenoids. It contains significant amounts of beta-carotene, capsaicin, and vitamins A and C (Anaya-Esparza et al., 2021). These components offer various health benefits, aiding in the prevention of numerous diseases. They are particularly beneficial for liver health and have potential advantages in addressing issues related to male reproductive health (Dini, 2018).

3.2.Foodborne bacteria related to meat, dairy and vegetables.

Nowadays foodborne bacteria are one of the main concerns related to the health of people, due to pathogens that constantly are evolving or due to emerges microorganisms that can represent a potential risk to human health. However, over time the most important pathogens related to food have been found in meat, dairy, and vegetables, mainly. Although during the time, from every outbreak investigation information and knowledge have been developing, the microorganisms may change over time, they can become more resistant to antibiotics, or they can become resistant to hard circumstances related to treatments that are applied in the food industry to try to control them to guarantee the food safety (Lianou et al., 2017).

In recent years, significant attention has been given to microorganisms implicated in foodborne outbreaks, with notable pathogens including *Salmonella, Listeria monocytogenes, E. coli, and*

Campylobacter jejuni. Specifically concerning meat and poultry, prevalent microorganisms associated with foodborne illness encompass *Salmonella, E. coli, Campylobacter, Yersinia, and Clostridium perfringens.* Certain food items exhibit a higher propensity for harboring. pathogenic microorganisms, such as raw and undercooked animal products like meat, poultry, eggs, unpasteurized milk, and dairy derivatives, as well as seafood. Additionally, raw vegetables, grains, fruits, and their respective products, including leafy greens, sprouts, and flour, are recognized as potential vectors for contamination. It is noteworthy that while these foods pose an increased risk of microbial contamination, any food may become compromised at various stages of production and distribution, including the potential for cross-contamination within kitchen environments (Centers for Disease Control and Prevention, 2023).

Additionally, it is recognized that dairy products are common foods in many countries, offering a favorable environment for the growth of numerous microorganisms due to their nutritional content. Dairy products derived from raw milk are frequently identified as harboring microorganisms such as *Staphylococcus aureus, Salmonella spp., Listeria monocytogenes,* and *E. coli*, which rank among the most prevalent pathogens (Asfaw et al., 2023).

Moreover, fresh vegetables are recognized to contain substantial populations of epiphytic microorganisms, predominantly non-pathogenic. Nevertheless, throughout the "farm to fork" production, particularly in open field cultivation, fresh vegetables are susceptible to environmental conditions or factors that may introduce various pathogenic microorganisms. The safety of vegetables and fruits can be compromised by the presence of various hazards, including microbiological (bacteria, viruses, parasites, fungi), chemical (mycotoxins, nitrate, pesticides, heavy metals), or physical (soil, stones, glass, pieces of metals) contaminants. Among these, physical hazards pose a lower risk to consumers compared to chemical or biological hazards, as they are more readily observable and removable. Contamination can occur at every stage of the supply chain. Sources of contamination in the preharvest phase include manure, compost, dust, soil, irrigation water, feces, pesticides (such as insecticides and fungicides), insects, wild or domestic animals, and human activities. Handling, storage, and transportation procedures can also lead to postharvest contamination, potentially caused by personnel, process equipment, transport containers, and water/ice (Macieira et al., 2021).

As previously mentioned, vegetables can serve as reservoirs for several pathogens. Enteric pathogens like *Escherichia coli* and *Salmonella* are particularly concerning during food-related outbreaks. There have been numerous cases linking typhoid fever outbreaks to the consumption

of contaminated vegetables grown in or fertilized with soil or sewage that is contaminated. Various bacterial pathogens, including *Salmonella, Shigella, Campylobacter, E. coli O157: H7, Listeria monocytogenes,* and *Staphylococcus aureus,* have been identified as contaminants commonly associated with vegetables (Degaga et al., 2022). Table 1 gives an overview of the common microorganisms from meat, dairy, and vegetable products.

Table 1. Reported isolated bacteria from bell pepper phyllosphere, raw pork meat and raw milk samples. Bell pepper phyllosphere: (Kimiran -Erdem et al., 2013; Mamphogoro et al., 2020; Tizhe & V, 2020)*;Raw pork meat:* (Bantawa et al., 2018; Wang et al., 2023) *Raw milk* (Calahorrano-Moreno et al., 2022; Taye et al., 2021; Vahedi et al., 2013).

Bell pepper phyllosphere	**Raw pork meat**	**Raw milk**
Bacillus spp.	*Pseudomonas spp.*	*Pantoea spp.*
Clostridium spp	*Escherichia coli*	*Paracoccus spp.*
Sphingobium spp.	*Brochothrix spp.*	*Sphingomonas*
Paenibacillus spp.	*Aeromonas spp.*	*Deinococcus spp.*
Lactococcus spp.	*Leuconostoc ssp.*	*Listeria spp.*
Acinetobacter spp.	*Streptococcus ssp.*	*Yersinia spp.*
Agrobacterium spp.	*Acinetobacter ssp.*	*Salmonella spp.*
Enterococcus ssp.	*Photobacterium ssp.*	*Escherichia coli*
Flavobacterium ssp	*Serratia spp.*	*Staphylococcus aureus*
Lactobacillus ssp.	*Macrococcus spp.*	*Brucella*
Weissella ssp.	*Klebsiella spp.*	*Lactobacillus spp.*
Microbacterium ssp	*Staphylococcus spp.*	*Lactococcus spp.*
Enterobacter sakazakii	*Flavobacterium spp.*	*Streptococcus spp.*
Enterobacter agglomerans	*Akkermansia ssp.*	*Leuconostoc spp.*
Pseudomonas oryzihabitants	*Serrata ssp.*	*Pediococcus spp.*
Pseudomas luteola	*Weissella ssp.*	*Bifidobacteria spp.*
Cedeea lapagei	*Salmonella enterica*	*Enterococcus spp.*
.	*Proteus mirabilis*	*Aerococcus* spp.
	Streptococcus pneumoniae	*Kokuria spp.*
	Pseudomonas fragi	
	Weissella viridans	

It is important to note that the microorganisms mentioned above have been commonly associated with foodborne illnesses over time. However, it is crucial to recognize that microorganisms can evolve and develop resistance to various stressful environments that have been modified to impede their growth throughout the food chain. While efforts are made to ensure food safety within the food system, new microorganisms may emerge as potential pathogens, creating new concerns for food safety and human health. Therefore, continued surveillance and adaptation of food safety measures are essential to address emerging microbial threats and maintain the safety of the food supply.

3.3.Emerging pathogens in food

The appearance or emergence of new or unexpected pathogens in foods has been identified as one of the most significant trends likely to impact food safety in the coming years. Various definitions for 'emerging pathogens' have been proposed, leading to confusion. For instance, an emerging pathogen has been defined as one linked to a novel and serious public health disease. Alternatively, the term 'emerging' has also been used to describe microbial strains that have developed enhanced resistance to stresses and adapted to new environments (Koutsoumanis et al., 2014).

Regarding foodborne pathogens, it has been suggested that terms like 'new,' 'evolving,' 'emerging,' and 're-emerging' should be distinguished and considered separately. 'New foodborne pathogens' are serious hazards for public health, previously unrecognized as agents of outbreaks. 'Evolving foodborne pathogens' become more potent or more associated with other food products over time. 'Emerging foodborne pathogens' are those that have newly arisen and may have been recognized as pathogens, but are now associated with foodborne transmission. Finally, 'emerging pathogens' were known for some time, fell to low levels, and are now showing increasing trends (Mor-Mur & Yuste, 2010).

The emergence or re-emergence of foodborne pathogens is a complex process influenced by multiple factors including changes in agricultural practices (e.g., increased antibiotic use in animal production), microbial adaptation and evolution, technological changes in the food industry, shifts in human behaviour and eating habits, demographic changes, healthcare infrastructure, environmental parameters such as climate change, global food trade, and advancements in pathogen detection methodologies. Essentially, any change in the food chain, whether direct or indirect, is expected to create selection pressure leading to the emergence of foodborne pathogens (Koutsoumanis et al., 2014).

3.4.Antibiotic resistance.

Antibiotic resistance is a growing concern across medical, veterinary, and food industries, impacting the quality and safety of the food supply chain. It poses one of the most significant threats to global health, food security, and development. The use of antibiotics is now common in modern medicine and has been a great help in the treatment of many diseases caused by foodborne microorganisms. Antimicrobials are vital in terrestrial and aquatic agriculture for therapeutic and growth-promoting purposes. Their availability to agriculture is crucial. However, global consumption estimates vary due to inadequate surveillance; only 42 countries monitor antimicrobial use in livestock. Although, crop production uses a small fraction of

antimicrobials. Concerningly, two-thirds of the projected increase in antimicrobial usage will be in animal production, with pig and poultry farming leading the rise. (FAO, 2016).

The primary consequence of these residues in animal-derived foods is the acceleration of antimicrobial resistance. Consuming food contaminated with antibiotic-resistant pathogens can lead to gastrointestinal disorders in humans. Additionally, these antibiotic-resistant pathogens can transfer genes to other microorganisms through both vertical and horizontal transmission, facilitating the spread of antimicrobial resistance (AMR). Previous studies have identified multi-resistant bacterial pathogens across various sources in the food chain, underscoring the urgency for responsible antibiotic use in both veterinary and human healthcare sectors. Humans can acquire antibiotic-resistant bacterial infections through various means, such as consuming contaminated food or coming into contact with colonized or diseased animals, their body fluids, excretions, or secretions. Additionally, these pathogens can cause illness when people consume undercooked food (Rafiq et al., 2022). Besides, there have been instances of antibiotic misuse, leading to the development of resistance among various microorganisms against commonly used doses. This has resulted in widespread antibiotic resistance, affecting both spoilage and pathogenic microorganisms (Wu-Wu et al., 2023).

In research conducted by Uddin et al. (2021), is explained the reasons behind antibiotic resistance where imply that the bacteria are living microorganisms that have the ability to adapt over the time. They have the replication, survive and spread as rapidly as possible as the main objective, resulting in evolved microorganisms that are adjusted to the environment to guarantee their continued existence. Bacteria naturally develop resistance to drugs when exposed to growth-inhibiting substances like antibiotics. This resistance can lead to genetic modifications that make them immune to medications. While antibiotic resistance is a natural process for bacteria, several factors contribute to its rise. These include antibiotic overuse and misuse, inaccurate diagnosis and inappropriate prescribing, patient misuse and self-medication, poor healthcare conditions, inadequate personal hygiene, and extensive use of antibiotics in agriculture (Uddin et al., 2021; Wu-Wu et al., 2023).

The most common antibiotic types reported as antibiotics that usually do not have notable effects on foodborne pathogens are β-lactam, aminoglycosides, tetracycline, and sulfonamides (Figure 1).

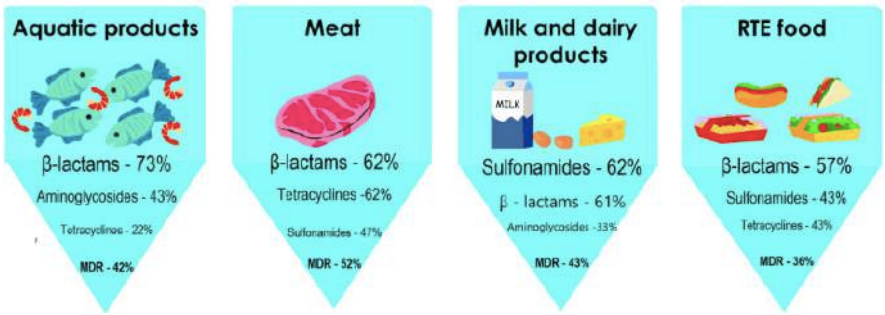

Figure 1. The most common type of the antibiotic resistance depending on the type of food product (Grudlewska-Buda et al., 2023).

In Figure 2 a timeline is presented where there is evidence of how the microorganisms have become resistant to the antibiotics that have been applied over time. For instance, *Staphylococcus* is resistant to penicillin, *Shigella* developed resistance to Tetracycline-R in nine years, *Enterococcus* presented resistance to Gentamicin after 12 years, and there are other microorganisms mentioned in the timeline that develop resistance to many antibiotics like Levoflaxin, Linezolid, Daptomycin, and Ceftaroline, even the periods to develop antibiotic resistances starts decreasing over the time (Ventola, 2015).

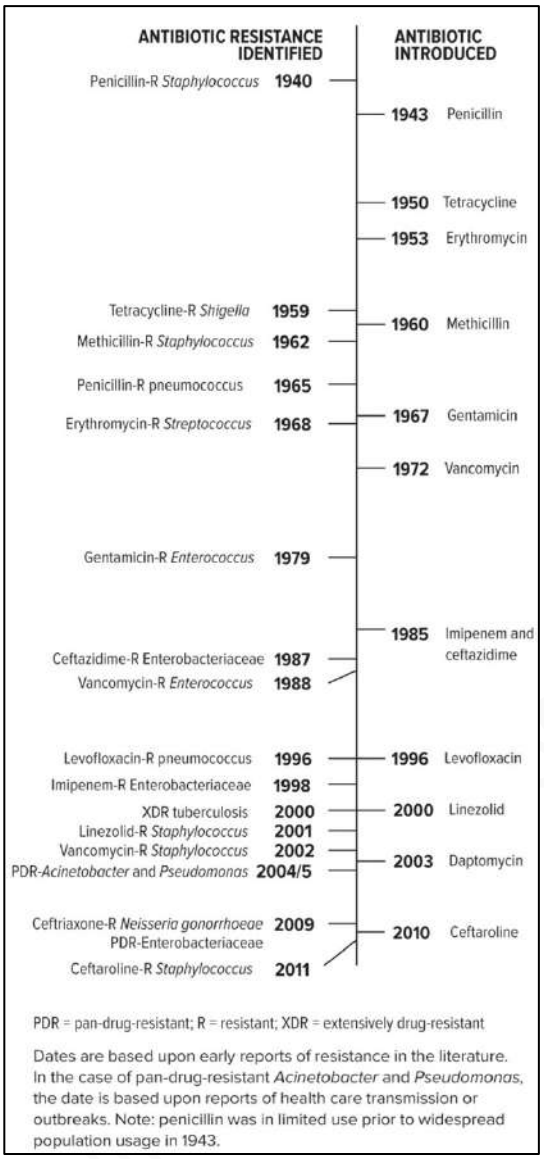

Figure 2. Developing Antibiotic Resistance: A timeline of Key Events (Ventola, 2015).

Among microorganisms that have presented resistance to traditionally used antibiotics are *Clostridium* spp. and *Salmonella enterica*, as well as *Campylobacter jejuni* isolated from retail food (Zhong et al., 2016), *Staphylococcus*, *Pseudomonas,* and *Enterococcus* (Ventola, 2015). Emerging foodborne pathogens present challenges beyond antibiotic resistance, including heightened virulence, lower infectious doses, and increased resistance to food-related stresses. Despite efforts by public health authorities, limited information about their virulence and stress responses impedes effective control and prevention (Lianou et al., 2017). Addressing the challenge of pathogen emergence requires robust surveillance programs and the development of innovative molecular techniques for studying foodborne pathogens.

3.5.Oxytetracycline-hydrochloride.

Oxytetracycline (Figure 3) is a versatile antibiotic used to treat various bacterial infections in both humans and animals. It is effective against infections of the respiratory tract, urinary tract, soft tissues, and skin caused by a wide range of bacteria, except for resistance commonly seen in certain strains. In cattle, it is commonly used to treat bovine respiratory disease caused by specific bacteria. In pigs, it has been utilized for conditions like atrophic rhinitis and pneumonic pasteurellosis (National Library of Medicine, 2024; Washington et al., 2012). Oxytetracycline hydrochloride interferes with the binding of aminoacyl-tRNA to the mRNA-ribosome complex, thereby preventing peptide elongation and inhibiting protein synthesis. It is often used to treat skin conditions (National Center for Biotechnology Information, 2024).

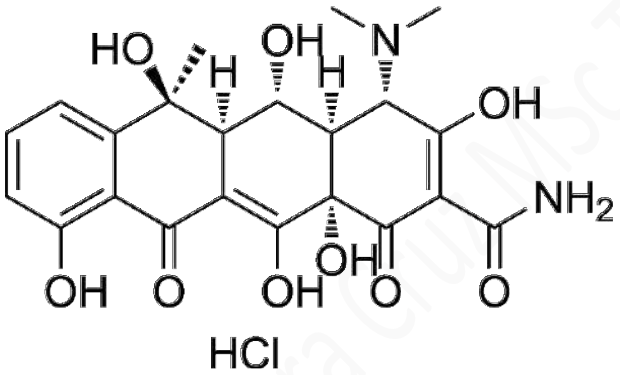

Figure 3. Oxytetracycline hydrochloride Chemical structure.(MCE, 2013).

Researchers observed a significant disturbance in the microbiome diversity of treated with oxytetracycline (OTC) cattle in the short term. This led to an increase in antibiotic resistance genes (ARGs) associated with tetracycline resistance compared to control animals that were not exposed to oxytetracycline (OTC). The most pronounced impact of OTC on fecal microbiome richness was seen on day 3 following antibiotic administration. While urine serves as the primary excretion route for OTC, fecal excretion can account for up to 40%, indicating a close interaction between the drug and the microbial population in bovine feces. Consequently, the fecal microbiome of the treated cattle exhibited reduced diversity values three days after OTC administration. Besides, this decline in bacterial community richness due to antibiotic treatment aligns with findings from previous studies involving humans (Dethlefsen & Relman, 2011; Rovira, 2023).

3.6.The Minimum Inhibitory Concentration Value of Antibiotics

The minimal inhibitory concentration (MIC) is defined as the lowest concentration of an antibiotic at which bacterial growth is completely inhibited. It serves to characterize the in vitro susceptibility or resistance of specific bacterial strains to a given antibiotic. The reliable determination of MIC values is crucial as it informs the choice of an appropriate therapeutic strategy, thereby influencing the effectiveness of infection therapy. To obtain accurate MIC values, several factors must be taken into account, including selecting the appropriate method, adhering to labelling guidelines, and interpreting the results competently (Kowalska-Krochmal & Dudek-Wicher, 2021). Table 2 indicates some estimated MIC ranges for ETEC, EPEC, and *Campylobacter* spp. which are known pathogens responsible for many outbakes in the food industry.

Table 2. Estimates MIC values for some foodborne bacteria (Mahindroo et al., 2024).

Bacteria name	Antibiotic	MIC range [µg/ml]
ETEC (Enterotoxigenic *E. coli*)	Ciprofloxacin	0.0625 -16
	Azithromycin	1-256
	Gentamicin	0.5-128
	Ertapenem	0.125-32
	Colistin	0.125-16
	Tetracycline	0.25-64
EPEC (Enteropathogenic *E. coli*)	Ceftriaxone	0.25-64
	Ciprofloxacin	0.0625 -16
	Azithromycin	1-256
	Gentamicin	0.5-128
	Ertapenem	0.125-32
	Colistin	0.125-16
	Tetracycline	0.25-64
Campylobacter spp.	Ceftriaxone	0.25-64
	Ciprofloxacin	0.002-32
	Azithromycin	0.016-256
	Gentamicin	0.016-256
	Tetracycline	0.016-256

4. Materials and methods

4.1.Location

The investigation described in this study was conducted at the Microbiology Lab of the Department of Food Microbiology, Hygiene, and Safety at the Institute of Food Science and Technology of the Hungarian University of Agriculture and Life Sciences.

4.2.Collection of samples:

Based on the scope of this project, samples of the phyllosphere of the bell pepper (*Capsicum annuum*), raw pork meat, and raw milk were collected. Isolation and identification of microorganisms of bell pepper phyllosphere are described in the following.

The bell pepper phyllosphere samples were obtained from an organic production system. Disinfected scissors and sterilized bags were employed for collection, ensuring the integrity of the samples. To obtain representative samples, a random sampling approach was adopted. Samples consisting of fruit, top leaves, bottom leaves, and flowers (phyllosphere) were gathered from a greenhouse situated in Kismaros, Hungary.

4.3. Colonies isolation

To isolate microorganisms from the phyllosphere of the bell pepper plant, 10 grams of each part of the bell pepper phyllosphere (fruit, top leaves, bottom leaves, flowers) were placed in new sterilized sample bags with filter included. 90 ml of peptone water was added as a diluent to create a dilution of 1:10. Subsequently, the samples were homogenized in a sample mixer during 1.5 – 2 minutes. Then, serial dilution (10^1, 10^3 and 10^5) was prepared and 0.1 ml were spread on PC (Plate Count) agar, Cetrimid agar, Chromocult agar and MRS (de Man, Rogosa and Sharpe) agar Also, a double layer PC agar was performed to isolate some facultative anaerobes. PC, Cetrimid and Chromoult plates were incubated at 37 °C for 24 hours and MRS plates were incubated at 30 °C in anaerobic condition (JAR) for 24 hours.

4.3.1. Culture medium preparation.

Plate Count Agar (PC agar):

- Composition: peptone 0.5%, yeast extract 0.25%, glucose 0.1% and agar 1.5%
- Manufacturer: Diagnostics Laboratory Inc.
- Diluent: Distilled water.
- Purpose: Determination of Total microbial count.

Cetrimid agar:

- Composition: 46.7 g of Cetrimid agar in one liter of distilled water and 10 ml of glycerol.
- Manufacturer: (Sigma-Aldrich).
- Diluent: Distilled water.
- Purpose: Isolation of *Pseudomonas* species.

Chromocult agar (ChromoBio Coliform):

- Composition: 30 g per liter of distilled water.
- Manufacturer: Biolab Diagnostics Laboratory Inc.
- Diluent: Distilled water.
- Purpose: A selective and differential chromogenic medium for the simultaneous detection of coliforms and *Escherichia coli* according to ISO 9308.

MRS Agar:

- 63 g of the medium powder and 10 ml of MRS supplement per liter of distilled water.
- Manufacturer: Biolab Diagnostics Laboratory Inc.
- Diluent: Distilled water.
- Purpose: A low selective medium for the isolation and cultivation of *Lactobacillus* spp. according to ISO 15214.

4.4. Colony identification

The microorganisms were counted, and then to the different colonies, a code was assigned for each one. Each colony was described according to its shape, elevation, and colour.

For identification, each different colony in all the plates with the different agar mediums was selected and given a code. Table 3 presents the description of the label for a better understanding of the given code.

Table 3. Description of the colony labels.

Type of sample*	Dilution	Medium type	Colony letter
1: Fruit	1: 10^1	1: PC	Different letters of the
2: Bottom leaves	2:10^2	2: MRS	alphabet were assigned to
3: Top Leaves	3:10^3	3: Cetrimid	a different colony to
4: Flowers	4: 10^3	4: Chromocult	follow a sequence.

***: Samples from bell pepper phyllosphere**

4.4.1. Colony morphological description

Once the colonies were selected and codified, the description was carried out, mentioning the form, elevation, margin, colour, and opacity.

After codification, pure cultures of the selected colonies were made to obtain single colonies, more microbial material, and perform in a better way the chemicals and morphological identification. For this purpose, the streak technique was performed in TSA Agar. After inoculation, the plates were incubated at 30 °C for 24 hours. MRS plates were incubated at anaerobic conditions at 30 °C for 24-48 hours.

To analyze the cell morphology of the isolates a simple staining using Chrystal violet stain was carried out.

4.4.2. Characterization of isolates by Biochemical tests

Using a microscope to identify microorganisms is important, but in most cases is not enough to get the entire information that is required for an accurate identification, that is why microscopic analysis is associated with other techniques such as biochemical techniques: catalase test, oxidase test, and Potassium hydroxide test.

4.4.2.1.Catalase test

The catalase test is a biochemical test for aerobic organisms that detects the production of catalase enzymes in the organism.

In the case of catalase with a sterilized loop a drop of bacteria was taken out and placed in a sterilized piece of glass into a Petri dish and then a drop of the reagent was added to the extracted colony. The test was positive in case bubbles were formed immediately after the colony was in contact with the reagent and if there was no bubble the test was negative.

4.4.2.2.Oxidase test

The test depends on the ability of certain bacteria to produce indophenol blue from the oxidation of dimethyl-p-phenylenediamine and α-naphthol.

In case of Oxidase test with a sterilized loop, a drop of bacteria was taken out and placed in a sterilized paper with the reagent added. If the blue change darker was positive and if any change happened was negative for catalase.

4.4.2.3. Potassium hydroxide test

The potassium hydroxide test (KOH test) is employed to identify Gram-negative bacteria. To start the procedure a drop of 3% of potassium hydroxide was placed in a sterile microscope slide and then a colony was taken from the petri dish and it was placed into the drop before added. Then, after 30 seconds was stirring and gently the loop was put away from the suspension formed. In the case of a positive result for the test, the viscosity changes to sticky, and the suspension can follow the loop when it is raised, and if the test was negative the consistency does not change and a separation between the suspension and the loop will be sawed when the loop is raised. A positive result refers to Gram-negative cell wall characteristic of the investigated bacterium.

4.4.3. Identification with MALDI TOF-MS

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is widely regarded as an excellent tool for bacterial identification. MALDI-TOF MS represents the latest generation tool utilized for the rapid identification and classification of microorganisms (Buszewski et al., 2017). This method relies on ionizing microbial cells using short laser pulses, followed by accelerating the resulting particles within a vacuum system using an electric field. Upon ionization, a unique molecular fingerprint in the form of a spectral profile is obtained for each microorganism. This spectrum is subsequently compared to an existing database, allowing for automated identification. Sample preparation for MALDI-TOF MS involves crystallizing the sample with an abundant molar excess of matrix, typically a UV-absorbing organic acid, on target plates (Buszewski et al., 2017; Franco-Duarte et al., 2019; Randell, 2014).

From each isolated microorganisms described before 24 hours pure culture was prepared. Then, with a sterile toothpick a single colony was taken from the agar plate onto de target place spot overlaid with 1 µl 70 % formic acid. After drying, a drop of matrix (alpha-4-cyano-4-hydroxycinnamic acid, CHCA) was added. Then, mass spectrum analysis was carried out by using MALDI-TOF-MS. Results were compared to a reference database. Results of scores higher than 2.00 were considered as high confident (Topić Popović et al., 2023).

4.5. MIC value determination

After identification by MALDI TOF-MS, the microorganisms from all three types of raw material (Bell pepper phyllosphere, raw pork meat, and raw milk) were selected to perform preliminary experiments to determine the MIC value.

4.5.1. Preliminary experiment

A suspension of 24 hours old pure culture from the selected bacteria was prepared with an OD (Optical density) value of 0.5-1 (1,5x108 – 3,0x108 CFU/ml). Then, 1 ml of suspension (OD=0.5-1) was transferred to a TSA Agar plate and moved to distribute the suspension along the surface and then the remaining was taken out with the pipette to prepare a lawn of bacteria. After drying, one filter paper disk of (0.5 cm in diameter) was placed in the middle of the plate. 10 µl of 3µg/µl oxytetracycline-hydroxide was transferred to the circular filter paper. The plates were incubated for 24 hours at 30 °C.

Considering the results of the preliminary test altogether, six isolates (two from each type of raw material) were chosen for further investigation.

4.5.2. Disk diffusion test

Suspensions with an OD value of 0.5-1 were prepared of 24 hours old cultures of the six selected bacteria. Then, 1 ml of suspension (OD = 0.5-1) was transferred to a TSA plate and moved to distribute the suspension along the surface and then the remaining was taken out with the pipette to prepare a lawn of bacteria. After drying, four filter paper disks of (0.5 cm of diameter) were placed on the plate in order to test four different oxytetracycline-hydroxide concentrations 3.0, 1.5, 0.750 and 0.375 µg/µl. Each isolate was tested in three repetitions for each concentration. The diameter of the inhibition zone was measured after incubation at 30 °C for 24 hours.

4.5.3. Thermo Scientific Multiskan Spectrum – UV/Vis microplate and cuvette spectrophotometer.

For this procedure, a suspension of 107 cells/ml was prepared using the Optical Densitometer (DEN-1B McFarland Densitometer). And 6.0, 3.0, 1.5, 0.750 and 0.375 µg/µl. oxytetracycline-hydrochloride was applied. In this procedure, a Tissue Culture Plate 96 well was used and it was divided for the treatment in triplicates (Figure 4), the sample controls without the antibiotic, antibiotic, and broth media (2xccTSB). 135 µl of double concentration of TSB was placed in the wells for treatments, blinds, control samples, and the well just with the TSB. Then, the sterilized distilled water was transferred, and then the

different antibiotic concentrations. Finally, the microbial suspension of the selected microorganism was transferred into the wells with the antibiotic treatments and into the control sample wells.

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1+O (6.0)	S1+O (6.0)	S1+O (6.0)	S2+O (6.0)	S2+O (6.0)	S2+O (6.0)	B (6.0)					
B	S1+O (3.0)	S1+O (3.0)	S1+O (3.0)	S2+O (3.0)	S2+O (3.0)	S2+O (3.0)	B (3.0)					
C	S1+O (1,5)	S1+O (1.5)	S1+O (1.5)	S2+O (1.5)	S2+O (1.5)	S2+O (1.5)	B (1.5)					
D	S1+O (0.75)	S1+O (0.75)	S1+O (0.75)	S2+O (0.75)	S2+O (0.75)	S2+O (0.75)	B (0.75)					
E	S1+O (0.375)	S1+O (0.375)	S1+O (0.375)	S2+O (0.375)	S2+O (0.375)	S2+O (0.375)	B (0.375)					
F	S1R1	S1R2	S1R3	S2R1	S2R2	S2R3	TSB					
G												
H												

S – sample; O- oxytetracycline-hydrochloride; B-blind R-repetition

	2xcc TSB (µl)	Antimicrobial (in proper conc.) (µl)	Dest water (µl)	Microbial suspension (µl)
Green marked wells	135	135	-	30
Violet marked wells	135	135	30	-
Orange marked wells	135	-	135	30
Blue marked well	135	-	165	-

Figure 4. Tissue Culture Plate 96 well for Multiskan measurement scheme.

After that, the Tissue Culture Plate 96 well was placed in the Multiskan Ascent equipment to be incubated at 37 ºC and check the turbidity each 30 min for 24 hours.

4.5.4. Interpretation of results

4.5.4.1.Disk diffusion test.

The reading guide of EUCAST disk diffusion method for antimicrobial susceptibility testing Version 10.0 January 2023 and European Committee on Antimicrobial Susceptibility Testing Breakpoint tables for interpretation of MICs and zone diameters Version 14.0 were used.

EUCAST/CLSI guidelines are used to interpret the zones of inhibition for each antibiotic tested. The diameter of the inhibition zone is compared with the breakpoint zone size. If the diameter of the zone of inhibition is equal to or greater than the size that indicates 'susceptible' in the guidance document, the isolate is considered susceptible (S) (Gaur et al., 2023).

4.5.4.2. Multiskan

Once the results from Thermo Scientific Multiskan Spectrum equipment were obtained the data was from each repetition of the different samples were considered separately to calculate the media and then create curves indicating the tendency of growth. The calculation of the media and the curves were made in Microsoft Excel.

5. Results and discussion

5.1.Isolation

Table 4 presents the microbial counts (CFU/g) of samples from the Bell pepper phyllosphere cultivated on different culture media. The samples include conventional Bell pepper fruit, organic bottom leaves, organic top leaves, and organic flowers.

Table 4. Microorganism count (CFU/g) of each type of sample of Bell pepper phyllosphere in different culture mediums.

	Bell pepper (Fruit)	**Bottom Leaves**	**Top Leaves**	**Flowers**
PCA double layer	$1{,}07 \times 10^4$	$3{,}35 \times 10^4$	$5{,}00 \times 10^4$	$5{,}00 \times 10^5$
PCA	$2{,}00 \times 10^4$	$8{,}00 \times 10^4$	$3{,}00 \times 10^6$	$1{,}00 \times 10^7$
Chromocult	$1{,}99 \times 10^4$	$7{,}96 \times 10^4$	$2{,}99 \times 10^6$	$9{,}95\times 10^6$
Cetrimid	$3{,}86 \times 10^1$	$7{,}72\times 10^2$	$1{,}60\times 10^3$	$6{,}56 \times 10^4$
MRS	$1{,}10 \times 10^3$	$2{,}52 \times 10^3$	$1{,}67 \times 10^3$	$3{,}46 \times 10^4$

*: All the samples are coming from the phyllosphere of the bell pepper plant.

The microbial counts varied across the different types of samples and culture media, indicating the presence of diverse microbial populations on the bell pepper plant surfaces. Based on the results, lactic acid bacteria, *Pseudomonas* spp, coliform bacteria were present on the plant. It can be concluded that the dominant microorganisms on the plant were the coliform bacteria, because total microbial count and bacteria on Chromocult culture medium have been detected in almost the same quantity, Showing that bell pepper phyllosphere presents a microbiome with a variety of microorganisms, although its quantity/ratio is influenced by the environmental conditions and agricultural practices that are carried out during the maintenance crop, including fertilization, irrigation, fertilization and pruning of leaves. In Table 4 the bottom leaves exhibited higher counts across all media type that could be due to that part is closer to the soil (rhizosphere) than the other parts, considering also that is an organic crop where usually manure is used as part of the compost process (Steele & Odumeru, 2004) But also, the irrigation water can be a source of different microorganisms, that can influence the safety of the produce. The quality of irrigation water can impact both food safety and health and has been recognized as a potential origin of pathogens in produce associated with outbreaks of disease. Some examples are an outbreak occurred in 2019 in romaine lettuce crops due to O145 Shiga toxin-producing *E. coli* (STEC) and 2006 involving the of E. coli O157:H7 in spinach (Gelting & Baloch, 2013).

Flowers also exhibit elevated counts across the culture media, likely attributed to frequent contact with insects seeking nectar., because in research presented by Keller et al. (2021) it is mentioned that the interaction between bees and plants is acknowledged as a common pathway

for microbial transmission in both directions. In contrast, the bell pepper fruit shows low microbial counts compared to the others, which is attributed to the composition of the bell pepper. Capsaicin and dihydrocapsaicin are bioactive compounds present in the bell pepper fruit, and several studies have revealed their antibacterial activity against *Staphylococcus aureus* (Ekom et al., 2021).

In summary, the microbial counts varied significantly across the different parts of the Bell pepper phyllosphere and culture media. Flowers consistently exhibited the highest microbial counts, followed by top leaves, bottom leaves, and fruit. Cetrimid medium which reveals the presence of *Pseudomonas spp.*, consistently yielded the lowest counts across all sample types. These findings indicate that the microbial composition of the Bell pepper phyllosphere is influenced by the plant part, due to different factors that take place during the whole agricultural practices applied for bell pepper production.

The macro- and micromorphological characteristics of all isolates detected on the various culture media are detailed in the Annex 1 (Table 1 to Table 11). Isolates have been identified by MALDI-TOF-MS method. Results giving high score identification are presented in Table 5. In order to give a better overview of the description of morphological characteristics in subchapter 5.2 the isolates are named already according to the identification results.

Table 5. Microorganisms isolates from bell pepper phyllosphere and identified by MALDI-TOF-MS.

SAMPLE SOURCE	CODE	Microorganism name
Fruit	P6	*Brachybacterium conglomeratum*
	P36	*Staphylococcus saprophyticus*
	P2	*Pseudomonas extremorientalis*
	P7	*Pseudomonas oryzihabitants*
Bottom leaves	P15	*Staphylococcus warneri*
	P16	*Micrococcus luteus*
	P17	*Moraxella osloensis*
	P10	*Bacillus cereus*
	P12	*Staphyloccoccus hominis*
Top leaves	P26	*Microbacterium arborescens*
	P21	*Pseudomonas flavescens*
	P19	*Pluralibacter pyrinus*
Flowers	P32	*Bacillus cereus*
	P33	*Microbacterium arborescens*
	P28	*Pseudonomas antarctica*
	P29	*Pseudomonas fulva*
	P30	*Pseudonomas oryzihabitans*

5.2.Morphological and biochemical characteristics of identified (by MALDI TOF-MS) bacterial isolates from the phyllosphere of Bell pepper plant.

5.2.1. Bell pepper (Fruit)

The morphological and biochemical characteristics of identified microorganisms from bell pepper (fruit) are presented in detail in Table 1 to Table 4 the Appendix 1. Table 6 summarize the main macro and micro morphological characteristics.

Table 6. Morphological and biochemical characteristics of identified bacterial isolates from Bell pepper (fruit).

	Identified isolates from Bell pepper (fruit)			
	Brachybacterium conglomeratum	*Staphylococcus saprophyticus*	*Pseudomonas extremorientalis*	*Pseudomonas oryzihabitants*
Morphological characteristics				
Form	Circular	Circular	Circular	Rhizoid
Elevation	Convex	Arise	Convex	Crateriform
Margin	Entire	Entire	Entire	Undulate
Color	Pale-yellow	White	White	Yellow
Opacity	Opaque	Opaque	Translucent	Dense
Shape	Cocci	Cocci	Bacilli	Bacilli
Arrangement	Sarcina	Sarcina	Bacillus	Diplobacilli
Biochemical characteristics				
Catalase test	+	+	+	-
Oxidase test	-	-	+	-
KOH test	Gram positive	Gram positive	Gram negative	Gram negative

+: Positive; -: negative

There are some relevant studies presented by Takeuchi *et.* al, (1995) that describe similar morphological characteristics to *Brachybacterium conglomeratum* (Figure 5), emphasizing that this microorganism is mainly cocci in the stationary phase with a 0.5 to 1 μm in diameter and it can change its shape during the growth phase and present a short rods shape of 2 μm long, that is the reason why in some cases this bacterium is described as coccobacilli. Also, is mentioned that it is a gram-positive bacterium and oxidase-negative which is certainly the same as the result found in the performed KOH test and coincides that this bacterium has a positive result for the catalase test (Table 6) which means that *Brachybacterium conglomeratum* is able to produce catalase in order to defend itself against attacks by hydrogen peroxide (Iwase et al., 2013).

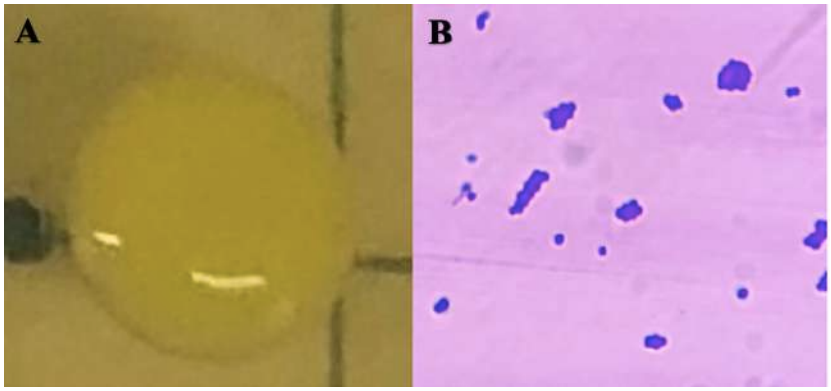

Figure 5. Isolate P6. Brachybacterium conglomeratum. A: Colony morphology on PC agar; B: Cell morphology under light microscope (100X).

In addition, *Brachybacterium conglomeratum* is considered an environmental bacterium but also can be found in the urinary and vaginal tract and is associated with patients with HPV (Human papillomavirus) (Cortés-Ortíz et al., 2023). However, *Brachybacterium conglomeratum* has been isolated from oil brine in Japan and reports an optimum temperature to grow 28-30 °C stratified as mesophilic microbe. Also, *Brachybacterium* species have been isolated from poultry deep litter, Beaufort cheese, oil-contaminated coastal sand, salt-fermented seafood, and seawater during a bacterial diversity of the marine environment in a region of India (Kaur et al., 2016).

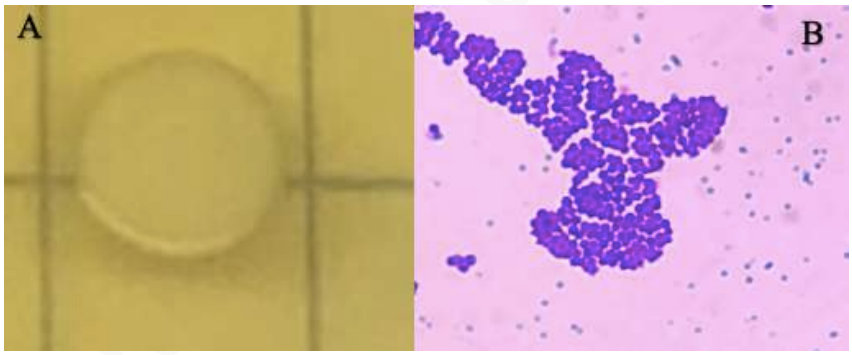

Figure 6. Isolate P36. Staphylococcus saprophyticus. A: Colony morphology on MRS agar (anaerobe conditions); B: Cell morphology under light microscope (100X).

In a similar case, *Staphylococcus saprophyticus* (Figure 6) is known as a urinary tract pathogen responsible for urinary tract infection (UTI) that can be transferred to food due to bad manufacture practices (Hedman et al., 1990; Pessoa et al., 2022). Also, it can be found naturally in animal and human skin, ready-made food as sandwiches and salads sold in retail shops, raw beef or pork carcass, ice-cream, items of confectionaries were ready-made pastries from retail bakeries, cooked food from restaurant and also from eggs. This mentioned bacterium was found in meat cutter protective gloves as well and *E. coli* was found in the same samples (Hedman et al., 1990), which means that *Staphylococcus saprophyticus* can be transmitted from human to food due to a bad handling during slaughtering and bad manufacture practices.

The genus *Pseudomonas* is a group of bacteria that commonly are present in soil and fresh water ecosystems. Specifically, *Pseudomonas extremorientalis* was isolated for first time from a drinking water reservoir in the Far East of Russia (Ivanova et al., 2002).

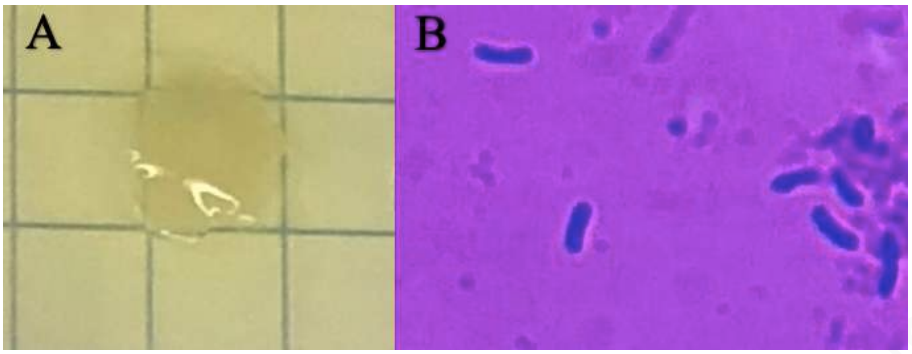

Figure 7. Isolate P2. Pseudomonas extremorientalis. A: Colony morphology on Cetrimid agar; B: Cell morphology under light microscope (100X).

Pseudomonas extremorientalis (Figure 7) is a fluorescent bacterium, considered a harmful microorganism to food, but is important to know that it can form biofilm on the surface even in dry environments, which can influence during the cleaning process (Ivanova et al., 2002). According to results reported by Buchana Imani (2021), *Pseudomonas extremorientalis* was found in isolates from wastewater but no reports were found about its presence in food sources.

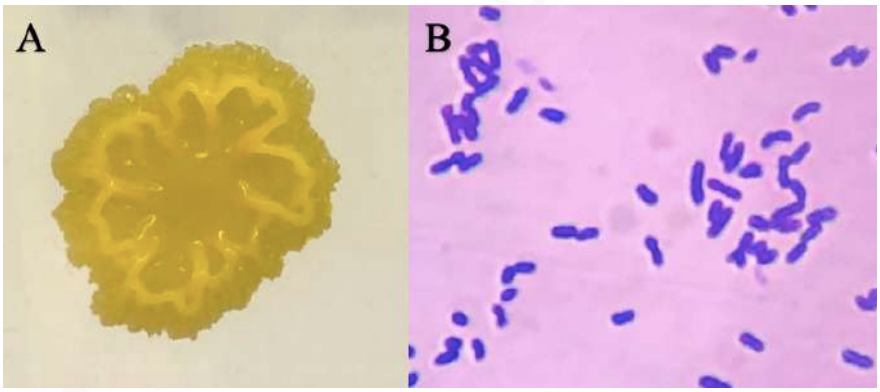

Figure 8. Isolate P7. Pseudomonas oryzihabitants. A: Colony morphology on Cetrimid agar; B: Cell morphology under light microscope (100X).

Pseudomonas oryzihabitants (Figure 8) commonly is isolated from moist environments such as soil and rice paddles (Panagopoulos et al., 2016). But also, it has been isolated from chicken meat products like breast, thigh, hamburger, and nuggets. The genus *Pseudomonas* is one of the significant microbes that lead to putrefaction in chilled meat (Elbehiry et al., 2022).

5.2.2. Bell pepper bottom leaves

The performed identification of the isolates from bell pepper bottom leaves resulted in six microorganisms: S*taphylococcus, Micrococcus, Moraxella, Bacillus,* and *Staphylococcus*. Table 7 shows the summarized morphological and biochemical descriptions.

Table 7. Morphological and biochemical characteristics of identified bacterial isolates from Bell pepper bottom leaves.

	Identified isolates from Bell pepper bottom leaves				
	Staphylococcus warneri	*Micrococcus luteus*	*Moraxella osloensis*	*Bacillus cereus*	*Staphylococcus hominis*
Morphological characteristics					
Form	Circular	Circular	Circular	Irregular	Circular
Elevation	Raised	Convex	Convex	Flat	Raised
Margin	Undulate	Entire	Entire	Undulate	Entire
Color	Pastel yellow and white in the center	Yellow	White	White	White
Opacity	Opaque	Opaque	Transparent	Opaque	Opaque
Shape	Micrococci	Cocci	Bacilli	Bacilli	Cocci
Arrangement	Sarcina	Tetrad, sarcina	Diplococci-bacillus	Streptobacilli	Sarcina
Biochemical characteristics					
Catalase test	+	+	+	+	+
Oxidase test	-	-	+	+	-
KOH test	Gram positive	Gram positive	Gram negative	Gram negative	Gram positive

+: Positive; -: negative

Staphylococcus warneri (Figure 9) was identified for the first time identified at Long Island Jewish Medical Center in New York between 1984 and 1989. It can cause endocarditis, and like other staphylococci it may be associated with significant morbidity and mortality in hospitalized patients, (Kamath et al., 1992; Rodríguez Montserrat, 2018). Most of the infections caused by *Staphylococcus warneri* are usually related to skin infections (Rodríguez Montserrat, 2018). In relevant research, Phukon et al. (2013) report the endophytic occurrence of *Staphylococcus warneri* in fresh apples and explain how the human pathogenic bacteria can contaminate both outside as epiphytes and inside as endophytes parts of fruits and vegetables, using the cellulose fibrils and aggregative fimbriae for attachment to the plant, similar to how the plant pathogenic bacteria does.

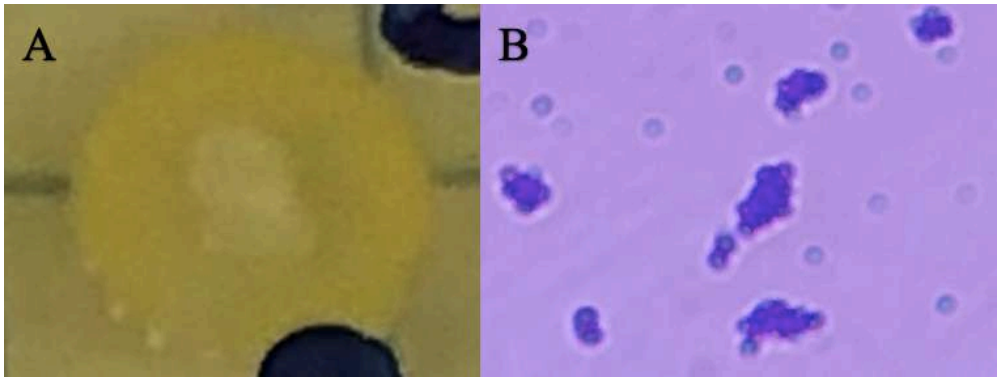

Figure 9. Isolate P15. Staphylococcus warneri. A: Colony morphology on PCA agar; B: Cell morphology under light microscope (100X).

*Micrococcus luteus (*Figure 10), an obligate aerobe, can be present in various environments including soil, air, and animals. Being an opportunistic pathogen, cases of infections caused by this microorganism are rare and it is usually reported in patients with compromised immune systems and suffering from malnutrition (Shi et al., 2023a). Regarding the biochemical test (Table 7) *M. luteus* is gram-positive, catalase-positive and, oxidase-negative bacteria, which differs from the results presented by Zhu et al. (2021) because they report that *Micrococcus luteus* is a catalase-negative microorganism which implies that it does not produce the enzyme catalase as mechanism protection, but it can adapt its cellular processes to thrive in oligotrophic conditions. An oligotroph is an organism that can survive in an environment with minimal nutrient levels (Davey et al., 1993).

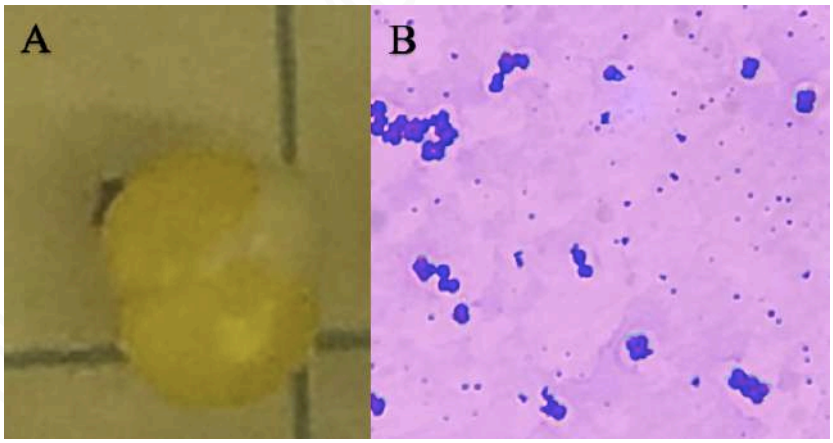

Figure 10. Isolate P16. Micrococcus luteus. A: Colony in PCA agar; B: Cell morphology under light microscope (100X).

Although, the UK Health Security Agency shows in the National Collection of Type Culture that *Micrococcus luteus* (Strain NCTC 4351) is catalase-positive, the same results for the research presented by Health Canada & Environment and Climate Change Canada (2018) expressing that this microorganism (*M. luteus* strain ATCC 4698) is a mesophilic microbe (optimum growth at 30-37 ºC). The reason for the different catalase results can be explained with because the fact that catalase activity can be influenced by several factors including growth stage, environmental conditions, genetic variability, metabolic state, culture conditions (pH,

temperature, media composition), and post-translational modifications such as phosphorylation, because when there is a high concentration of H_2O_2, catalase may undergo phosphorylation, which can reduce its activity. This reduced activity occurs because phosphorylation changes the conformation of the enzyme, decreasing its efficiency in breaking down H_2O_2. However, the exact mechanism by which phosphorylation inhibits catalase activity still remains unclear (Hadwan et al., 2024).

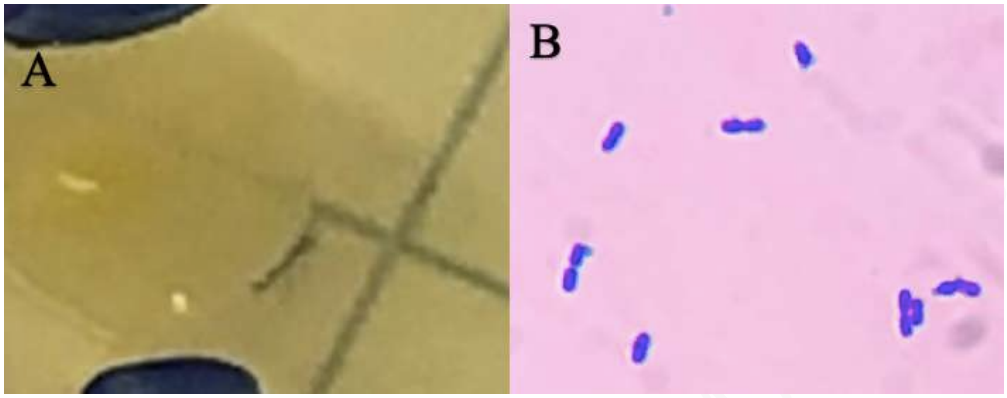

Figure 11. Isolate P17. Moraxella osloensis. A: Colony in PCA agar; B: Cell morphology under light microscope (100X).

Macro- and micromorphological characteristics of *Moraxella osloensis* (Figure 11) are similar to the investigation performed by Phe (2015).

Moraxella osloensis, an aerobic microorganism, belongs to Moraxellaceae family that has been isolated from meat, fish, and dairy products, but also from mucous membranes of humans and animals (Betts, 2006). Consequently, it is associated with spoilage development in food especially in those products with high protein content help in air at refrigeration temperature (Santos et al., 1999).

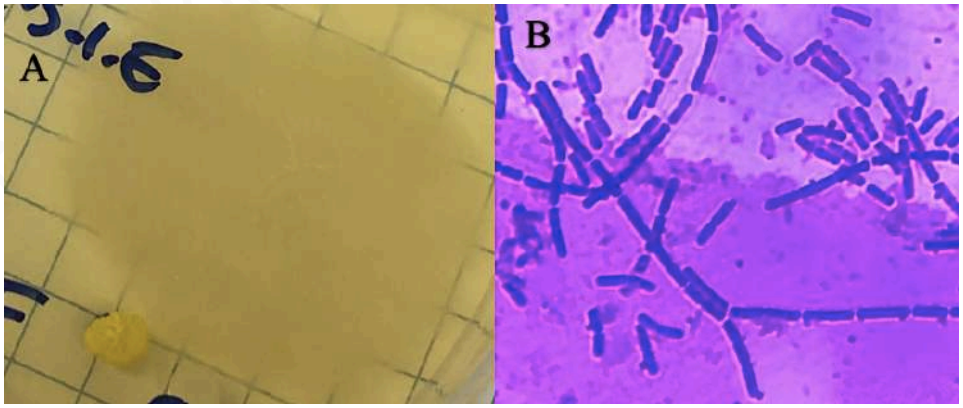

Figure 12. Isolate P10. Bacillus cereus isolate. A: Colony (white) morphology in PCA agar; B: Cell morphology under light microscope (100X).

Bacillus cereus is known as a facultative anaerobic, gram-positive bacterium found in soil, vegetation, and food. However, the result for KOH test presented in Table 6 said that *Bacillus cereus* is gram-negative, which supports the statement declared in research presented by (Noonan L & Freeman J, 2024) that, occasionally, *B. cereus* may appear gram-variable or even

gram-negative with age. Regarding its morphological characteristics, *B. cereus* presented an irregular form, flat elevation, undulate margin and white opaque color-opacity, in its colony growth in PCA agar (Figure 12A), and its cells present a bacilli shape with a streptobacilli arrangement mainly (Figure 12B). Noonan L & Freeman J (2024) describe the *B. cereus* as straight or slightly curved with square ends arranged either alone or in short chains.

Bacillus cereus a positive catalase and oxidase microbe is able to produce spores that are resistant to heat and desiccation, which makes it a real concern for food safety because in the presence of spores and favorable conditions (pH > 4.8 and temperature range of 8 – 55 °C), the spores will grow causing food poisoning. *B. cereus* can cause emetic syndrome because of the consumption of cereulide. Cereulide is an emetic toxin produced by *B. cereus* bacteria found in contaminated food. To induce vomiting, the food typically needs to have *B. cereus* levels exceeding 10,000 per gram. This toxin is heat-resistant and cannot be eliminated by cooking. It is commonly associated with starchy foods like pasta and rice. On the other hand, the diarrheal form of *B. cereus* poisoning occurs when high levels of vegetative cells of this microorganism (also over 10,000 per gram) are consumed, leading to enterotoxin production in the small intestine. This form can be linked to a wider range of foods, including meats, soups, vegetables, and dairy products (Centre for Disease Control, 2024).

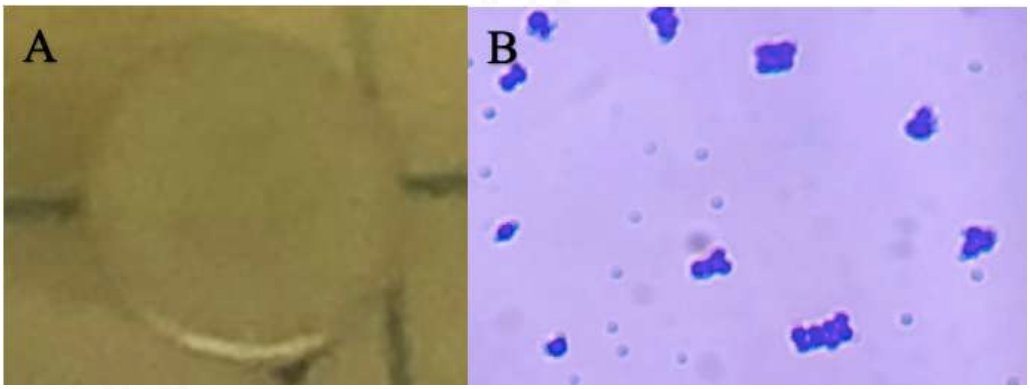

Figure 13. Isolate P12. Staphylococcus hominis isolate. A: Colony (white) morphology in PCA agar; B: Cell morphology under light microscope (100X).

Staphylococcus hominis (*S. hominis*) (Figure 13) is a gram-positive, coagulase-negative bacteria that occurs as a normal commensal organism on the skin (Vasconcellos et al., 2022). *Staphylococcus hominis* have been reported also as microbiota present in salame Napoli (Aspri & Tsaltas, 2020). It belongs to *Staphylococcus* species, recognized as a significant pathogen responsible for food poisoning outbreaks (Aspri & Tsaltas, 2020).

5.2.3. Bell pepper top leaves

From Bell pepper top leaves were identified 14 different strains but just three could be identified by MALDI-TOF-MS: *Microbacterium arborescent, Pseudomonas flavescens,* and *Pluralibacter pyrinus.* All the morphological and biochemical characteristics are detailed in Table 8.

Table 8. Morphological and biochemical characteristics of identified bacterial isolates from Bell pepper top leaves.

	Identified isolates from Bell pepper top leaves		
	Microbacterium arborescens	*Pseudomonas flavescens*	*Pluralibacter pyrinus*
Morphological characteristics			
Form	Circular	Circular	Circular
Elevation	Flat	Umbonate	Arise
Margin	Entire	Entire	Undulate
Color	Orange	Yellow	Purple
Opacity	Opaque	Opaque	Opaque
Shape	Bacilli	Bacilli	Bacilli
Arrangement	Palisades	Coccobacillus	Coccobacillus
Biochemical characteristics			
Catalase test	+	+	+
Oxidase test	-	+	-
KOH test	Gram positive	Gram negative	Gram negative

+: Positive; -: negative

Recently, *Microbacterium spp.* has been identified as a pathogen in humans, attributed to the growing number of immunocompromised patients and increased awareness of the pathogenic potential of coryneform bacteria (Kesarwani et al., 2021). *Microbacterium arborescens* (Figure 14) have been found in aquatic, soil, animal, and plant samples, and they can be associated with plants, fungi, animals, and clinical samples. Many species and subspecies within this family are either plant pathogens or have been suggested to have plant pathogenic properties (Evtushenko & Takeuchi, 2006).

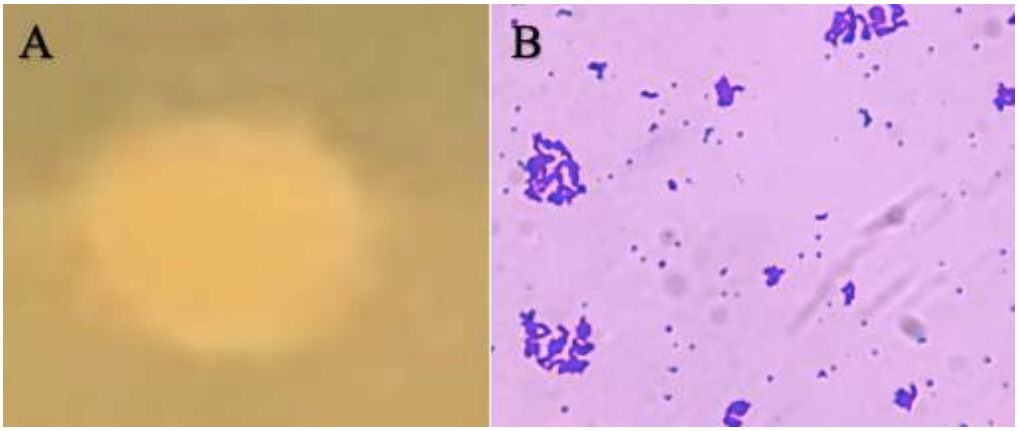

Figure 14. Isolate P25. Microbacterium arborecens isolate. A: Colony in PCA agar; B: Cells seen under light microscope (100X).

A research performed by H Kang et al. (2018), describes nine novel candidates microorganism isolated in Korea, between those microorganism were found *Pseudomonas flavescens* HME8118 in an isolation from seawater of Yellow sea, and it presented the same morphological and biochemical characteriscs founded in the present research. However, there are not any reports regarding the ocurrance of *Pseudomonas flavescens* (Figure 15) in food, but in general, *Pseudomonas* spp. have been isolate as an abudanmt member of microbiota in milk, beef, pork, chicken (Stellato et al., 2017) and vegetables (Ruiz-Roldán et al., 2021).

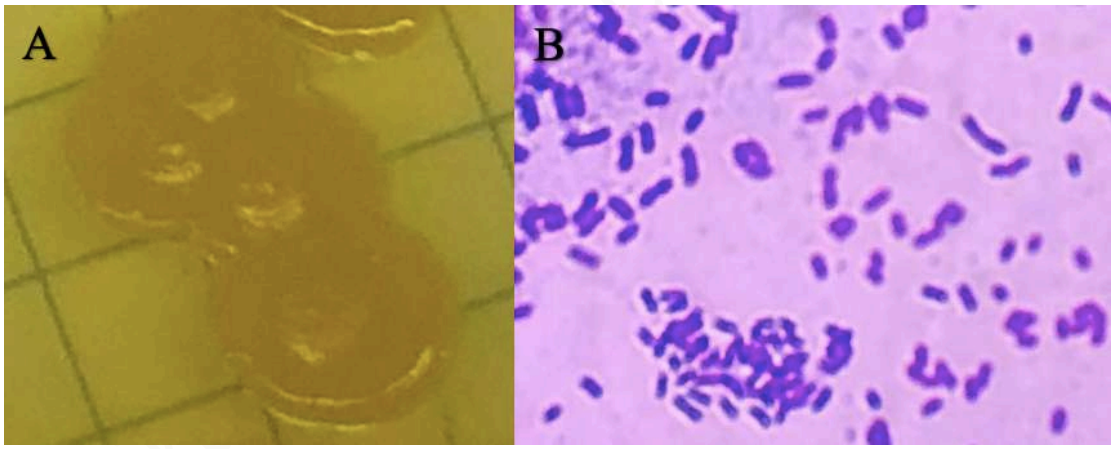

Figure 15. Isolate P21. Pseudomonas flavescens isolate. A: Colony morphology on Cetrimid agar; B: Cell morphology under light microscope (100X).

Pluralibacter pyrinus (Figure 16) have been isolate from pear tree in a brown leaf spot (Phyllosphere) in Korea (Reimer et al., 2022).

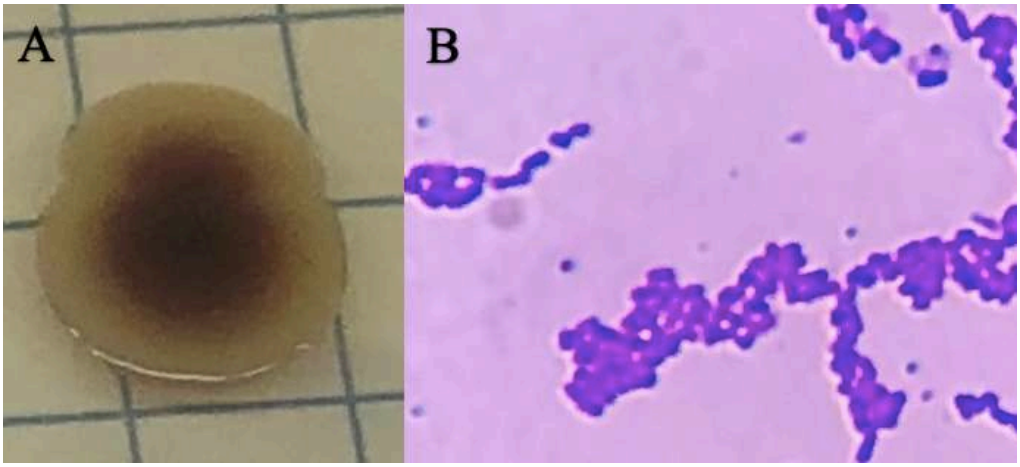

Figure 16. Isolate P20. Pluralibacter pyrinus isolate. A: Colony morphology on Chromocult agar; B: Cell morphology under light microscope (100X).

Pluralibacter pyrinus called before *Enterobacter pyrinus* is facultative-anaerobic that was reclassified in 2013 into the novel genus *Pluralibacter* (Brady et al., 2013). Besides of pear trees, surveillance data and outbreak reports from South America, North America, Europe, and Asia, show that *Enterobacter* spp. is a significant opportunistic pathogen affecting newborns and weakened patients in intensive care units (ICU). Data from the Centers for Disease Control and Prevention (CDC) in the United States indicate that the percentage of resistant strains has remained relatively stable. Nonetheless, there is widespread awareness of a global crisis involving multidrug-resistant gram-negative bacteria (Lopardo et al., n.d.).

5.2.4. Bell pepper flowers

The isolates identified from Bell pepper flowers were *Bacillus cereus*, *Microbacterium arborescens, Metschnikowia pulcherrima, Pseudonomas antarctica, Pseudomonas fulva,* and *Pseudonomas oryzihabitants,* although *Metschnikowia pulcherrima* is a ubiquitous species of yeast, with numerous strains, belonging to the family *Metschnikowiaceae*, and found in grapes, cherries, flowers, spoiled fruits, and consequently carried by fruit flies and this microorganism will not be describe deeply. All the morphological and biochemical characteristics of the microorganisms of bell pepper flowers are presented in Table 9.

Table 9. Morphological and biochemical characteristics of identified bacterial isolates from Bell pepper flowers.

	Identified isolates from Bell pepper flowers				
	Bacillus cereus	*Microbacterium arborescens*	*Pseudomonas antarctica*	*Pseudomonas fulva*	*Pseudomonas oryzihabitants*
Morphological characteristics					
Form	Irregular	Circular	Circular	Circular	Circular
Elevation	Flat	Flat	Raised	Convex	Crateriform
Margin	Undulate	Entire	Entire	Entire	Lobate
Color	White	Orange	Beige	Yellow	Yellow
Opacity	Opaque	Opaque	Translucent	Opaque	Translucent
Shape	Bacillus	Bacilli	Bacilli	Bacilli	Bacilli
Arrangement	Single	Palisades	Diplobacilli	Coccobacilli	Diplobacilli
Biochemical characteristics					
Catalase test	+	+	+	+	-
Oxidase test	+	-	+	-	-
KOH test	Gram positive	Gram positive	Gram negative	Gram negative	Gram negative

+: Positive; -: negative

Bacillus cereus (Figure 17) was already described, due to it was found also in bell pepper bottom leaves. It can produce spores, enabling it to withstand extreme temperatures and survive for extended periods. It often contaminates a variety of foods, such as beef, turkey, rice, beans, and vegetables (McDowell et al., 2023). Factors influencing the growth and survival of *B. cereus* include water activity (Aw), pH, and temperature of the surrounding environment. While *B. cereus* spores germinate more effectively at higher Aw levels, some can even germinate at Aw values below 0.80, and they thrive at Aw values of 0.90 or higher (Staack et al., 2008).

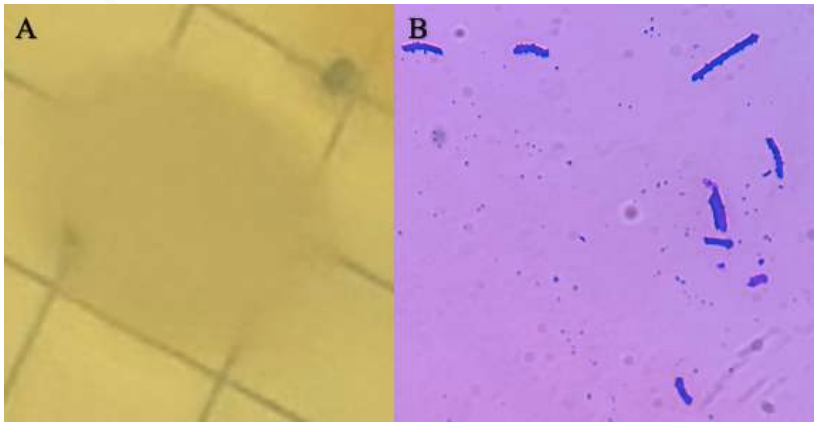

Figure 17. Isolate code: P32. Bacillus cereus isolate. A: Colony morphology in PCA agar; B: Cell morphology under light microscope (100X).

Microbacterium arborescens (Figure 18) can be found in diverse terrestrial and aquatic environments and can be associated with plants, fungi, animals, and clinical samples. Many

species and subspecies within this family are either plant pathogens or have been suggested to have plant pathogenic properties (Evtushenko & Takeuchi, 2006), which support the present isolation from two different parts of the Bell pepper plant phyllosphere: top leaves and flowers.

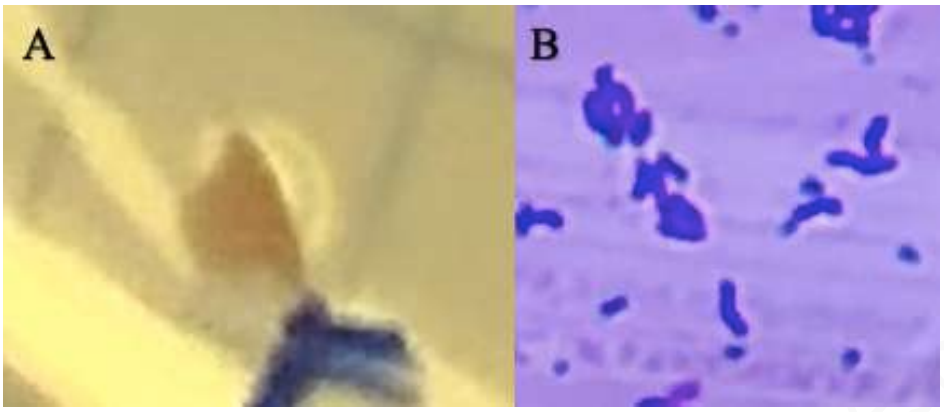

Figure 18. Isolate code: P33. Microbacterium arborescens isolate. A: Colony morphology in PCA agar (Double layer); B: Cell morphology under light microscope (100X).

Pseudomonas antarctica (Figure 21), as its name suggests, was isolated from Antarctica (Reddy et al., 2004). This isolate was psychrophilic. In contrast, the microorganisms reported in this case were isolated from Bell pepper flowers collected from a warehouse during the autumn season (13-23 °C). This gram-negative bacterium, which is positive for catalase and oxidase test, has been reported from various habitats, including Antarctica (Reddy et al., 2004).

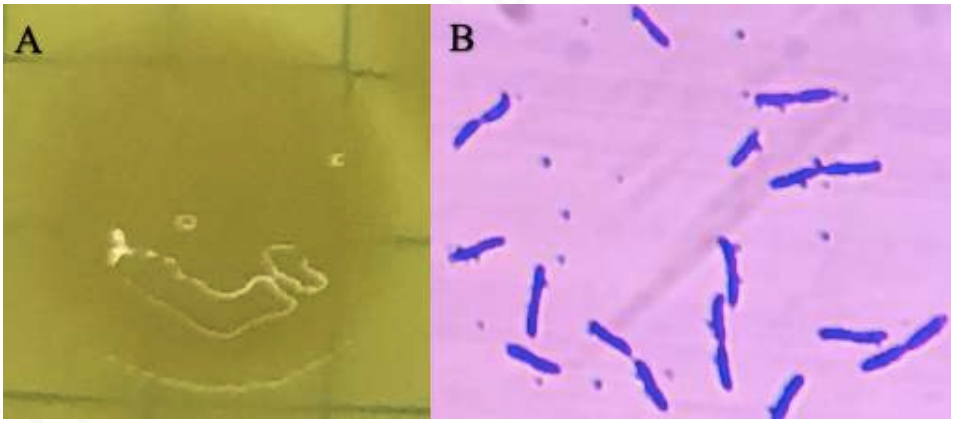

Figure 19. Isolate P28. Pseudomonas antarctica isolate. A: Colony morphology on Cetrimid agar; B: Cell morphology under light microscope (100X).

The first report of its existence in Antarctica was in 1976. However, were not identified at species level until 1989, when *Pseudomonas* spp. isolated from Antarctic soil and water samples were identified as psychrophilic strains of *P. aeruginosa, P. fluorescens, P. putida*, and *P. syringae* (Reddy et al., 2004). It is important to mention that *Pseudomonas* spp. have been isolated from seawater and freshwater samples from Terra Nova Bay and Wanda Lake (Reddy et al., 2004).

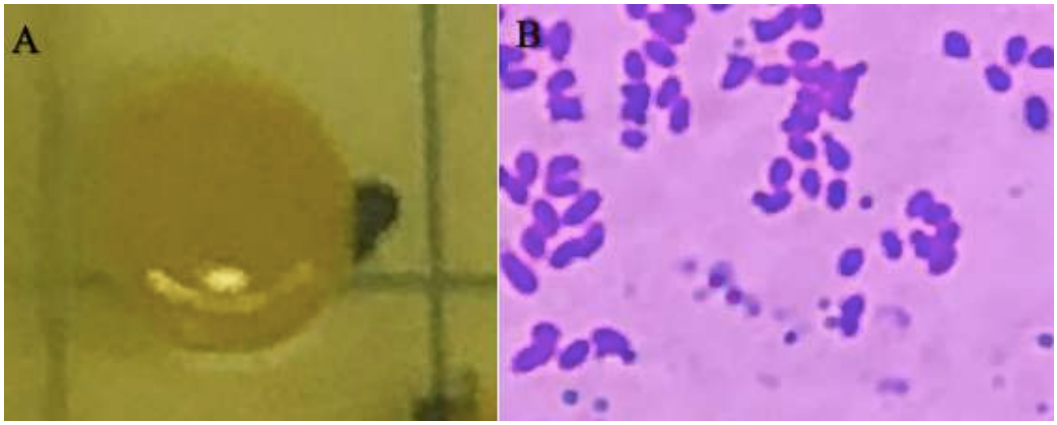

Figure 20. Isolate P29. Pseudomonas fulva isolate. A: Colony morphology on Cetrimid agar; B: Cell morphology under light microscope (100X).

Pseudomonas fulva (Figure 20) is part of the *Pseudomonas* fluorescent group and also, it is associated with occurrence in rice plants (Uchino et al., 2001) and can cause meningitis (Almuzara et al., 2010).

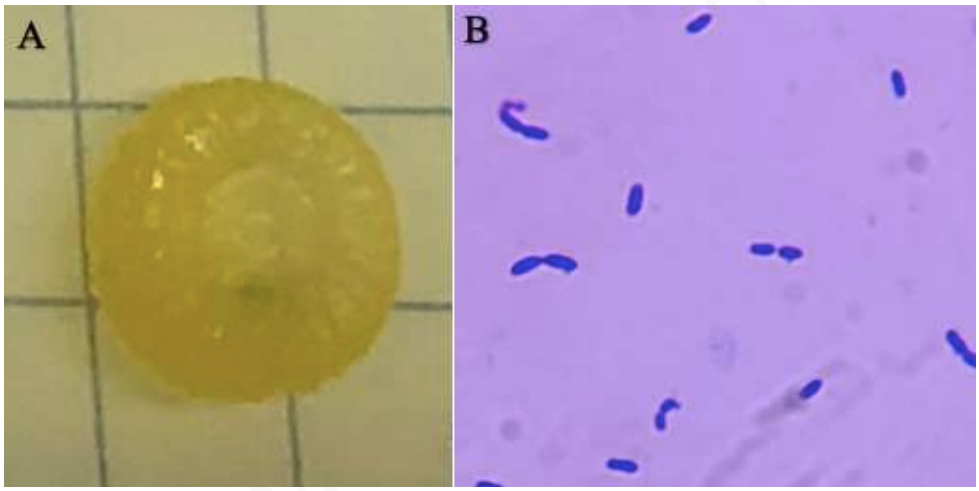

Figure 21. Isolate P35. Pseudomonas oryzihabitants isolate. A: Colony morphology on Chromocult agar; B: Cell morphology under light microscope (100X).

Pseudomonas oryzihabitants is a gram-negative isolated from Bell pepper flowers which have been reported as part of the isolation of ready-to-eat salads (Lupan, n.d.).

Based on the results, is important to remark on how many of these microorganisms are commonly reported as commensal in the human body. Showing us how there are emerging new microbes as potential causes for foodborne illnesses.

5.3.Interpretation of susceptibility test results.

The following results correspond to all the identified microorganisms by MALFI-TOF-MS from the three different food sources that are part of the whole PhD project.

5.3.1. Preliminary results

The preliminary antimicrobial susceptibility testing conducted on various microorganisms isolated from bell pepper phyllosphere, raw milk, and pork raw meat has provided valuable insights into their susceptibility to different antimicrobial agents. The results indicate varying degrees of susceptibility among the tested microorganisms which are presented based on the diameter of the inhibition zone in Table 10.

*Table 10. Inhibition Zone Diameter [mm] of oxytetracycline-hydrochloride (Disk content 30 ug) applied to isolates from Bell pepper phyllosphere, raw milk and pork raw meat. S: Susceptible; R: Resistant *:* (European Committee on Antimicrobial Susceptibility Testing, 2024).

Microorganism	Inhibition Zone Diameter [mm]	Interpretation based on common breakpoints *
Bell pepper phyllosphere		
Brachybacterium conglomeratum	18	-
Pseudomonas extremorientalis	31	S
Pseudomonas oryzihabitants	34	S
Micrococcus luteus	35	S
Moraxella osloensis	23	
Bacillus cereus	25	S
Staphylococcus hominis	34	S
Microbacterium arborescens	26	S
Pseudomonas flavescens	30	S
Pluralibacter pyrinus	24	
Pseudonomas antarctica	36	S
Pseudomonas fulva	29	S
Raw milk		
Kocuria salsicia	20	-
Staphylococcus chromogenes	33	S
Macrococcus caseolyticus	10	R
Acinetobacter johnsonii	21	-
Buttiauxella noackiae	19	R
Stenotrophomonas maltophilia	14	R
Hafnia alvei	33	S
Pantoea agglomerans	26	S
Pork raw meat		
Buttiauxella gaviniae	20	S
Aeromonas sp.	19	-
Kocuria salsicia	25	S
Pseudomonas lundensis	23	S
Pseudomonas fluorecens	31	S

In the bell pepper phyllosphere samples, several *Pseudomonas* species (*P. extremorientalis, P. oryzihabitants, P. flavescens, P. antarctica, and P. fulva*) and other bacteria such as *Micrococcus luteus*, *Bacillus cereus,* and *Staphylococcus hominis* exhibited sensitivity to the tested antimicrobial agent (oxytetracycline-hydrochloride). This suggests that these antimicrobial agents could be effective in controlling these microorganisms.

For the microorganisms isolated from raw milk, *Staphylococcus chromogenes* and *Hafnia alvei* showed sensitivity to the antibiotic tested. Isolates of *Staphylococcus* resistant to tetracycline have not been documented. Resistant isolates are either rare or have not been reported to date. According to EUCAST guidelines, *Staphylococcus* spp. is considered susceptible if the inhibition zone diameter is 22 mm or greater, and resistant if the diameter is less than 23 mm (European Committee on Antimicrobial Susceptibility Testing, 2024).

However, *Macrococcus caseolyticus*, *Buttiauxella noackiae*, and *Stenotrophomonas maltophilia* were found to be resistant to oxytetracycline-hydrochloride. This highlights the potential challenges in controlling certain bacterial species from raw milk.

In the case of pork raw meat samples, *Pseudomonas fluorecens, Buttiauxella gaviniae, Kocuria salsicia*, and *Pseudonomas lundensis* were sensitive to the antibiotic. While for *Aeromonas* spp., specific information was not regarding the effect of the tetracycline antibiotics, but *Aeromonas* spp. isolates from wastewater plant were 100% resistant to penicillin, oxacillin, ampicillin, and vancomycin (Igbinosa & Okoh, 2012).

Based on those results and the results achieved in the project carried out with two other antimicrobials six microorganisms (*Micrococcus luteus, Pseudomonas antarctica, Kocuria salsicia, Macrococcus caseolyticus, Buttiauxella gaviniae,* and *Pseudomonas lundensis)*, two from each food source, were selected to perform the Disk diffusion test and microdilution in Multiskan.

5.3.2. Determination of the MIC value.

The susceptibility of six different microorganisms isolated from food to oxytetracycline-hydrochloride treatment was evaluated across four concentrations: 3.00 µg/µl, 1.50 µg/µl, 0.75 µg/µl, and 0.375 µg/µl (Table 11). The inhibition zone diameters resulting from each concentration provide insights into the effectiveness of oxytetracycline-hydrochloride against these microorganisms.

Table 11. Inhibitory effect of oxytetracycline-hydrochloride in case of six different microorganisms isolated from food.

		Concentration of OTC			
		3,00 µg/µl	**1,50 µg/µl**	**0,75 µg/µl**	**0,375 µg/µl**
Source	**Microorganism**	**Diameters of inhibition zones [mm]**			
Bell pepper	*Micrococcus luteus*	43,70 ± 3,50	40,70 ± 3,10	37,0 ± 1,00	29,3 ± 2,30
	Pseudomonas antarctica	31,00 ± 2,60	27,70 ± 2,10	23,7 ± 1,20	19,0 ± 3,00
Raw milk	*Kocuria salsicia*	37,70 ± 0,60	34,30 ± 1,50	31,3 ± 0,60	28,3 ± 1,50
	Macrococcus caseolyticus	9,30 ± 0,60	9,00 ± 0,00	8,00 ± 0,00	0,0 ± 0,00
Raw pork meat	*Buttiauxella gaviniae*	30,00 ± 1,00	26,00 ± 0,00	22,7 ± 1,50	21,7 ± 0,60
	Pseudomonas lundensis	26,30 ± 0,60	21,00 ± 1,00	18,5 ± 0,70	14,3 ± 0,60

Micrococcus luteus exhibited a dose-dependent response to oxytetracycline-hydrochloride treatment, with the largest inhibition zone observed at 3.00 µg/µl. *Pseudomonas antarctica* also showed a dose-dependent sensitivity to the treatment. The inhibition zone diameter decreased from 31.00 mm at 3.00 µg/µl to 19.0 mm at 0.375 µg/µl (Table 11).
Similarly, *Kocuria salsicia* displayed a decreasing trend in inhibition zone diameter with decreasing concentrations of oxytetracycline-hydrochloride, ranging from 37.70 mm at 3.00 µg/µl to 28.3 mm at 0.375 µg/µl.

On the other hand, *Macrococcus caseolyticus* exhibited very limited sensitivity to oxytetracycline-hydrochloride (Figure 22), with minimal inhibition zones observed across all concentrations, indicating potential resistance to oxytetracycline-chloride.

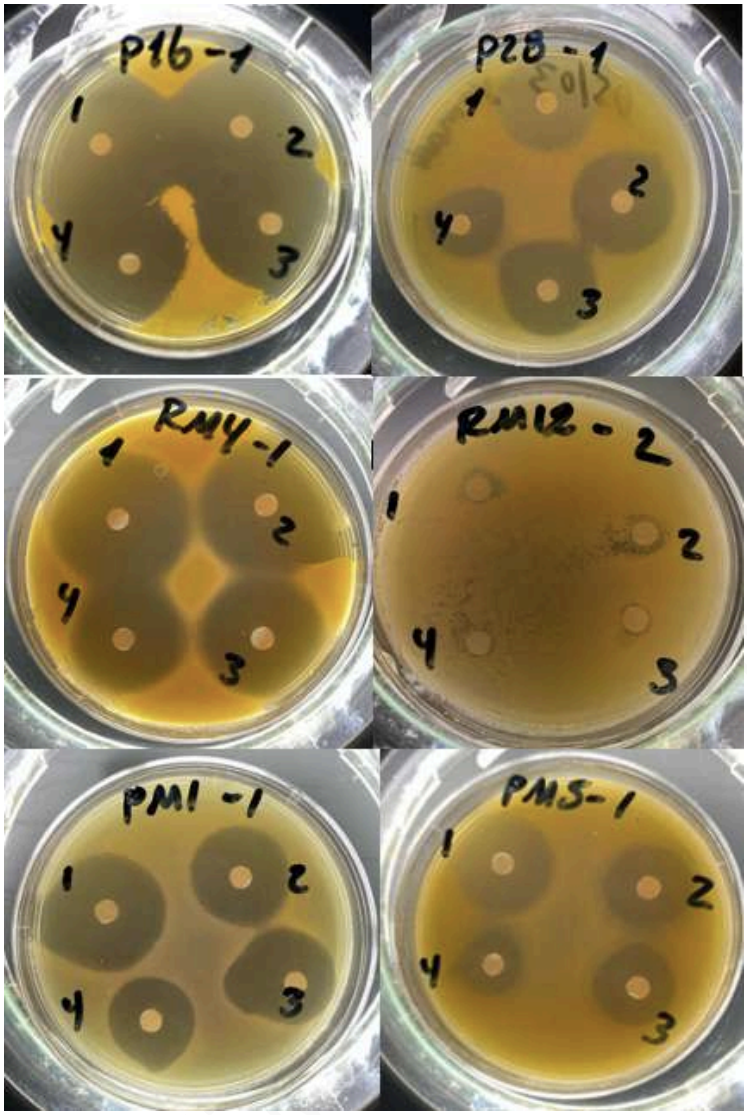

Figure 22. Disk diffusion test. Oxytetracycline-hydroclhoride concentrations: 1: 3 µg/µl; 2: 1.5 µg/µl; 3:0.750 µg/µl; 4: 0.375 µg/µl. P16: Micrococcus luteus; P28: Pseudomonas antarctica; RM4: Kocuria salsicia; RM12: Macrococcus caseolyticus; PM1: Buttiauxella gaviniae; PM5: Pseudomonas lundensis.

Buttiauxella gaviniae and *Pseudomonas lundensis* both showed decreasing inhibition zone diameters as the concentration of oxytetracycline-hydrochloride decreased. However, the sensitivity of these microorganisms was notably lower compared to *Micrococcus luteus* and *Kocuria salsicia*.

In summary, the effectiveness of oxytetracycline-hydrochloride varied among the tested microorganisms. While *Micrococcus luteus* and *Kocuria salsicia* demonstrated relatively higher sensitivity to the treatment, *Macrococcus caseolyticus* appeared to be resistant. These findings underscore the importance of understanding the antimicrobial susceptibility profiles of specific microorganisms to optimize treatment strategies and combat antimicrobial resistance effectively. Further research is warranted to explore the underlying mechanisms of resistance and to evaluate alternative antimicrobial agents or treatment strategies for resistant microorganisms.

5.3.3. Multiskan growth modelling curves.

Figure 23 illustrates the growth modeling curves of *Micrococcus luteus* and *Pseudomonas antarctica* over time exposed to varying concentrations of oxytetracycline-hydrochloride. The x-axis represents time in hours, while the y-axis represents the optical density (OD) or absorbance readings, indicating microbial growth.

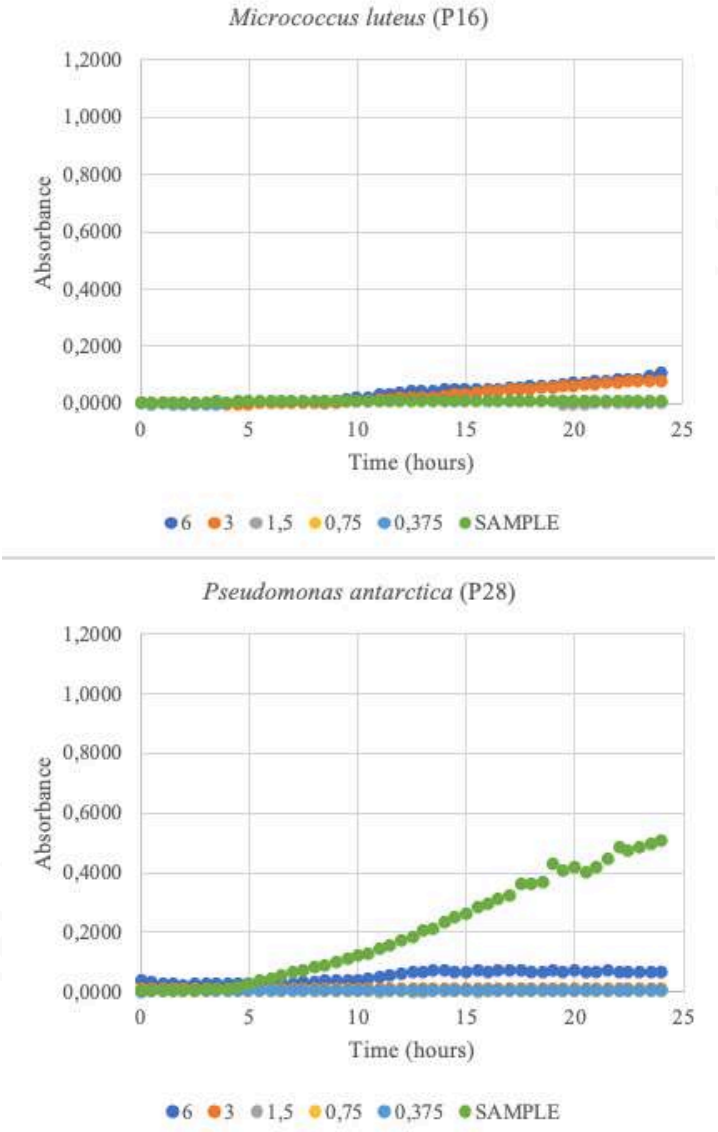

Figure 23. Multiskan growth curves of isolated microorganisms from bell pepper phyllosphere in an applied antibiotic (oxytetracycline-hydrochloride) treatment solution at 6, 3, 1,5, 0,75 and 0,375 µg/µl.

The growth curve shows a clear dose-dependent response, where the four applied concentrations of the antibiotic (oxytetracycline-hydrochloride) inhibit the microbial growth. Similar to the findings of the agar disk diffusion test *Micrococcus luteus* was inhibited by all the concentrations of the applied antibiotic. *Micrococcus luteus* presented susceptibility to 15

antibiotics and no resistance genes were detected (Shi et al., 2023b). *M. luteus* can enter a dormant state without forming spores. In contrast to other actinobacteria, *M. luteus* produces only one resuscitation-promoting factor needed to exit dormancy and possesses a limited number of other proteins related to dormancy (Wickham Micro, 2018).

Pseudomonas antarctica presents a sensitiveness of all concentrations of the tested antibiotics, which is similar to results presented by Reddy et al. (2004) where exposed that the mentioned microorganism is sensitive to ampicillin amoxycillin, bacitracin, carbenicillin, chloramphenicol, chlortetracycline, colistin, cotrimoxazole, erythromycin, kanamycin, gentamicin, lincomycin, nitrofurazone, nitrofurantoin, nystatin, oxytetracycline, penicillin, polymyxin B, rifampicin, tetracycline and tobramycin, but resistant to furazolidone, and trimethoprim.

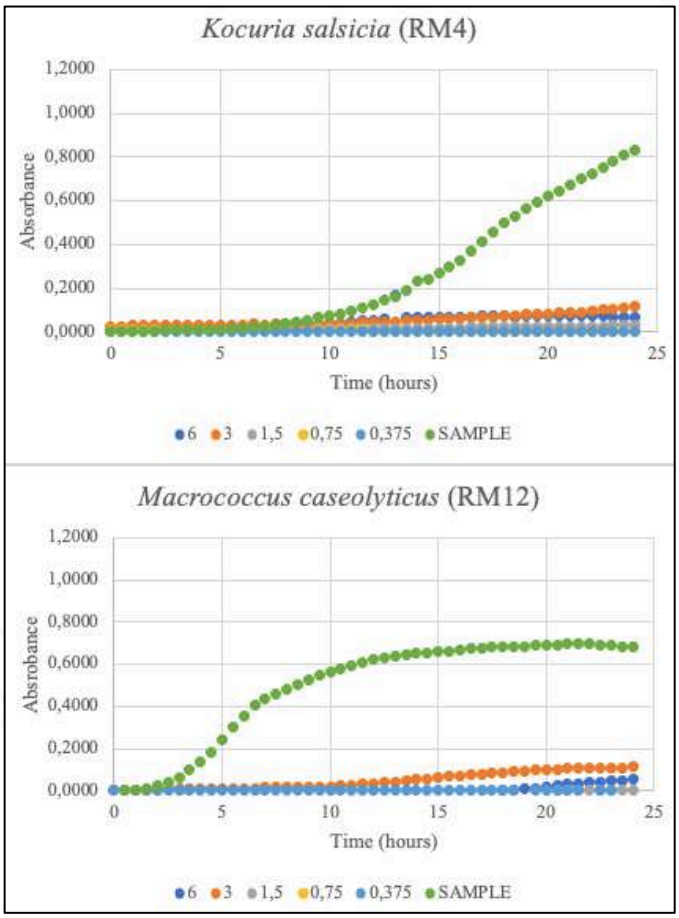

Figure 24. Multiskan growth curves of isolated microorganisms from raw milk in an applied antibiotic (oxytetracycline-hydrochloride) treatment at 6, 3, 1,5, 0,75 and 0,375 µg/µl.

Figure 24 emphasizes the effectiveness of oxytetracycline-hydrochloride against *Kocuria salsicia* and *Macrococcus caseolyticus*, indicating potential resistance or insensitivity due to a slight change in the curve belonging to 3 µg/µl. In research, the most frequently isolated species was *Kocuria kristinae* (46.1%) presents an antimicrobial resistance lower for vancomycin (7%)

and tetracyclines (6.7%) comparing to Vancomycin (47%), cephalosporins (39.6%), and quinolones (36.6%) (Ziogou et al., 2023).

In *M. caseolyticus* isolates from retail meat in China, were screened for phenotypic antimicrobial-resistant profiles. The isolates were resistant to ampicillin, cefazolin, ceftazidime, lincomycin, piperacillin, penicillin, streptomycin, and tetracycline, while a majority were sensitive to amikacin, cefuroxime, and gentamicin, indicating a broad but complex multi-drug resistance (Zhang et al., 2022).

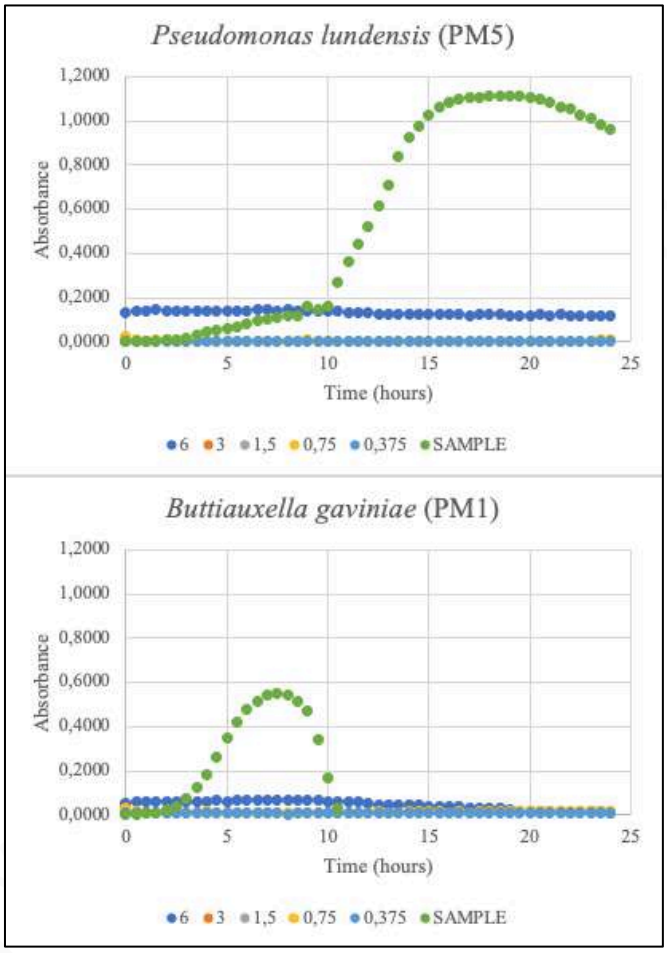

Figure 25. Multiskan growth curves of isolated microorganisms from raw pork meat in an applied antibiotic (oxytetracycline-hydrochloride) treatment at 6, 3, 1,5, 0,75 and 0,375 µg/µl.

In Figure 25, again is presented how the antibiotic treatment inhibits the growth of the bacteria, in this case, *Pseudomonas lundensis* and *Buttiauxella ganiniae*. In a study, it was found that about 95% of *Pseudomonas* strains demonstrated susceptibility to gentamicin and ciprofloxacin. This was closely followed by 93.0% showing susceptibility to tetracycline and 91.9% to levofloxacin which support the presented results. In contrast, a significant proportion of *Pseudomonas* strains exhibited resistance to imipenem, with 95.3% being resistant. (Meng et al., 2020). In the case of *Buttiauxella* strains are mostly sensitive to antibiotics.

This could be attributed to the milk originating from a production system where antibiotics are regularly administered, potentially impacting the resistance of naturally occurring microorganisms in that environment, as well as those isolated from pork (*Kocuria salsicia* and *Macrococcus caseolyticus*). In animal production, antibiotics are necessary for animal welfare, unlike in agricultural settings where antibiotic use is typically lower, as evidenced by the bacteria isolated from Bell pepper (*Micrococcus luteus* and *Pseudomonas antartica*), which show greater susceptibility. Furthermore, considering the samples were obtained from an organic production facility what can explain the susceptibility of those microorganisms (Lauková et al., 2018).

6. Conclusion

The main objectives of this research were to isolate and identify microorganisms from the bell pepper phyllosphere and analyze antibiotic resistance patterns of three different food sources (bell pepper phyllosphere, raw pork meat, and raw milk) as part of a PhD research. The antibiotic used was Oxytetracycline-hydrochloride. Through achieving these objectives, we aimed to contribute to assessing the potential risks these microorganisms pose to food safety and public health, emphasizing the importance of investigating antibiotic resistance patterns in foodborne microorganisms.

The bell pepper phyllosphere presents a variety of microorganisms (Brachybacterium conglomeratum, Pseudomonas extremorientalis) that are not commonly found in food sources. Most have been reported in hospital environments or that are naturally presented in the human body as commensals. This indicates a huge concern regarding food safety because those microorganisms can be considered as potential pathogens among immunosuppressed people. That is why based on the results this study recommended following good agricultural practices to keep out of potential pathogens the produces, concerning more in the source and the safety of the compost, irrigation water, and coworkers who are in contact with the plants and fruit during the maintenance and harvesting activities.

The incidences of microorganisms in raw pork meat and raw milk are explained in the other projects that were carried out at the same time. Regarding the antibiotic resistance of isolates from the bell pepper phyllosphere, raw pork meat, and raw milk. Almost all isolates were susceptible to the applied antibiotic (Oxytetracycline-hydrochloride). Notably, *Macrococcus caseolyticus*, isolated from milk, demonstrated significant resistance to the antibiotic. These findings suggest performing the same assessment considering higher and lower antibiotic concentrations and other types of antibiotics.

Consequently, the whole research emphasizes the importance of continued surveillance and management strategies to address antibiotic resistance in foodborne microorganisms, thereby safeguarding public health and food safety.

7. **Summary**

The widespread use of antibiotics in agriculture, animal husbandry, and food production has led to the selection and proliferation of antibiotic-resistant strains of bacteria in the food supply chain. These resistant bacteria pose a threat to human health, as they can contaminate food products and cause infections that are difficult to treat with conventional antibiotics. Understanding antibiotic resistance in foodborne bacteria is crucial for planning effective strategies to control its spread and safeguard public health. Additionally, ongoing exploration of emerging pathogens in food production and their response to commonly used antibiotics is essential. This research describes the microbiota of bell pepper and investigates the antibiotic resistance of bacteria that have been isolated from raw pork meat, raw milk, and bell pepper phyllosphere; Food samples were collected and processed to isolate bacteria, followed by microscopic investigations and biochemical identification methods (Oxidase, Catalase, and Potassium hydroxide tests) to characterize the bacterial strains. The bacteria selected were identified in genus and species level using MALDI-TOF-MS. The isolates were subjected to antibiotic susceptibility testing using Oxytetracycline hydrochloride at four different concentrations between 0,375 µg/µl – 3,00 µg/µl to assess their resistance profiles using the method disk diffusion test and Spectroscopy UV/Vis (Multiskan Ascent Microplate Reader) to find the MIC (Minimum Inhibitory Concentration) value. The findings reveal unexpected bacteria in the samples, concerning the prevalence of antibiotic resistance among foodborne bacteria. Through biochemical identification methods, various bacterial species were identified (*Micrococcus luteus, Pseudomonas antarctica, Kocuria salsicia, Macrococcus caseolyticus, Buttiauxella gaviniae Pseudomonas lundensis*) highlighting the diverse microbial populations present in the food samples, due to the different environments that the food is exposed along the production chain. Antibiotic susceptibility testing revealed that *Macrococcus caseolyticus* was the most resistant bacteria among the others it showed minor inhibition zones (8-9 mm) at different concentrations of the antibiotic, in contrast to *Micrococcus luteus* which was the most sensitive microorganism with large inhibition zones (29-43 mm) at all applied concentrations. *Pseudomonas antarctica, Kocuria salsicia, Buttiauxella gaviniae,* and *Pseudomonas lundensis* showed similar resistance with inhibition zones of 19-37 mm. These findings underscore the importance of monitoring antibiotic resistance in foodborne bacteria and implementing measures to mitigate its spread, such as prudent antibiotic use in agriculture and food production, as well as surveillance programs to track resistance trends. Continued research in this area is vital for safeguarding public health and ensuring the efficacy of antibiotic treatment in foodborne illnesses, encouraging to continue with this study with Multiskan using lower antibiotic concentrations.

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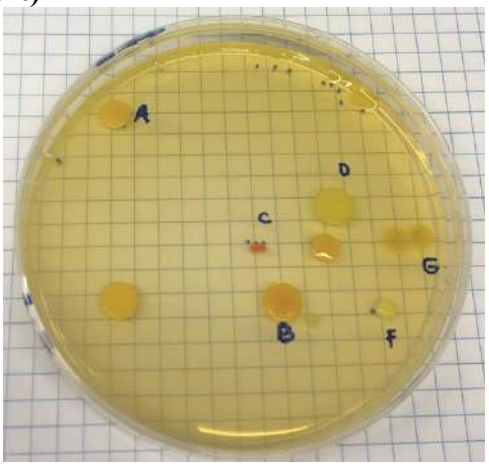
9. Annexes

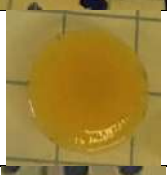
1. Description of isolates from each sample and each type of agar.

1.1.Bell pepper (Fruit)



1. Colonies isolated from Bell pepper (fruit) and cultured in PC Agar

Table 1. Morphological description of the isolated colonies of Bell pepper using PC Agar.

Bell pepper (Fruit)						
Colony image	**Colony code**	**Colour**	**Opacity**	**Form**	**Elevation**	**Margin**
	1.3.1.A	Orange	Opaque	Circular	Flat	Entire
	1.3.1.B	Orange	Translucent	Circular	Raised	Entire
	1.1.3.C	Red	Opaque	Punctiform	Convex	Entire
	1.3.1.D	Neon yellow	Opaque	Circular	Convex	Entire
	1.3.1.E	Yellow	Transparent	Circular	Flat	Entire
	1.3.1.F	Pale yellow	Opaque	Circular	Flat	Entire

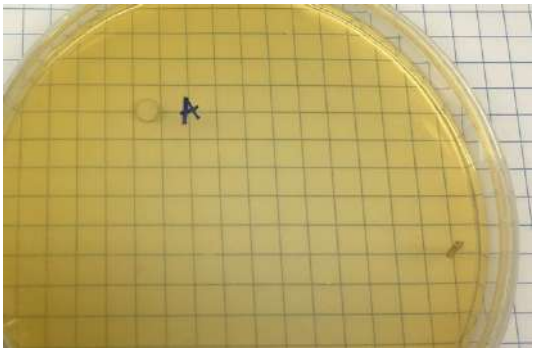


Figure 2. Colonies isolated from Bell pepper and cultured in MRS Agar

Table 2. Morphological description of the isolated colonies of Bell pepper using MRS Agar.

Conventional Bell pepper (Fruit)						
Colony image	**Colony code**	**Colour**	**Opacity**	**Form**	**Elevation**	**Margin**
	1.3.2.A	White	Opaque	Circular	Raised	Entire

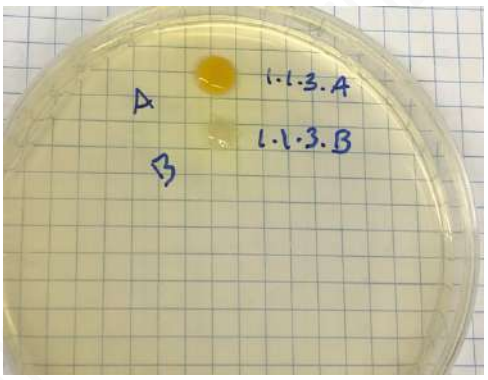


Figure 3. Colonies isolates from Bell pepper and cultured in Cetrimid Agar

Table 3. Morphological description of the isolated colonies of Bell pepper using Cetrimid Agar.

Conventional Bell pepper (Fruit)						
Colony image	**Colony code**	**Colour**	**Opacity**	**Form**	**Elevation**	**Margin**
	1.1.3.A	Yellow	Opaque	Circular	Convex	Entire
	1.1.3.B	White	Translucent	Circular	Convex	Entire

Figure 4. Colonies isolated from Conventional Bell pepper and cultured in Chromocult Agar

Table 4. Morphological description of the isolated colonies of Conventional Bell pepper using Chromocult Agar.

Bell pepper (Fruit)						
Colony image	**Colony code**	**Colour**	**Opacity**	**Form**	**Elevation**	**Margin**
	1.3.2.A	Yellow	Opaque	Rizhoid	Chrateriform	Undulate

1.2.Bell pepper Bottom Leaves

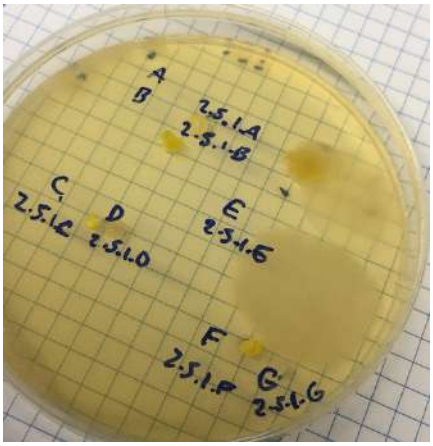


Figure 5. Colonies isolated from Bell pepper Bottom Leaves and cultured in TGE Agar

Table 5. Morphological description of the isolated colonies of Bell pepper Bottom Leaves and cultured in TGE Agar.

Bell pepper (Fruit)						
Colony image	**Colony code**	**Colour**	**Opacity**	**Form**	**Elevation**	**Margin**
	2.5.1.A	Yellow and white in the centre	Opaque	Circular	Raised	Undulate
	2.5.1.B	Yellow	Translucent	Circular	Flat	Entire
	2.5.1.C	Yellow	Opaque	Circular	Raised	Undulate
	2.5.1.D	White	Transparent	Circular	Raised	Entire
	2.5.1.E	White	Opaque	Irregular	Flat	Undulate
	2.5.1.F	Yellow	Opaque	Irregular	Umbonate	Lobate
	2.5.1.G	White	Opaque	Circular	Flat	Entire

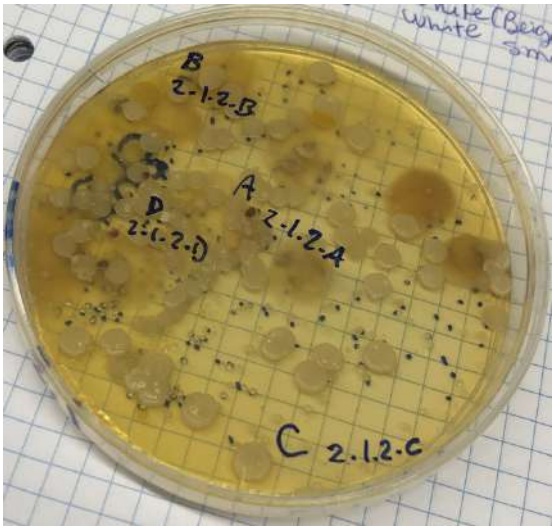


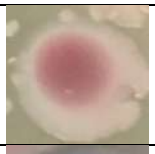
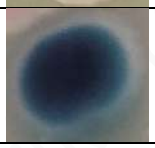
Figure 6. Colonies isolated from Bell pepper Bottom Leaves and cultured in MRS Agar.

Table 6. Morphological description of the isolated colonies of Bell pepper Bottom Leaves cultured in MRS Agar.

Bell pepper Bottom Leaves						
Colony image	**Colony code**	**Colour**	**Opacity**	**Form**	**Elevation**	**Margin**
	2.1.2.A	Purple	Opaque	Circular	Raised	Entire
	2.1.2.B	Yellow	Opaque	Circular	Flat	Entire
	2.1.2.C	White	Opaque	Circular	Raised	Entire
	2.1.2.D	White	Opaque	Circular	Raised	Entire

Figure 7. Colonies isolated from Bell pepper Bottom Leaves and cultured in Chromocult Agar.

Table 7. Morphological description of the isolated colonies of Bell pepper Bottom Leaves cultured in Chromocult Agar.

Bell pepper Bottom Leaves						
Colony image	**Colony code**	**Colour**	**Opacity**	**Form**	**Elevation**	**Margin**
	2.1.4.A	Pink	Opaque	Circular	Raised	Entire
	2.1.4.B	Blue	Opaque	Circular	Flat	Entire

1.3.Bell pepper Top Leaves

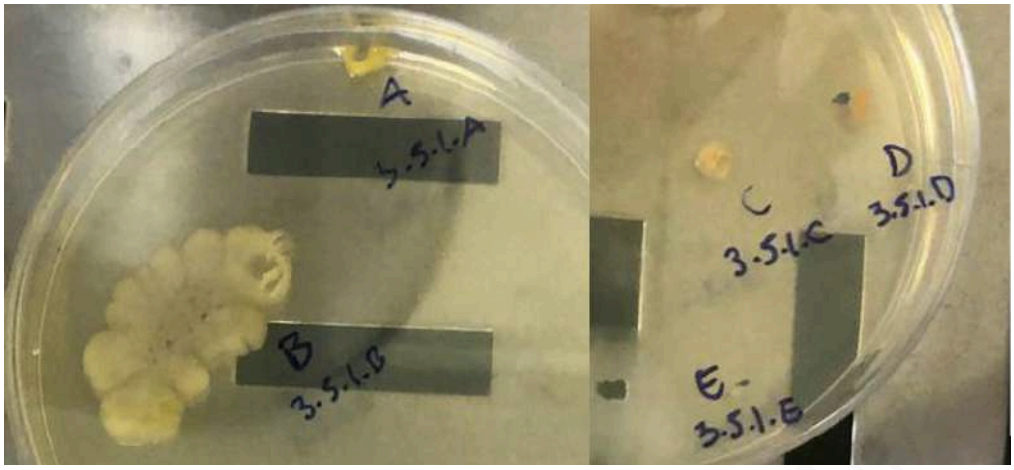
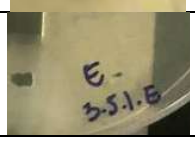


Figure 8. Colonies isolated from Bell pepper Top Leaves and cultured in TGE Agar. (Left: TGE spread; Right: TGE double layer)

Table 8. Morphological description of the isolated colonies of Bell pepper Top Leaves cultured in TGE Agar.

Bell pepper Top Leaves						
Colony image	**Colony code**	**Colour**	**Opacity**	**Form**	**Elevation**	**Margin**
	3.5.1.A	Yellow	Opaque	Irregular	Raised	Entire
	3.5.1.B	White	Translucent	Rizhoid	Flat	Undulate
	*3.5.1.C	Orange	Opaque	Circular	Flat	Entire
	*3.5.1.D	Orange	Opaque	Circular	Flat	Entire
	*3.5.1.E	White	Translucent	Irregular	Flat	Entire

***:** These colonies were obtained by culturing with the technique of double layer.

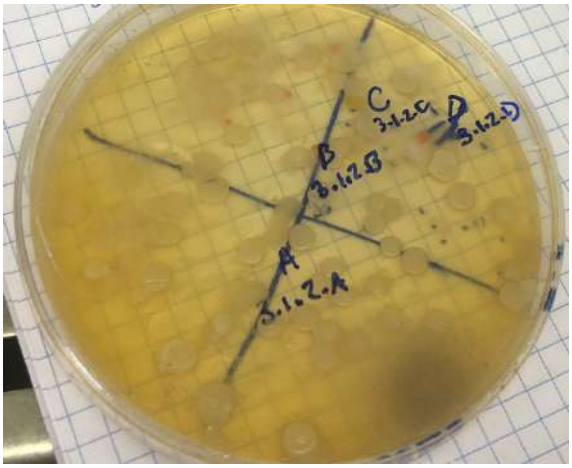


Figure 9. Colonies isolated from Bell pepper Top Leaves and cultured in MRS Agar.

Table 9. Morphological description of the isolated colonies of Bell pepper Top Leaves cultured in MRS Agar.

Bell pepper Top Leaves						
Colony image	**Colony code**	**Colour**	**Opacity**	**Form**	**Elevation**	**Margin**
	3.1.2.A	White	Opaque	Circular	Raised	Entire
	3.1.2.B	Orange	Opaque	Circular	Raised	Entire
	3.1.2.C	Yellow	Opaque	Circular	Flat	Entire
	3.3.2.D	Yellow	Opaque	Circular	Raised	Entire

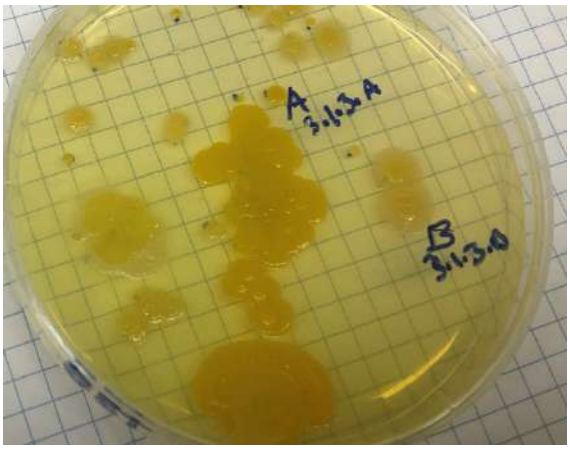


Figure 10. Colonies isolated from Bell pepper Top Leaves and cultured in Cetrimid Agar.

Table 10. Morphological description of the isolated colonies of Bell pepper Top Leaves cultured in Cetrimid Agar.

Bell pepper Top Leaves						
Colony image	**Colony code**	**Colour**	**Opacity**	**Form**	**Elevation**	**Margin**
	3.1.3.A	Yellow	Opaque	Circular	Umbonate	Entire
	3.1.3.A	Yellow	Translucent	Circular	Umbonate	Curled

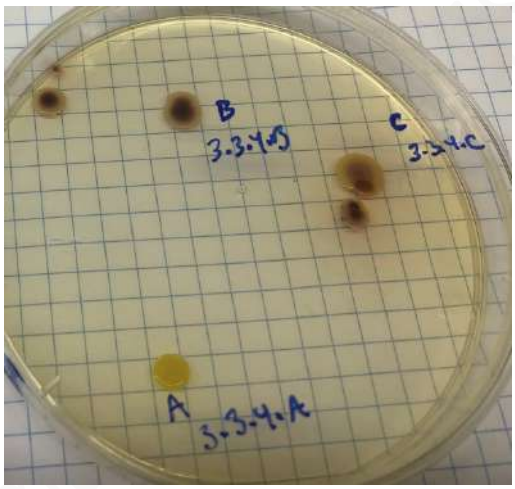


Figure 11. Colonies isolated from Bell pepper Top Leaves and cultured in Chromocult Agar.

Table 11. Morphological description of the isolated colonies of Bell pepper Top Leaves cultured in Chromocult Agar.

Bell pepper Top Leaves						
Colony image	**Colony code**	**Colour**	**Opacity**	**Form**	**Elevation**	**Margin**
	3.3.4.A	Yellow	Opaque	Circular	Raised	Entire
	3.3.4.B	Darker purple	Opaque	Circular	Flat	Curled
	3.3.4.C	Lighter purple	Opaque	Circular	Flat	Curled

1.4.Bell pepper flowers

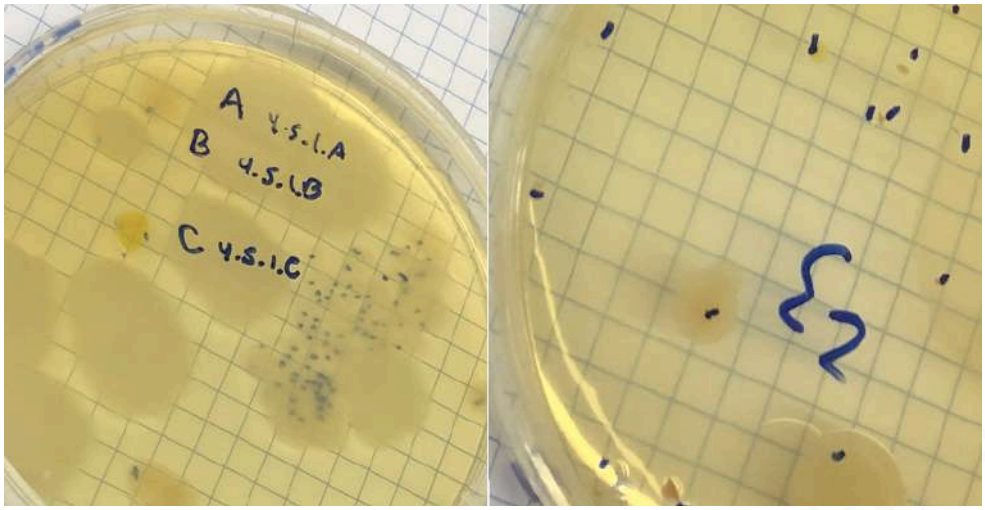


Figure 12. Colonies isolated from Bell pepper Flowers and cultured in TGE Agar.

Table 12. Morphological description of the isolated colonies of Bell pepper Flowers cultured in TGE Agar.

Bell pepper flowers						
Colony image	**Colony code**	**Colour**	**Opacity**	**Form**	**Elevation**	**Margin**
	4.5.1.A	Beige	Transparent	Circular	Flat	Entire
	4.5.1.B	White	Opaque	Irregular	Flat	Ondulate
	4.5.1.C	Yellow	Transparent	Irregular	Flat	Entire
	*4.5.1.D	Orange	Opaque	Irregular	Flat	Entire

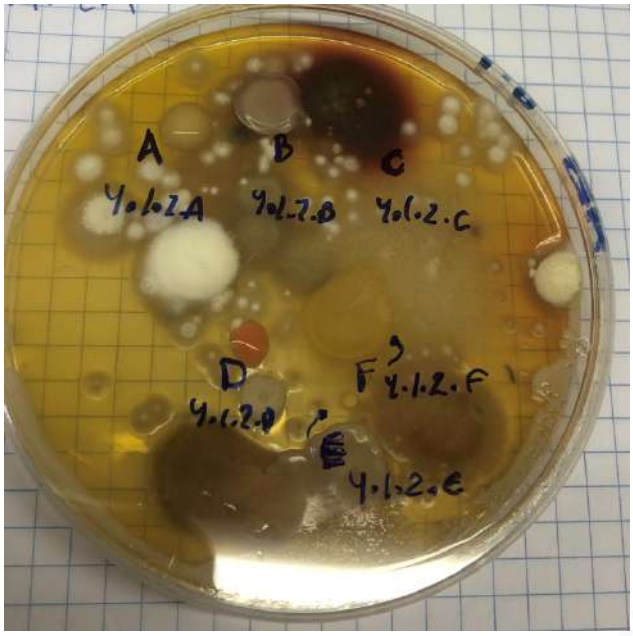


Figure 13. Colonies isolated from Bell pepper Flowers and cultured in MRS Agar.

Table 13. Morphological description of the isolated colonies of Bell pepper Flowers cultured in MRS Agar.

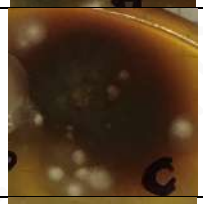
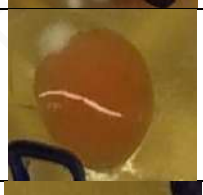
Bell pepper flowers						
Colony image	**Colony code**	**Colour**	**Opacity**	**Form**	**Elevation**	**Margin**
	4.1.2.A	Yellow	Opaque	Circular	Convex	Entire
	4.1.2.B	White	Opaque	Circular	Raised	Curled
	4.1.2.C	Purple	Opaque	Circular	Flat	
	4.1.2.D	Orange	Opaque	Irregular	Convex	Entire
	4.1.2.E	Yellow	Opaque	Circular	Raised	Entire
	4.1.2.F	Beige	Opaque	Circular	Raised	Entire

Figure 14.. Colonies isolated from Bell pepper Flowers and cultured in Cetrimid Agar.

Table 14. Morphological description of the isolated colonies of Bell pepper Flowers cultured in Cetrimid Agar.

Bell pepper flowers						
Colony image	**Colony code**	**Colour**	**Opacity**	**Form**	**Elevation**	**Margin**
	4.3.3.A	White	Translucent	Circular	Raised	Entire
	4.3.3.B	Yellow	Opaque	Circular	Convex	Entire

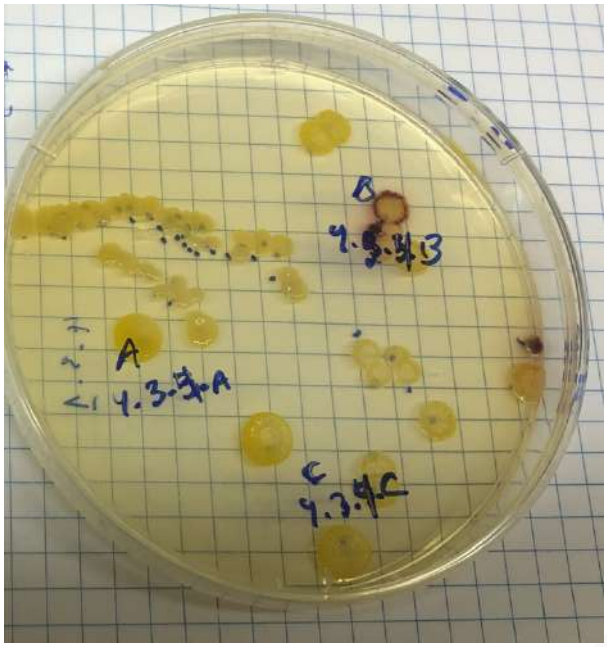


Figure 15. Colonies isolated from Bell pepper Flowers and cultured in Chromocult Agar.

Table 15. Morphological description of the isolated colonies of Bell pepper Flowers cultured in Cetrimid Agar.

Bell pepper flowers						
Colony image	**Colony code**	**Colour**	**Opacity**	**Form**	**Elevation**	**Margin**
	4.3.4.A	Yellow	Opaque	Circular	Flat	Entire
	4.3.4.B	Yellow	Opaque	Circular	Raised	Entire
	4.3.4.C	Yellow	Opaque	Circular	Crateriform	Lobate

***:** These colonies were obtained by culturing with the technique of double layer.

2. Microbial morphology and biochemical tests.

Table 1. Microbial morphology description, Biochemical tests (Catalase, Oxidase and Potassium hydroxide tests) and identification by MALDI TOF-MS of Bell pepper (fruit).

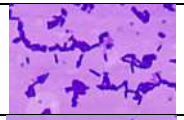
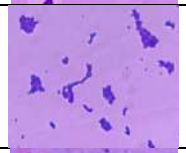
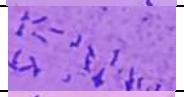
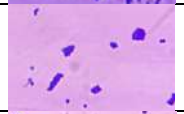
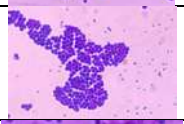
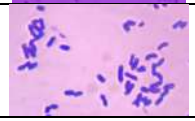
Bell pepper (Fruit)								
Microscopical description					Biochemical tests			Identification
Microscopy image	Colony code	Isolate code	Shape	Arrangement	Catalase	Oxidase	Potassium hydroxide	MALDI TOF-MS
	1.3.1.A	P4	Bacilli	Palisades	Positive	Positive	Negative	NI
	1.3.1.B	P3	Bacilli	Palisades	Positive	Negative	Negative	NI
	1.3.1.D	P5	Bacilli	Bacillus	Positive	Negative	Positive	NI
	1.3.1.F	P6	Cocci	Sarcina	Positive	Negative	Negative	*Brachybacterium conglomeratum*
	1.3.2.A	P36	Cocci	Sarcina	Positive	Negative	Negative	*Staphylococcus saprophyticus*
	1.1.3.A	P1	Bacilli	Coccobacillus	Positive	Negative	Negative	NI
	1.1.3.B	P2	Bacilli, slightly curve rods	Bacillus	Positive	Positive	Negative	*Pseudomonas extremorientalis*
	1.1.4.A	P7	Bacilli	Diplobacillli	Negative	Negative	Negative	*Pseudomonas oryzihabitants*

Table 2. Microbial morphology description, Biochemical tests (Catalase, Oxidase and Potassium hydroxide tests) and identification by MALDI TOF-MS of Bell pepper bottom leaves.

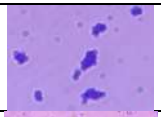
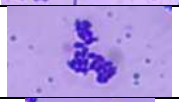
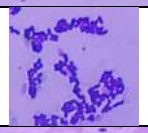
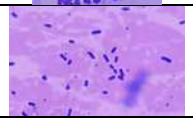
Bell pepper Bottom Leaves								
Microscopical description					Biochemical tests			Identification
Microscopy image	**Colony code**	**Isolate code**	**Shape**	**Arrangement**	**Catalase**	**Oxidase**	**Potassium hydroxide**	**MALDI TOF-MS**
	2.5.1.A	P15	Micrococci	Sarcina	Positive	Negative	Negative	*Staphylococcus warneri*
	2.5.1.C	P16	Cocci	Tetrad, sarcina	Positive	Negative	Negative	*Micrococcus luteus*
	2.5.1.D	P17	Cocci	Diplococci bacillus	Positive	Positive	Positive	*Moraxella osloensis*
	2.5.1.E	P10	Bacilli	Streptobacilli	Positive	Positive	Positive	*Bacillus cereus*
	2.5.1.F	P11	Cocci	Sarcina	Positive	Negative	Negative	NI
	2.5.1.G	P12	Cocci	Sarcina	Positive	Negative	Negative	*Staphylococcus hominis*
	2.1.2.B	P38	Cocci	Staphylococci/ micrococci	Positive	Negative	Positive	NI
	2.1.2.C	P39	Bacilli	Coccobacillus	Positive	Negative	Negative	NI
	2.1.4.A	P13	Bacilli	Coccobacillus	Positive	Negative	Positive	NI
	2.1.4.B	P14	Bacilli	Bacillus	Positive	Negative	Positive	NI

Table 3. Microbial morphology description, Biochemical tests (Catalase, Oxidase and Potassium hydroxide tests) and identification by MALDI TOF-MS of Bell pepper top leaves.

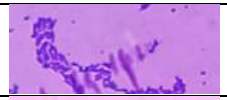
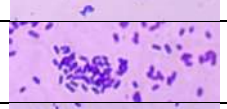
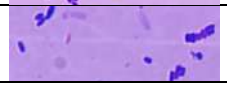
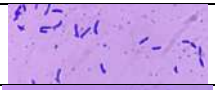
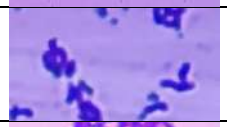
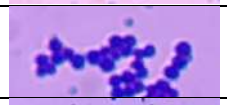
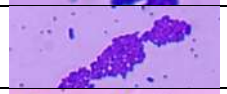
Bell pepper Top Leaves								
Microscopical description					Biochemical tests			Identification
Microscopy image	**Colony code**	**Isolate code**	**Shape**	**Arrangement**	**Catalase**	**Oxidase**	**Potassium hydroxide**	**MALDI TOF-MS**
	3.5.1.A	P23	Bacilli	Palisades	Positive	Negative	Positive	NI
	3.5.1.B	P24	Cocci	Diplococci	Positive	Negative	Positive	NI
	3.5.1.C	P25	Bacilli	Palisades	Negative	Negative	Negative	*Microbacterium arborescens*
	3.5.1.D	P26	Bacilli	Palisades	Positive	Negative	Negative	*Microbacterium arborescens*
	3.5.1.E	P27	Bacilli	Palisades	Positive	Positive	Positive	NI
	3.1.2.A	P47	Micrococci	Sarcina	Negative	Negative	Negative	NI
	3.1.2.B	P41	Micrococci	Sarcina	Positive	Negative	Negative	NI
	3.3.2.D	P49	Cocci	Diplococci	Negative	Negative	Negative	NI
	3.1.3.A	P21	Bacilli	Bacillus	Positive	Positive	Positive	*Pseudomonas flavescens*
	3.1.3.B	P22	Bacilli	Bacillus	Positive	Positive	Positive	NI
	3.3.4.A	P18	Bacilli	Coccobacillus	Positive	Negative	Positive	NI
	3.3.4.C	P20	Bacilli	Coccobacillus	Positive	Negative	Positive	*Pluralibacter pyrinus*

Table 4. Microbial morphology description, Biochemical tests (Catalase, Oxidase and Potassium hydroxide tests) and identification by MALDI TOF-MS of Bell pepper flowers.

Bell pepper Flowers								
Microscopical description					Biochemical tests			Identification
Microscopy image	**Colony code**	**Isolate code**	**Shape**	**Arrangement**	**Catalase**	**Oxidase**	**Potassium hydroxide**	**MALDI TOF-MS**
	4.5.1.A	P31	Bacilli	Palisades	Positive	Positive	Positive	NI
	4.5.1.B	P32	Bacilli	Bacillus	Positive	Positive	Negative	*Bacillus cereus*
	4.5.1.D	P33	Bacilli	Bacillus	Negative	Negative	Negative	Microb*acterium arborescens*
	4.1.2.A	P42	Cocci	Staphylococci	Positive	Negative	Negative	NI
	4.1.2.E	P45	Cocci	Sarcina	Positive	Negative	Negative	NI
	4.1.2.F	P46	Cocci	Staphylococci	Negative	Negative	Positive	NI
	4.3.3.A	P28	Bacilli	Diplobacillus	Positive	Positive	Positive	*Pseudomonas antarctica*
	4.3.3.B	P29	Bacilli	Coccobacillus	Positive	Negative	Positive	*Pseudomonas fulva*
	4.3.4.A	P34	Bacilli	Coccobacillus	Positive	Negative	Positive	NI
	4.3.4.B	P35	Bacilli (Slightly curve rods)	Bacillus	Negative	Negative	Positive	*Pseudonomas oryzihabitans*
	4.3.4.C	P30	Bacilli	Diplobacilli	Negative	Negative	Positive	*Pseudonomas oryzihabitans*

Acknowledgements

I would like to express my sincere gratitude to my supervisor Andrea Taczmanné Brückner PhD and Gabriella Kiskó PhD for their guidance, their insightful feedback and constructive criticism, which greatly enriched the quality of this work. Thanks to God for the wisdom that He gave to me during this process. I am deeply thankful to my family and friends for their unwavering support, encouragement, and understanding throughout this endeavor. Their love and encouragement have been my source of strength and motivation.

Finally, I would like to acknowledge the funding provided by Hungarian University of Agriculture and Life Science, which made this research possible.

This thesis would not have been possible without the contributions of all those mentioned above, and for that, I am truly grateful.

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