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DEPARTMENT OF AGRONOMY, FOOD, NATURAL RESOURCES, ANIMALS AND ENVIRONMENT

MASTER'S DEGREE IN
FOOD SCIENCE AND TECHNOLOGY

The spread of antibiotic-resistant bacteria: Analysis of water sources and "ready to eat" salads.

Supervisor
Prof. Giulio Cozzi
Co-supervisor
Prof. Bjørn-Arne Lindstedt

Graduand:
Davide Martini
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n. 2025212

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Abstract

Antibiotic resistance is becoming a major concern for human health. Several studies have shown that antibiotic resistant bacteria can be found in the environment, where aquatic ecosystems act as a reservoir as well as food products, such as ready to eat foods, milk, and meat products.

This project aims to assess the presence of antibiotic resistant bacteria in water sources in both Norway and Italy, and in ready to eat salads. Between March and June 2022, a total of 7 water sources were sampled. Five of them were sampled in Norway, of these four were sampled in Ås and 1 in Ullesvang municipalities. The 2 water samples from Italy were obtained in two different municipalities of the province of Vicenza (Marostica and Pojana Maggiore).

In June, 3 samples of ready to eat salads were sampled in three different Norwegian supermarkets in Ås. From both water and salad samples, a total of 26 different bacteria were isolated (n_{water} = 20; n_{salad} = 6) using two different selective media, extended spectrum β-lactamase agar and carbapenem resistant Enterobacteriaceae agar and later identified by 16s rDNA sequencing.

The bacteria were tested for the presence of 12 commonly known genes for antibiotic resistance through PCR. None of the samples showed the presence these genes.

Sixteen samples (n_{water} = 14; n_{salad} = 2) were subjected to Minimum Inhibitory Concentration test for 7 different classes of antibiotic for a total of 9 antibiotics (ampicillin, amoxicillin, penicillin G, cefepime, cefotaxime, meropenem, ciprofloxacin, erythromycin, and tetracycline). Ten samples were found to be multi-drug resistant (n_{water} = 9; n_{salad} = 1), 3 of which were part of the *Pseudomonas non aeruginosa group*, 1 was part of the *Acinetobacter* group, and 6 were part of the *non-specie related* group. Four samples were subjected to full genome sequencing using Illumina Miseq. Results show the presence of a β-lactamase (OXA-334) but also novel genes (CpxR and MexB) that code for efflux pumps that can effectively remove various classes of antibiotics including carbapenems. Two samples also show the presence of several novel virulence factors, closely related to the ones found in *Pseudomonas aeruginosa*.

Riassunto

L'antibiotico resistenza è un tema che sta attirando attenzioni sempre maggiori dati i possibili risvolti negativi per la salute umana ad essa associata. Diversi studi hanno mostrato la presenza di batteri antibiotico resistenti sia nell'ambiente, dove le sorgenti d'acqua rappresentano i principali reservoir, sia in diversi alimenti come vegetali, latte, prodotti a base di carne, e altri alimenti pronti al consumo.

Questo progetto si pone come obbiettivo la verifica della presenza di batteri antibiotico resistenti in diverse fonti d'acqua e in insalate *ready to eat*.

Tra marzo e giugno 2022 sono state dunque campionate sette diverse fonti d'acqua, cinque delle quali in Norvegia e le restanti due in Italia.

Nel corso del mese di giugno poi, sono state campionate tre insalate *ready to eat* da tre supermercati locati nella città di Ås.

Dal totale delle 10 unità campionarie mediante due diversi terreni di coltura, l'*extended spectrum β-lactamase agar*, e il *carbapenem resistant enterobacteriaceae agar* sono stati isolati 26 ceppi diversi di batteri antibiotico resistenti (n_{acqua}=20; $n_{insalata}$=6) i quali sono stati successivamente identificati mediante il sequenziamento del DNA 16s.

I campioni sono stati sottoposti a PCR per verificare la presenza di 12 geni di antibiotico resistenza comunemente presenti in batteri isolati nell'ambiente ospedaliero. Tuttavia, nessuno dei campioni ha mostrato la presenza dei suddetti geni.

16 campioni (n_{acqua} = 14; $n_{insalata}$ = 2) sono stati sottoposti al *Minimum inhibitory concentration* test (test della minima concentrazione inibente) per sette diverse classi di antibiotici per un totale di nove antibiotici (ampicillina, amoxicillina, penicillina G, cefepima, cefotaxima, meropenem, ciprofloxacina, eritromicina e tetraciclina).

Dai risultati è emersa la presenza di 10 batteri multi-farmaco resistenti (n_{acqua} = 9; $n_{insalata}$ = 1), di cui 3 facenti parte del gruppo *Pseudomonas non aeruginosa*, 1 facente parte del gruppo *Acinetobacter*, e i restanti 6 facenti parte del gruppo non legato ad una specifica specie.

Di questi, quattro campioni sono stati sottoposti al sequenziamento genetico completo con tecnica *Illumina miseq*.

I risultati hanno mostrato la presenza di un enzima, la β-lattamasi (OXA-334) e di diversi geni che codificano per pompe di efflusso per svariate classi di antibiotici mai descritte in precedenza. Il sequenziamento genetico ha inoltre dimostrato la presenza di diversi fattori di virulenza, anch'essi mai descritti in precedenza.

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1 INTRODUCTION

Before the era of modern medicine, the treatment of diseases was entrusted to plants and traditional remedies that included various toxic substances such as mercury or arsenic. The first antimicrobials per se, sulphonamides, were discover only in the 1900s, but the true revolution in the field of antimicrobial compounds was Penicillin G, the very first antibiotic which were discovered by Alexander Fleming in 1928. The discovery of antibiotic compounds represents one of the most important steps in human medicine as they allowed the treatment of several diseases by selectively targeting the bacteria without compromising human cells and tissues de facto setting new health standards (Ola Sköld, 2011a). Anyway, due to a reckless use of this drug less than twenty years later, in the 1940s, the first antibiotic resistant strains of *Staphylococcus aureus* made their appearance in London civilian hospitals. During the next decade, because of the need to find a treatment for the "white plague" better known as tuberculosis, caused by *Mycobacterium tuberculosis*, streptomycin was discovered. Even though this antibiotic presented some problems related to toxicity it became the main treatment for tuberculosis. However, due to the aggressive treatments needed to cure this disease it did not take long for antibiotics resistant strains to make their appearance. To overcome the problem of penicillin-resistant strains more antibiotics like tetracycline, chloramphenicol and erythromycin were developed, but as for their predecessor, resistant bacteria made their appearance shortly after (Miao et al., 2011). A crucial milestone for antibiotic resistance is the appearance of the first strains of multidrug-resistant Enterobacteriaceae, namely *Escherichia coli*, *Shigella*, and *Salmonella* in the 1960s (Levy & Marshall, 2004). The abuse of antibiotics in both humans and animals, especially in intensive livestock farming, where antibiotics are not only used to treat diseases, but also as prophylaxis and growth promotors, combined with the incorrect treatment of human waste, animal waste and manufacturing waste.

led to the spread of antibiotics in the environment. Even though the residues of antibiotics in treated effluents and sewage sludge are present in concentrations way lover than the minimum inhibitory concentration, with the highest being in the order of micrograms per liter, it promoted the selection of bacteria resistant to these substances. As already happened in the past, thanks to bacteria's inherent capability of adapting, the resistant strain rapidly developed new methods to avoid the action of antibiotics (Finley et al., 2013). As a result, today, antibiotic resistant bacteria are almost ubiquitous in the environment, and of all sources, Aquatic ecosystems play a major role not only as a reservoir for antibiotic resistant bacteria

(ARB) but also for the development and transfer of antibiotic resistance to other species, including ones that are pathogenic for humans (Baquero et al., 2008). The presence of ARB in the environment also means that these bacteria can be virtually spread anywhere including food chains and it cannot be stopped. As Kirbis and Krizman (2015) reported, ARB were found in food products such as vegetables, milk, meat products and ready to eat food.

The outcome of the antibiotic resistance phenomenon is rather worrisome, the CDC report shows that in 2019 ARB were directly responsible for the death of at least 1.27 million people worldwide and they were also associated with about 5 million deaths (ANTIMICROBIAL RESISTANCE Global Report on Surveillance, 2019). Moreover, the discovery of new antibiotics is difficult as most compounds do not reach the clinical trials due to the lack of activity even in highly permeable bacteria and even if a compound shows a promising antimicrobial activity it must deal with other problems such as potency, useful pharmacokinetics, low protein binding, adsorption, distribution, metabolism, and excretion (Silver, 2013).

Ever since penicillin G was discovered, new β-lactams and other classes of antibiotics have been produced, such as macrolides, quinolones and tetracyclines; however, they all, suffer from resistance-related problems. Currently, β-lactams, which include several classes of antibiotics such as penicillins, 3rd generation cephalosporines, are the most used drugs to treat both humans and animals (Bush & Bradford, 2016). Carbapenems, which are also part of the family of β-lactams represent instead the last resort for emerging resistance when it comes to antibiotics (Meletis, 2016).

To come a conclusion, the importance given to ARB lies in the low number of alternatives or even the complete lack of alternatives when it comes to carbapenems. Therefore, the absence of alternative treatments options can lead to longer periods of illnesses or worse, the inability to treat an infectious disease.

1.1 Antibiotic resistance mechanisms

1.1.1 Intrinsic resistance

Antibiotic resistance (AR) is closely linked to genetics, and it can be intrinsic or acquired. Intrinsic resistance essentially derives from a natural insensitivity of a given strain to the action of the antibiotic. The simplest example of intrinsic AR is the absence of the target of the antibiotic. Typically, gram-negative bacteria are inherently resistant to a wider variety of

antibiotics when compared to gram-positive bacteria (Badino & Odore, 2009). This is mainly due to the differences in the outer membrane composition. The outer membrane of Gram-negative bacteria presents saturated lipid molecules covalently bound to polysaccharides units. Since the fatty acids that compose the chain are saturated, they can be tightly packed thus giving a less fluid outer membrane and therefore, decreasing the permeability (**Figure 1**) (Cox & Wright, 2013).

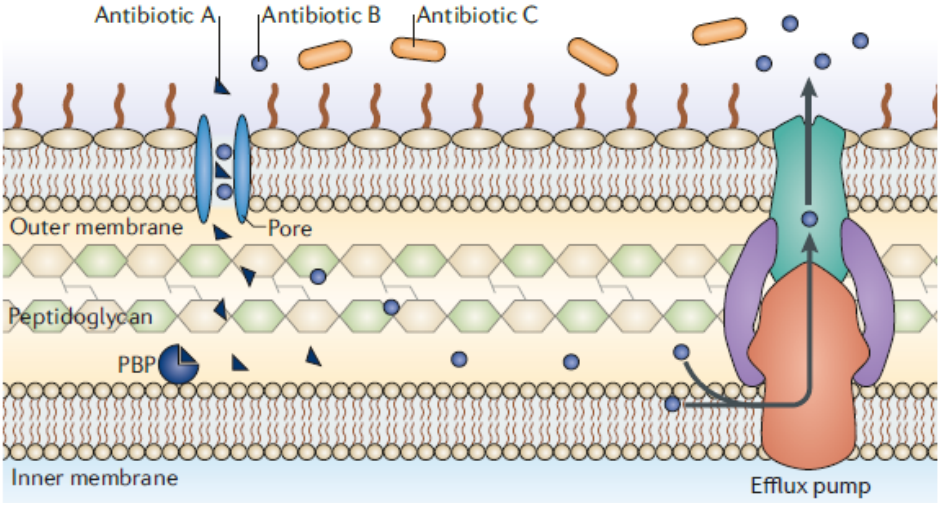

Figure 1 intrinsic mechanisms of resistance: The picture shows two different mechanisms of intrinsic resistance. Antibiotic A can pass through the porin of the outer membrane and reaches the penicillin-binding protein (PBP). Antibiotic B also passes through the porin, but it cannot bind to the PBP therefore, it is forced out by the efflux pump. Antibiotic C cannot enter the outer membrane and so it cannot link to the PBP (Blair et al., 2015).

The cytoplasmic membrane is also a suitable candidate for inherent resistance. For example, the lipopeptide *daptomycin* is effective only on gram-positive bacteria, this is due to the gram-negative's lower proportion of anionic phospholipids when compared to the gram-positives. The lack of abundance of these phospholipids interferes with the insertion of the daptomycin in the cytoplasmic membrane therefore, making it ineffective (Blair et al., 2015).

1.1.2 Acquired resistance

As outlined below, bacteria can also acquire or develop new mechanisms for AR. These mechanisms can be summarized in three categories:

1) Prevention of access to target: this can be achieved by directly reducing the penetration of the antibiotic into the bacterium or by pumps that efflux out the antibiotic.

2) Modification of the target of the antibiotic: this is usually related to a genetic mutation or a post-translational modification of the target.
3) Direct inactivation of the antibiotic: this process involves enzymes, such as β-lactams, that can hydrolase or change the structure of the antibiotic making it ineffective (Blair et al., 2015).

1.1.2.1 Prevention of access to target

As discussed earlier, gram-negative bacteria are inherently less permeable than gram-positive bacteria. However, the permeability could be further limited by downregulating the porins or by replacing the porins with more selective channels.

This mechanism is commonly found in *Pseudomonas spp.* and *Acinetobacter spp.*, where the lower expression of porins makes some strains resistant to newer antibiotics such as carbapenems or cephalosporins.

On the other hand, overexpression of efflux pumps could also lead to an increased level of AR resistance. The substrate specificity of the efflux pumps can vary as some pumps are able to transport only one class of antibiotic, but the majority of said pumps present a low substrate specificity therefore, they can transport compounds whose structure is very different. These kinds of pumps are known as multi-drug resistance efflux pumps (MDR).

It is worth noticing that the genes that encode for MDR pumps are not only found in bacterial chromosome, but some genes have been mobilized onto plasmid, making the transfer easier (Blair et al., 2015).

1.1.2.2 Modification of the target of the antibiotic

Most of the times, the action of an antibiotic is strictly related to its affinity with the target, therefore, a change in the structure of the target can confer resistance. The changes in the structure of the target might emerge as a random mutation, and if the antibiotic is present in the environment only the bacteria that present said mutation can proliferate.

Bacteria are also able to change the antibiotic structure, without involving changes in the genes. For example, the erythromycin ribosome methylase (ERM). ERM can add methyl groups in a specific target, making it impossible for macrolides to interact with the target.

Previously, this mechanism was not considered as clinically relevant, but recent studies point out that *armA* genes, that encode for a methyltransferase, were found in clinical isolates in Europe, India, and North America (Blair et al., 2015).

1.1.2.3 Direct inactivation of the antibiotic

The presence of enzymes that can modify the structure of antibiotic has been the leading cause of resistance. There are plenty of enzymes that can hydrolase antibiotics, such as β-lactams, aminoglycosides, and macrolides. Moreover, there are some antibiotics that can hydrolase specific classes of antibiotics, such as carbapenems or monobactams.

Is worth noticing that a single gene might show hundreds of variants, effectively making it impossible to overcome the problem.

Another way bacteria can counter an antibiotic is by the addition of chemical groups to the structure of the antibiotic that effectively make it ineffective.

Aminoglycoside antibiotics are large molecules that present many exposed amide and hydroxyl groups. Therefore, these antibiotics can be easily modified by aminoglycoside-modifying enzymes (Blair et al., 2015).

1.1.3 Biofilm

Biofilm is a non-closely genetic related mechanism of resistance that consists in a community of one or more bacterial species that is held together by a polymeric matrix often composed of polysaccharides. Biofilms are widespread in the environment and are commonly found in water sources such as lakes, rivers, ponds etc. (Muhammad et al., 2020). When it comes to antibiotics, the biofilms that are formed to resist these drugs are commonly identified as a community of more than one species. The resistance to antibiotics comes from several factors such as the density of the population, higher the density, higher the resistance, the kind of antibiotic present, for example, positively charged aminoglycosides and peptides which do not diffuse in biofilms and the more importantly, the number of cells that produce the enzymes that confer resistance. Usually, the species that produce the antibiotic degrading enzymes are in the top layer of the biofilm, in close contact with the drugs. The reason for that is that the production of these enzymes costs energy, and on the top layer is where the presence of nutrients and eventually oxygen is highest. Meanwhile, less resistant bacteria occupy the lower end of the niche where the ambient is detoxified, also the slower or arrested growth rate can inhibit the action of the antibiotics that somehow manages to get to the lower layer of the niche. Besides that, the antibiotic can be ineffective due to conditions created by bacterial metabolisms, such as presence of solutes, different pH, and gradient in oxygen (Vega & Gore, 2014).

1.2 The ways of the spread of antibiotic resistance

As discussed in the previous chapter, bacteria have lots of ways to avoid the action of antibiotics. However, a major concern how efficient they become in transmitting these resistance genes.

The transmission of antibiotic resistance genes can be divided into two main ways, vertical, where the transfer of the antibiotic-resistance gene occurs from the mother cell to the daughter cell, and horizontal, where the transmission occurs mainly via extrachromosomal carriers.

1.2.1 Vertical transmission

During vertical transmission (also called chromosomic resistance), the genetic material which is responsible for resistance passes from the mother cell to the daughter cell during replication. The resistance genes involved are primarily chromosomal genes, which may result from a spontaneous mutation even in the absence of the antibiotic in the environment. Vertical transmission is usually a gradual process as it requires a succession of several mutations, although in some rare cases the mutation may be a point mutation. This type of resistance is considered not very dangerous as the emergence of a resistance character is often correlated with other less advantageous characteristics that therefore make the bacterium less competitive. Although the appearance of the mutation may occur in the absence of the antibiotic, the proliferation of antibiotic-resistant bacteria only occurs in the presence of the antibiotic (Badino & Odore, 2009)

1.2.2 Horizontal transmission

Bacteria have an efficient system for transmitting and storing the genetic material responsible for AR. Some bacterial genes can be transferred from the chromosome to extrachromosomal DNA thus acquiring the ability to be acquired even by bacteria belonging to different genera. In this case the genetic material is transferred horizontally which means that it can be transferred to other strains. The horizontal transfer is an essential way for the spread of a new antibiotic resistance gene and is considered more threatening than the vertical transfer. Horizontal transfer is mostly carried out in strains that are phylogenetically close to each other, but sometimes it may also occur between bacteria that belong to different species which share the same habitat. However, for the transfer of an antibiotic resistance gene from one environmental strain to another strain, it is necessary that they share, at least momentarily, a given habitat. For example, environmental bacteria with AR genes may be temporarily present

within the human microbiota following the ingestion of contaminated water or food and thus share their resistance with the pathogenic bacteria already present. Horizontal gene transfer is induced by the presence of stress, such as the presence of the antibiotic itself in the environment. However, the transfer of genes for AR that reside on extra-chromosomal DNA fragments is a normal condition for bacteria that are part of the bacterial flora of a given habitat, and not all these genes originated because of contact of these microorganisms with antibiotics induced in the environment by humans (Bengtsson-Palme et al., 2018).

There are at least three mechanisms that bacteria use to transfer AR genes, the most important one is bacterial conjugation. Bacterial conjugation is also referred to as bacterial infection since the donor bacterium de facto infects the receiving bacteria with the AR gene via a cytoplasmic bridge. Other mechanisms for the passage of genetic material between bacteria are represented by transduction, which involves bacteriophages, and transformation, during which bacteria internalize genetic material present in the environment derived for instance, from the lysis of other bacterial cells.

The main vehicles mediating the acquisition of extra-chromosomal resistance are plasmids, transposons and integrons. In contrast to chromosomal resistance, precisely because of the ease of propagation between different bacterial populations, these vectors can persist within a given ecosystem even in the absence of antibiotics (Badino & Odore, 2009).

1.2.2.1 Plasmids

Plasmids consist in circular segments of DNA that can replicate independently from the bacterial chromosome. Although they are not essential for the life of the micro-organism, these organelles are of great importance in bacterial evolution as they can influence replication, metabolism, and reproductive capacity. Plasmids can also confer resistance to bacterial toxins, antibiotics, and bacteriophages, thus providing bacteria that have them more favorable conditions for survival and propagation. Plasmids can be transmitted from one bacterium to another either conjugatively or through other mechanisms (e.g., bacteriophages). Those containing the genetic material for conferring AT are called R-plasmids and can transfer the resistance character even against more than 10 different molecules (Badino & Odore, 2009).

1.2.2.2 Transposons

Transposons are short DNA sequences that can pass from plasmid to plasmid, from plasmid to chromosome or from plasmid to bacteriophage, and are therefore referred to as jumping genes; unlike plasmids, they are not able to self-replicate. Transposons present in gram-

negatives are normally inserted into plasmids that do not contain the information for the conjugative passage of genetic material (r-plasmids), whereas those present in gram-positive can be inserted into either r- or R-type plasmid. A single plasmid can carry more transposon at the same time, thus mediating simultaneous resistance to several antibiotics. Jumping genes are of fundamental importance in the spread of AR precisely because of the ease with which they can pass from one DNA chain to another, facilitating the involvement of a wide range of bacterial species (Badino & Odore, 2009).

1.2.2.3 Integrons

Integrons or gene cassettes consist of two conserved portions of DNA separated by a variable portion containing resistance-related genes. They are DNA elements containing a single gene and a site that allows recombination of the genetic material. Integrons can be in found the bacterial chromosome, but much more frequently they are integrated into plasmids or transposons and consequently can be easily transmitted from bacterium to bacterium (Badino & Odore, 2009).

2 AIMS OF THE STUDY

This study aims at assessing the presence of ARB in various water sources such as lakes, ponds, rivers, and underground water, and ready to eat salads (RTE). The water sources that were sampled are either used for irrigation, or as drinking water for both animals and humans. Therefore, the bacteria that might be present in these samples can eventually come into indirect or direct contact with both humans and animals. Among food products, RTE salads were found to be a great candidate for the spread of ARB, as for their nature, unlike milk or meat products, they are not suitable for thermic treatments or the use of chemical preservatives.

The study also wants to figure out the level of resistance of ARB to the main antibiotics used to treat humans and animals by subjecting them to the Minimum Inhibitory Concentration test. Lastly, attempt to identify what are the main mechanisms of resistance that bacteria use to avoid the action of antibiotics will be presented.

3 MATERIAL AND METHODS

3.1 Sampling sites and collection

Between March 17th and June 10th, 2022, seven water sources were sampled. Four water samples were collected in Ås Norway. The first two samples were obtained on the 17th of March from the Norwegian University of Life Science (NMBU) campus using a sterile Pyrex© water bottle. The places sampled were the Andedammen pond and a smaller pond located close to the veterinary faculty (**figure 2**).

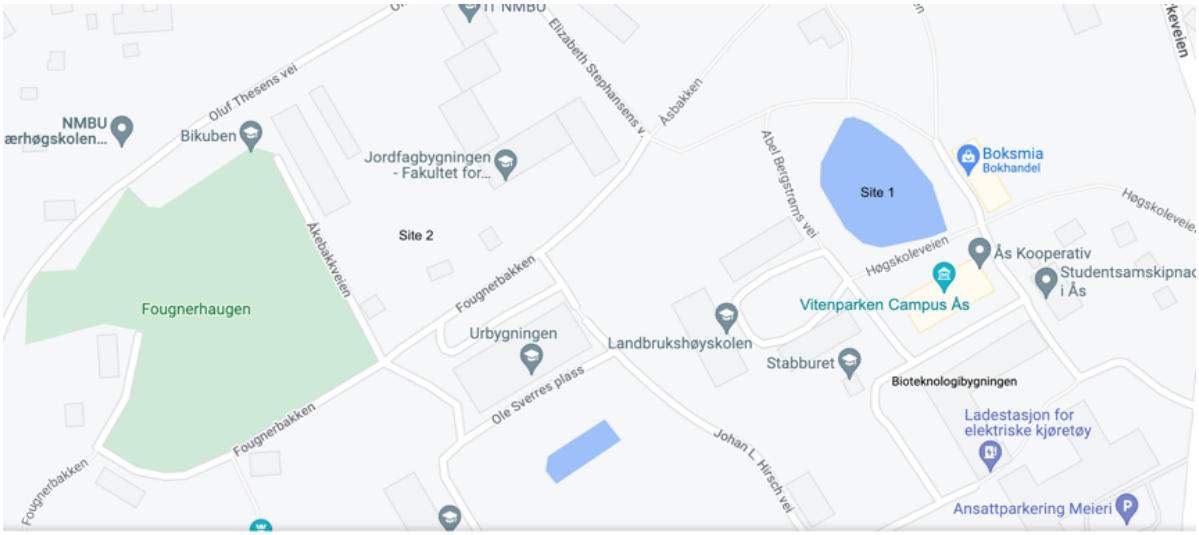

Figure 2: map showing water sampling sites on the NMBU campus. Sampling site 1 (59°40'01.4"N 10°46'09.9"E) sampling site 2 (59°40'00.5"N 10°45'55.1"E). note: the map does not show any water source in site 2 since the imagines online are out of date.

The 3rd and the 4th sample were collected the same way on the 24th of March from two lakes, the Årungen and the Pollevannet (**Figure 3)**.

The 5th sample was collected on the 10th of June, from Tysso river located along the "Trolltunga" route in the Municipality of Ullensvang using an unsterile plastic water bottle (**Figure 4**). The river ends up in the Ringedalsvatnet lake and its water represents the main source for tap water in the Municipality of Ullesvang. This procedure, however, could limit the significance of the results since it's impossible to differentiate the microorganisms present before the samples were taken. To try to overcome this problem, the water

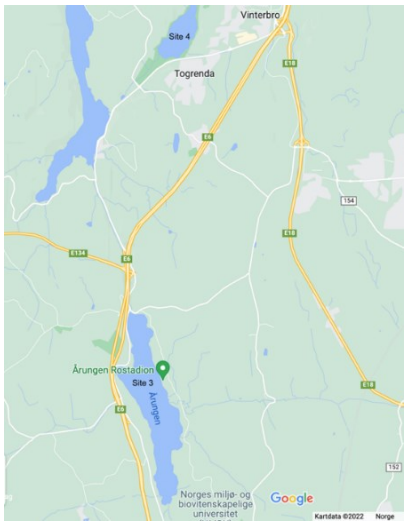

Figure 3: map showing water sampling sites in Ås. Sampling site 3 (59°41'19.4"N 10°44'36.2"E), site 4 (59°44'12.3"N 10°45'03.5"E).

bottles were washed a couple of times with the sampling water to sort of wash away the persisting flora.

Figure 4: map showing water sampling site in Ullensvang (60°08'12.2"N 6°45'25.8"E).

The water samples from Italy were instead collected on April 1st, using unsterile plastic water bottles. This choice was made because the samples were collected by another operator, and because of shipping limits, and due to shipping restrictions, glass water bottles were not allowed. Therefore, the same limits as sample number five must be considered.

The samples were collected in two different farms located in the province of Vicenza. The first sample was collected in a farm in the municipality of Marostica meanwhile the 2nd one was collected in a farm in the municipality of Pojana Maggiore (**Figure 5**).

Unlike the previous samples, which were obtained from surface water sources like ponds and rivers, these two were collected from groundwater, namely, rainwater that seeps into the ground and accumulates between the soil and rocks.

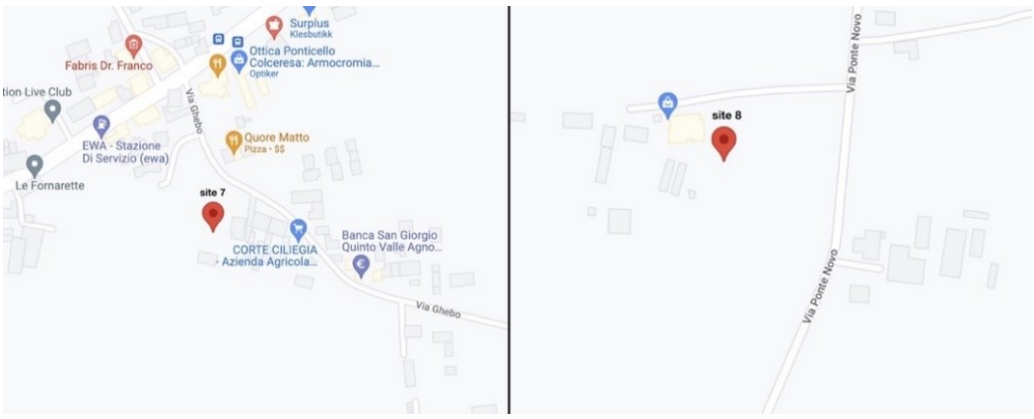

Figure 5: map showing water sampling sites in the province of Vicenza, on the left Marostica (45°43'23.9" N 11°36'59.7" E); on the right Pojana Maggiore (45°16'44.5" N 11°31'41.4E).

Regarding the salad samples, three ready to eat salads were bought from three different supermarkets located in Ås on June 27th, 2022.

3.2 Sample preparation

Although the water samples were collected on different days, they were all subjected to the same protocol, as outlined below.

Initially, to remove impurities and coarse debris, the water was filtered through a sterile paper filter into a sterile Pyrex© water bottle. Then, 1 mL of filtered water was pipetted and spread directly on a Brilliance™ Extended-spectrum beta-lactamase (ESBL) agar plate, a chromogenic screening plate for the detection of extended spectrum β-lactamase-producing organism.

The same procedure was carried out on Brilliance™ Carbapenem-resistant Enterobacteriaceae (CRE) agar plate, a chromogenic screening plate for the detection of carbapenem-resistant Enterobacteriaceae. These two media were chosen because of their high selectivity for antibiotic-resistant bacteria. Afterwards, 100 mL of the remaining water was subjected to a vacuum filtration on a filter membrane (**Figure 6)**.

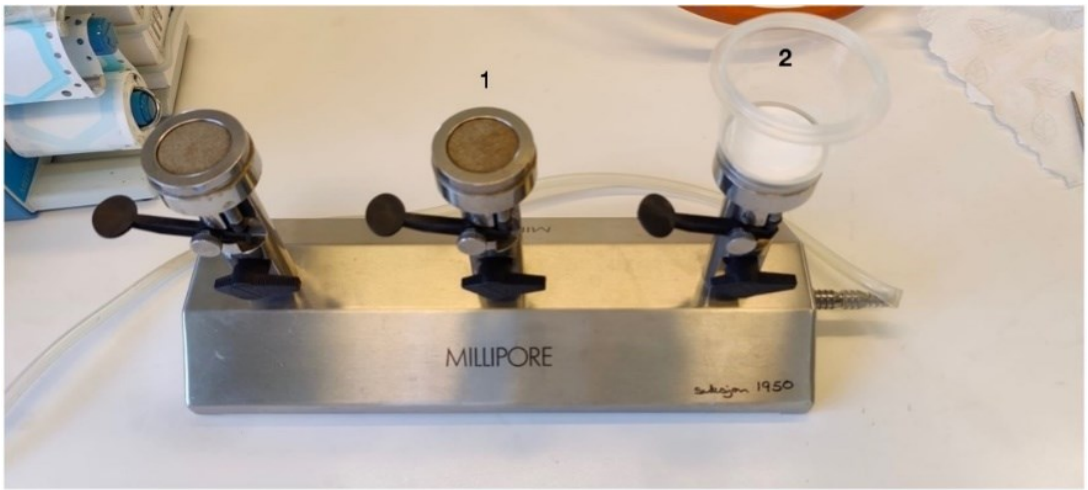

Figure 6: vacuum pump used to filtrate the water samples, before the filter in place (1) and with both filter and fennel in place (2).

Once all the water had passed through the filter, the membranes were transferred onto both ESBL plate and CRE plate using sterile tweezers.

The plates were then incubated at 37 °C for 48-72 hours, until colonies were visible. The temperature of 37°C was chosen to select only the bacteria that could proliferate inside the human body.

The salad samples were submitted to a slightly different protocol. Initially the surface of the confection was rubbed with a 70% alcohol solution, then, using sterile tweezers, 20 grams of product were picked up and diluted with 99 mL of sterile Ringer solution. The mixture was then transferred into a stomacher and run at a high speed for 2 minutes. The resulting solution was filtered through a paper filter. Finally, the filtered solution underwent the same treatments previously described for water samples.

3.3 Bacterial isolation

In both ESBL and CRE agar the presence of white colonies was presumably related to *Pseudomonas spp*. or *Acinetobacter spp*., meanwhile colonies showing green/dark blue or pale pink colours represent other antibiotic-resistant bacterial species. Since the antibiotic resistance of *Pseudomonas spp.* is largely described in literature, coloured colonies have been prioritized as the presence of AR strains not belonging to *Pseudomonas spp*. is of greater interest. Anyway, a small number of white colonies were picked to test the differences between Norwegian and Italian strains of *Pseudomonas spp*. and, the possible presence of *Acinetobacter spp*. towards which there is increasing attention.

Therefore, From the initial 40 plates a total of eight plates did not show any growth at all, seven other plates did not show further growth during the purification process and two more plates were unpickable due to the extended presence of mold that covered most of the surface of the plate (**Figure 7**).

Thus, from the remaining 25 starting plates single colonies were randomly picked using a sterile inoculation loop and inoculated again in a new plate which was then incubated again at 37°C for 48 to 72 hours. Some exceptions were made, mainly when both the unfiltered and filtered samples presented identical colonies or when only white colonies were present. In these cases, the starting sampling plates where united into a single plate thus, presenting only one or two colonies per plate instead of four.

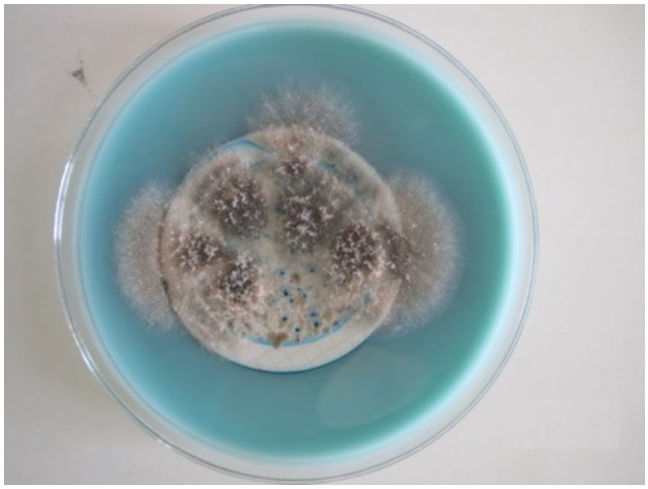

Figure 7: large presence of mold in sample S2F (veterinary pond).

The process was repeated until pure colonies were obtained. In the end, a total of 16 plates were obtained (**Table 1).**

Finally, a pure colony was picked from one media and transferred to the opposite media to preliminary test a possible cross-resistance.

Table 1: ESBL and CRE plates after purification. Where S is related to water samples and I to salad samples. Where, S1: Andedammen, S2 veterinary pond, S4: Årungen, S5: Pollevanet, S7: Pojana Maggiore, S8: Marostica and S10: Trolltunga. The letter F refers to the samples that were subjected to filtration. The following letters refer to the color of the colony picked: P = pink, W = white, G = green, R = red. Meanwhile the appendix was given when a phenotypic difference in color was visible (L = light and D = dark).

Water samples					
Medium	Sample plate	Cleaned plate			
ESBL	S1, S2W	S1FP	S1FP	S1W	S2W
	S1FW	No further growth			
	S2FW	No further growth			
	S4	No colonies			
	S4F	No colonies			
	S5	No colonies			
	S5F	No further growth			
	S7	No colonies			
	S7F	S7FW	S7FW	S7FW	S7FW
	S8	No colonies			
	S8F	No colonies			
	S10	No further growth			
	S10F	S10FR	S10FR	S10FR	S10FR
CRE	S1, S1F	S1G	S1G	S1FG	S1FG
	S2	S2R	S2R	S2R	S2R
	S2F	Mould			
	S4	No colonies			
	S4F	$S4FG_L$	$S4FG_L$	$S4FG_D$	$S4FG_D$
	S5	S5G	S5G	S5G	S5R
	S5F	Mould			

Medium	Sample plate	Cleaned plate			
CRE	S7	No colonies			
	S7F	$S7FG_L$	$S7FG_L$	$S7FG_D$	S7FW
	S8	S8W	S8W	S8W	S8W
	S8F	S8FG	S8FG	S8FW	S8FW
	S10	S10G	S10G	S10G	S10W
	S10F	S10FG	S10FG	S10FG	S10FG
Salad samples					
Medium	Sample plate	Cleaned plate			
ESBL	I1	No further growth			
	I1F	No further growth			
	I2, I2F, I3, I3F	I2W	I2FW	I3W	I3FW
CRE	I1	No further growth			
	I1F	I1FG	I1FG	I1FG	I1FG
	I2, I2F	I2R	I2G	I2G	I2FG
	I3, I3F	I3G	I3G	I3FW	I3FW

3.4 Identification of known antibiotic resistance genes

3.4.1 DNA extraction

DNA extraction was performed on a total of 28 colonies (**Table 2**) by using the GenElute™ Bacterial Genomic DNA Kit. Only one colony per colour was sampled in each plate with the exception being plate I2W; I2FW, I3W, I3WF where only I2W and I3W quarters were sampled. This decision was made since the filtered samples presumably contained the same strains present in the non-filtered samples.

Firstly, using a sterile inoculation loop, a pure colony was transferred into a 5 mL Eppendorf containing 1mL of Ringer solution. Each sample was then washed by vigorously mixing with a vortex until the cells were fully disperse in the solvent.

Samples were then centrifuged at 16.000 x g (m/s^2) for one minute. To fully remove any residual impurities, the cleaning step was carried out a second time. The cleaned pellet was then suspended in 180 µL of lysis T solution, a buffer solution that breaks the cells, Then, 20 µL of proteinase K enzyme were added. This serine protease cleaves the peptide bond adjacent to the carboxyl group of aliphatic or aromatic amino acids with amine group and helps the

purification of the nucleic acids by digesting contaminating proteins. After that, the samples were incubated at 55°C for 30 minutes.

After the incubation period, to maximize the later extraction, the cells were permeabilized by using 200 µL of lysis solution C, to assure a homogeneous mix, the solution was vortexed for about 10-15 seconds. The step was followed by another incubation at 55°C for 10 minutes.

During this incubation period, the columns for the next steps were prepared by adding 500 µL of column solution, a solution that helps to retain the DNA in the column, to a 2 mL tube containing the column. The tubes were then centrifuged at 12.000 G x g for 1 minute. After that, the eluate was discarded, and the column was placed back into the tube.

After incubation 200 µL of solution of 90% ethanol and 10% DNase free water was added to the samples and mixed with the vortex to dissolve any residual salts and minimizing DNA solubility. After that, all the volume present in the tube was transferred in the column and centrifuged at 6500 x g for 1 minute. The eluate was then discarded, and the column containing the DNA was placed again into a new collecting tube.

Subsequently, 500 µL of wash solution 1 was added and followed by centrifugation at 6500 x g for 1 minute. After discarding the eluate, 500 µL of washing solution 2 was added then, the samples were centrifuged at 12.000 x g for 3 minutes and the eluate was discarded. A 1-minute centrifugation was carried out to fully remove the wash solution.

The column was then placed in a new tube where 100 µL of elution solution was added. The samples were then incubated at room temperature for 5 min and subsequently centrifuged at 6500 x g for 1 minute. The eluate containing the DNA was stored in the freezer at -24°C until use.

Table 2: deriving plate and colony picked for DNA extraction.

Water samples					
Medium	Plate				Colony picked
ESBL	S1FP	S1FP	S1W	S2W	S1FP
	S1FP	S1FP	S1W	S2W	S1W
	S1FP	S1FP	S1W	S2W	S2W
	S7FW	S7FW	S7FW	S7FW	S7FW
	S10FR	S10FR	S10FR	S10FR	S10FR
CRE	S1G	S1G	S1FG	S1FG	S1G

Water samples					
Medium	Plate				Colony picked
CRE	S1G	S1G	S1FG	S1FG	S1FG
	S2R	S2R	S2R	S2R	S2R
CRE	$S4FG_L$	$S4FG_L$	$S4FG_D$	$S4FG_D$	$S4FG_L$
	$S4FG_L$	$S4FG_L$	$S4FG_D$	$S4FG_D$	$S4FG_D$
	S5G	S5G	S5G	S5R	S5G
	S5G	S5G	S5G	S5R	S5R
	S7FG	S7FG	S7FG	S7FW	S7FW
	$S7FG_L$	$S7FG_L$	$S7FG_D$	S7FW	$S7FG_L$
	$S7FG_L$	$S7FG_L$	$S7FG_D$	S7FW	$S7FG_D$
	S8W	S8W	S8W	S8W	S8W
	S8FG	S8FG	S8FW	S8FW	S8FW
	S8FG	S8FG	S8FW	S8FW	S8FG
	S10G	S10G	S10G	S10W	S10G
	S10G	S10G	S10G	S10W	S10W
	S10FG	S10FG	S10FG	S10FG	S10FG
Salad samples					
Medium	Plate				Colony picked
ESBL	I2W	I2W	I3W	I3W	I2W
	I2W	I2W	I3W	I3W	I3W
CRE	I1FG	I1FG	I1FG	I1FG	I1FG
	I2R	I2G	I2G	I2FG	I2R
	I2R	I2G	I2G	I2FG	I2G
	I3G	I3G	I3FW	I3FW	I3G
	I3G	I3G	I3FW	I3FW	I3FW

3.4.2 Polymerase chain reaction

The polymerase chain reaction (PCR) is a laboratory technique invented by Kary Mullis in 1983 that allows to reproduce thousands of copies of a given segment of DNA using proper primers. The procedure consists in three main phases, starting with the denaturation step, where the DNA is denaturized by using temperatures between 90-99°C. this causes separation

of the two helices of the DNA. The denaturation step is followed by an annealing step where the temperature is lowered to between 50 to 60°C, during this phase, the primer is paired with the RNA polymerase. Finally in the extension phase, the temperature is raised to 72°C to maximise the action of the DNA polymerase. This cycle is then repeated for a variable number of times.

To perform the amplification of the genes the Qiagen™ kit was used. The volumes needed for each reaction were the following:

- 2x Qiagen multiplex PCR master mix: 12,5 µL
- Primer mix 5 mMol: 2,5µL
- RNAse-free water: 9 µL
- Template DNA: 1 µL

In total, four different primer mixes and a control containing positive for bacteria DNA and positive for Enterobacteriaceae were used (**appendix 1).**

Each primer consisted in a solution of three known antibiotic-resistant genes often isolated in hospital environment. Every primer contained a front portion (F) and a rear portion (R).

The volume of genes in each primer mix solution was calculated using the following formula (1):

$$V_1 \, x \, C_1 = V_2 \; x \, C_2$$

Where: V_1 = volume 1; V_2 = volume 2; C_1 = concentration 1; C_2 = concentration 2

Solving the equation for V_1 (formula 1.1):

$$V_1 = \frac{2_{pmol/\mu L} \, x \, 200_{\mu L}}{100_{pmol/uL}} = \; 4_{\mu L}$$

Therefore, 4 µL of each primer needed for the mix was pipetted into an Eppendorf and diluted with RNAse-free water until a total volume of 200 µL was reached (**Table 3**).

The control was prepared by diluting 1 µL of positive for DNA and a 1 µL of positive for Enterobacteriaceae in 23 µL of RNAse-free water.

Table 3: example of volumes needed for the preparation of ESBL-1 primer mix solution.

Target gene	Primer sequence	Volume
blaCTX-m2	F- CGTTAACGGCACGATGAC	4 µL
	R- CGATATCGTTGGTGGTTCCAT	4 µL
blaOXA	F- GGCACCAGATTCAACTTTCAAG	4 µL
	R- GACCCCAAGTTTCCTGTAAGTG	4 µL
bla-SHV	F- AGCCGCTTGAGCAAATTAAAC	4 µL
	R- ATCCCGCAGATAAATCACCAC	4 µL
Total volume primers		24 µL
RNase-free water		176 µL
Total volume mix		200 µL

As for the DNA extraction, the PCR was carried out in different days, but the same protocol was followed.

Firstly, the master mix solutions were prepared according to the volumes previously described. Then, for each master mix 24 µL was dispensed into a 0,2 mL PCR tube. Lasty, 1 µL of DNA sample was added.

The samples where then run in the PCR with the program "*master ESBL*", contained in the "car" folder.

The Amplification conditions followed where the ones suggested by the constructor (**Table 4**).

Table 4: master ESBL program steps and time-temperature combinations.

Step	Time	Temperature
Initial heating	15 min	95 °C
Denaturation	30 s	94 °C
Annealing	90 s	62 °C
Extension	90 s	72
Number of cycles	30-45	
Final extension	10 min	72 °C
End	Forever	4 °C

3.4.3 Gel electrophoresis

Electrophoresis is an analytical technique capable of separating charged molecules in particular proteins, peptides but also nucleotides such as DNA and RNA. The operating principle behind electrophoresis is that negatively charged molecules move towards the positive pole with a velocity inversely proportional to their mass or their electric charge. However, since molecule with different mass and electric charge might present the same electrophoretic mobility only one of the two is used during an electrophoretic run, in this case, molecular mass.

The electrophoresis was carried out using agarose gel (1,5% agarose). In total eight gels were prepared as follows first, depending on the size of the mold, either 210 mL or 50 mL of 1x tris acetate-EDTA (TAE) buffer solution was measured in a graduated cylinder and then transferred in a clean Pyrex© bottle. After that, either 3,15 or 0,75 g of agarose powder were weighted and transferred in the bottle containing the 1x TAE solution.

To avoid breakage, the bottles were placed in the microwave with the lid not completely closed. The solution was then brought to a boil and left it boiling until the agarose completely dissolved, resulting in a clear solution.

After cooling the bottles under running water, Gelred® nucleic acid stain in a percentage equal to 5% (10,5 μL for 210mL gels and 2,5 μL for 50 mL gels) was added.

Lastly, the solution was poured into the mould and allowed to set for about 15-25 minutes depending on the size of the mould.

During the setting time, the samples were prepared by pipetting 2 μL of PCR product in a well which was previously added with 6 μL of RNAse-free water and 2 μL of dye solution.

Once set, the gel was transferred into the electrophoresis chamber, fully covered with 1x TAE buffer solution and the comb that shaped the wells was carefully removed. Then, each well was filled with 5 μL of the sample solution just described. To allow size comparison, 5 μL of 1kb DNA ladder solution was used as standard. To allow a better comparison the well chosen for the standard solution was the one in the middle as it is less sensible to convective motions. The electrophoresis was then run at 80 volts for one hour followed by 20 minutes more at 100 volts. Lastly, the gel was read under an ultraviolet (U.V.) light source.

3.5 Bacterial identification

The 16s DNA presents variable zones and some fixed zones typical of a given specie. As these zones are highly conserved since even a small mutation of said sequence is often fatal. Thanks to these fixed zones, the 16s DNA sequence can be used to identify bacteria (Janda & Abbott, 2007).

3.5.1 16s DNA extraction

The 16s DNA was extract using the Q5® protocol. According to the manufacturer's instructions a master mix solution containing the following reagents was needed for each reaction:

- 12,5 µL Q5© 5x High-fidelity DNA polymerase
- 1,25 µL primer 1F
- 1,25 µL primer 5R
- 9 µL RNAse-free water

The extraction was carried out on three different lots. Regardless of the lot to which a sample belonged 24 µL of master mix solution were pipetted into a 0,2 mL PCR tube. After that, 1 µL of DNA sample was added to the tube.

16s DNA amplification was performed with the PCR using the program "Q5" (**Table 5**) found in the "16s" folder.

Table 5: Q5 program steps, and time-temperature combinations.

Step	Time	temperature
Denaturation	30 s	94 °C
Annealing	90 s	60 °C
extension	90 s	72 °C
Number of cycles	35	
Final extension	10 s	72 °C
End	Forever	4 °C

3.5.2 Gel electrophoresis

To determine the presence of 16s DNA, the samples were subjected to gel electrophoresis. For both the gel and the run the same methods previously described in paragraph 3.3 were followed.

Correct extraction is confirmed by the presence of a band at around 1550 kDa when subjected to U.V. light.

3.5.3 Cleaning of PCR products

To quantify the amount of 16s DNA present in the samples the primers of the PCR (1F and 5R) needed to be removed. To do so GenElute^{tm} PCR Clean-Up kit was used. First 0,5 mL of column preparation solution was added to an Eppendorf tube containing the column.

The columns were then centrifuged at 12.000 x G for one minute. After that, the eluate was discarded, and the column was placed back in its tube. Afterwards, to allow the 16s DNA to stick to column, a volume of 115 μL of binding solution (equal to five times the remaining volume of the PCR product) was added in every sample well. The samples were then centrifuged at 12.000 x G for 1 minute and the eluate was again discarded. Subsequently, to remove any residue of binding solution, the samples were washed using 0,5 mL of wash solution. This step was again followed by a centrifugation at 12.000 x G for 3 minutes and the eluate was discarded. To remove the washing solution completely, the samples were centrifuged again at 12000 x G for 1 minute.

Lastly, 35 μL of elution solution was added to each well and incubated at room temperature for 5 minutes. The samples were finally eluted with a centrifugation at 12.000 x G for 1 minute. The eluate was stored at -24°C until use.

3.5.4 16s DNA quantification

The amount of DNA 16s present in the samples was measured using Qubit 2.0. kit® which uses a fluorometer to quantify the amount of DNA present in the sample. This technique uses the phenomenon of fluorescence in which certain substances, after being excited, emit a different radiation from the radiation used for the excitement and thus fluorescence is directly proportional to the amount of analyte, in this case 16s DNA, present in the sample.

First, the two standards solutions were prepared by pipetting 190 μL of buffer solution and 10 μL of standard solution (1 or 2) in two separate 0,5 μL Eppendorf.

The samples were prepared by pipetting 197 µL of buffer solution and 3 µL of cleaned 16s DNA sample into a 0,5 µL Eppendorf. Both the standard and the samples were briefly vortexed for around 10-15 seconds to ensure a homogenous mix.

To measure the concentration of 16s DNA, the dsDNA High Sensitivity program was used. Firstly, the calibration line was made by sequentially running the two standard solutions.

After the calibration of the fluorimeter all the samples were then run using the same program used to calibrate the system. The results (**Table 6**) were taken after using "calculate stock concentration" function to adjust the reading to the concentration of the 16s DNA present in each sample (3 µL).

Table 6: 16s DNA content in ng/µL in each water and salad samples.

Water samples								
sample	S1G	S1W	S1FG	S1FP	S2W	S2R	S5R	$S5R_G$
Concentration	8,20	8,27	9,67	10,6	14,2	5,08	7,87	8,53
Sample	$S4FG_D$	$S4FG_L$	$S7FW^E$	$S7FW^C$	$S7FG_D$	$S7FG_L$	S8W	S8FW
Concentration	7,2	0,183	31,7	25,1	23,3	22,7	36	31,8
Sample	S8FG	S10FR	S10G	S10W	S10FG			
Concentration	18,2	20,7	26,3	28,1	26,1			
Salad samples								
Sample	I1FG	I2W	I2R	I2G	I3W	I3G	I3FW	
Concentration	22,8	20,5	17,5	7,73	24,7	26,1	32,3	

3.5.5 Bacteria identification

Bacterial identification was entrusted to an external laboratory so before shipping, the samples were prepared according to the instructions provided by this laboratory.

Every Eppendorf tube, labelled with a univocal barcode, had to contain the following volumes:

- 5 µL 16s DNA containing approximately 10-20 ng/µL of DNA
- 5 µL Primer 5 µ/mol (1F or 5R)
- 5 µL RNAse-free water

Due to the low 16s DNA concentration in samples: S1G, S1W, S1FG, S2R, $S4F_D$, $S4FG_L$, S5R, $S5R_G$, and I2G the following volumes were measured instead:

- 7 µL 16s DNA
- 5 µL primer 5µ/mol
- 3 µL water

Meanwhile, to match the suggested concentration, the remaining samples were diluted with RNAse-free water (**Table 7**). After the dilution, the samples were prepared using the suggested volumes.

Table 7: dilution of each sample and final 16s DNA concentrations (ng/µL).

Water samples									
Sample	$S7FW^E$	$S7FW^C$	$S7FG_D$	$S7FG_L$	S8W	S8FW	S10G	S10W	S10FG
Sample volume (µL)	4	4	4	4	4	4	4	4	4
RNAse-free water (µL)	12	8	8	8	12	12	8	8	8
Final concentration (ng/µL)	10,56	12,55	11,65	11,35	12	10,6	13,15	14,05	13,05

Salad samples				
Sample	I1FG	I3W	I3G	I3FW
Sample volume (µL)	4	4	4	4
RNAse-free water (µL)	8	8	8	8
Final concentration (ng/µL)	11,4	12,35	13,05	16,25

The sequences returned from the laboratory were read using BioEdit (version 7.2), a software that allows to align biological sequences.

The first step was to load the biological sequences by simply uploading the files (.rasta format) inside the program. Then, the two coupled sequences with the opposite primers (1F and 5R) were merged using the function "cap contig assembly program" located in accessory application tab.

The resulting contig sequence that contained the coupled sequences of both primers was then copied using the "copy sequences to clipboard (fast format)" function. Lastly, each ".rasta" sequence was run on: https://blast.ncbi.nlm.nih.gov/Blast.cgi (**Table 8**).

Table 8: bacteria identified with 16s DNA sequence.

Water samples		
Sample	*Scientific name*	Percentual identity
S1G	*Caulobacter sp.*	99,85%
S1W	*Pseudomonas protegens*	99,51%
S1FG	*Caulobacter sp.*	99,56%
S1FP	*Pseudomonas reinekei*	99,51%
S2W	*Pseudomonas moraviensis*	99,65%
S2R	*Xanthomonas translucens pv. cerealis*	99,79%
S5R	*Caulobacter sp.*	99,49%
$S5R_G$	*Cohnella xylanilytica*	99,45%
$S4FG_D$	*Cohnella xylanilytica*	99,12%
$S4FG_L$	None	None
$S7FW^E$	*Pseudomonas chlororaphis subsp. aureofaciens*	99,01%
$S7FW^C$	*Sphingopyxis alaskensis RB2256*	100%
$S7FG_D$	*Caulobacter sp. SS14.14*	99,59%
$S7FG_L$	*Phenylobacterium sp. V7*	99,93%
S8W	*uncultured Acinetobacter sp.*	99,72%
S8FW	*uncultured Acinetobacter sp.*	99,65%
S8FG	*Brevundimonas sp. AbaT-2*	99,82%
S10FR	*Pseudomonas multiresinivorans*	99.91%
S10G	*Stenotrophomonas maltophilia*	99.65%
S10W	*Brevundimonas sp. AbaT-2*	99.23%
S10FG	*Stenotrophomonas sp.*	99.79%
Salad samples		
Sample	Scientific name	Percentual identity
I1FG	*Stenotrophomonas maltophilia*	99.82%

Salad samples		
Sample	Scientific name	Percentual identity
I2W	*Pseudomonas putida*	99.91%
I2R	*Herbaspirillum huttiense*	99.82%
I2G	None	None
I3W	*Pseudomonas corrugata*	99.74%
I3G	*Stenotrophomonas sp.*	100%
I3FW	*Acinetobacter calcoaceticus*	99.82%

3.6 Minimum Inhibitory Concentration (MIC) testing

MIC test is a quantitative method widely used for measuring the minimum concentration that inhibits bacterial growth (Annis & Craig, 2005).

In total, 16 samples were subjected to the MIC testing: SIW, S1FP, S2R, $S5R_G$, $S7FW^E$, $S7FW^C$, $S7FG_D$, $S7FG_L$, S8W, S8FW, S8FG, S10G, S10W, S10FG, I1FG and I2R. The samples S2W, $S4FG_D$, SF10R, I2W and IW3 were not subject to the MIC test since the same specie was already present in other samples.

For what concerns *Pseudomonas spp.*, usually, there is no statistically significant difference between strains. However, two samples coming from Norway water sources were tested to be later compared with the strain found in Italy.

An exception was made for the *Acinetobacter spp.* (S8F and S8FW) and Stenotrophomonas sp. (S10G, S10FG and I1FG) due to their relevance as emerging pathogens.

Meanwhile, samples S1G, S1FG, and S5R could not be tested because, at the time of testing, the bacteria were not vital anymore. In the end, sample $S4FG_L$ and I2G were not tested due to unsuccessful identification via 16s DNA.

In total, a panel of nine antibiotics which included six different classes was used: ampicillin, amoxicillin (with the addition of clavulanic acid), Penicillin G (Penicillins), cefotaxime (cephalosporines), meropenem (carbapenems), ciprofloxacin (fluoroquinolones), erythromycin (macrolides) and tetracycline (tetracyclines).

3.6.1 Antibiotics

3.6.1.1 β-lactams

B-lactams are one of the most common antibiotic classes used in both human and animals.

They usually inhibit the production of the proteins of the cell wall, therefore, depending on the drug used, the action can be either bacteriostatic or bactericidal.

B-lactams can be divided into four different categories:

- Penicillins
- Cephalosporins
- Monobactams
- Carbapenems

Penicillins can be further divided into three more groups:

- Natural: Penicillin G and penicillin V
- Anti-staphylococcal: methicillin, oxacillin and nafcillin, cloxacillin and dicloxacillin
- Extended spectrum: ampicillin, amoxicillin and met ampicillin.

Penicillin G is the forefather of antibiotics, its structure consists in a nucleus of *6-aminopenicillanic acid* ring, and one amino group (**Figure 8**).

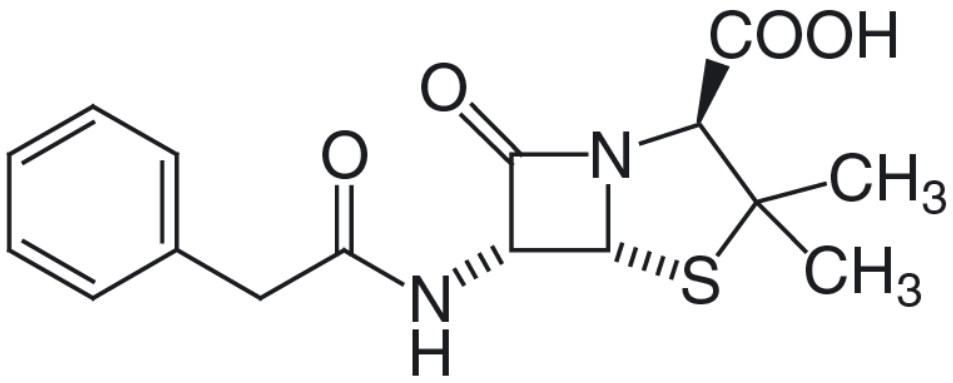

Figure 8: chemical structure of Penicillin G (Ola Sköld, 2011b).

Penicillin G is active only on gram-positive bacteria and on very few species of gram-negative bacteria (Nebbia Carlo, 2009a). The effect of penicillin G on gram – bacteria is limited because penicillin G acts by interfering with the synthesis of the bacterial cell wall during bacterial growth, thus the thicker layer of lipopolysaccharide makes it almost impossible for this drug to actively penetrate the cell wall. Besides antibiotic resistance, a big downside of penicillin G comes from its way of administration. Since this molecule is sensitive to low pH, it cannot be administered orally as it would be degraded in the stomach thus, it needs to be administered parenterally (Ola Sköld, 2011b).

Therefore, given the limits of penicillin G itself and the wide presence of resistant bacteria, the side chain of the native molecule has been modified to obtain a new semi-synthetic molecule: ampicillin (Etebu Ebimieowei & Ibemologi Arikekpar, 2016). This molecule was acid stable and was not affected by the enzymatic activity of penicillin-degrading enzymes and presented a much higher activity against gram – bacteria at the cost of their activity on some gram + cocci. The addition of a hydroxyl group in the side chain gave as result another semi-synthetic β-lactam, amoxicillin. We can think of this drug as a sister of ampicillin as it shares with this molecule most of the characteristics with the main difference being that amoxicillin is rapidly absorbed in the gastrointestinal tract hence it does not interfere with the microbiota present in the colon. Sometimes, amoxicillin is coupled with clavulanic acid, a non-antibiotic compound that inhibits some B-lactamase enzymes therefore, prolonging the antibacterial activity of the antibiotic.

Another major member of β-lactams are cephalosporins, this class of molecules is closely linked with penicillins, with the difference being the bacteria they are isolated from. Like the later generation of penicillins, cephalosporins resist most β-lactamases and are widely used to treat infection carried out by gram-negative bacteria. Most cephalosporins must be administered parentally, but there are some exceptions to the rule such as cephalexin and cefixime that can be administered orally (Ola Sköld, 2011b). The main difference in structure between penicillin G and cephalosporins is that the nucleus consists of a molecule of *7-aminocephalosporanic acid* which is more stable if compared to the *6-aminopenicillanic acid* contained in penicillin G. Cephalosporins can be further divided into four generations (Nebbia Carlo, 2009a). As for penicillins, cephalosporins interfere with the bacterial cell wall during growth.

Lastly, carbapenems, are the last generation of B-lactams and possess a large activity spectrum. The very first carbapenem discovered is thienamycin which was isolated from *streptomyces cattleya* but since it decomposes in water its use as a drug is practically impossible. To solve the problems of thienamycin new semisynthetic molecules were produced: imipenem and carbapenem. The first one can only be administered parentally but since it is sensible to an enzyme, the dihydropeptidase must be combined with the inhibitor cilastin. Carbapenem on the other hand is resistant to renal dihydropeptidase hence it does not need to be combined with the inhibitor. Contrary to the other β-lactams, the way of administration is not that relevant since both these drugs are used as a last resort when it comes to antibiotic treatments (Ola Sköld, 2011b).

3.6.1.2 Quinolones

Quinolones are a family of synthetic antimicrobials discovered in the early 1960s, and the first quinolone discovered was the Nalidixic acid. They are a cheap and efficient antibiotic drug and are commonly used in urinary infections carried out by gram – bacteria where they exert a bactericidal activity as they interfere with the DNA during the replication by inhibiting the DNA gyrase, an enzyme responsible for compacting the double strand of bacterial DNA. Quinolones can be further divided into three different generations, the 3rd of which contains the fluoroquinolones whose chemical structure differs from quinolones for the addition of a *6-fluoro* and *5-piperidine* groups. From this addition several fluoroquinolones were discovered, such as ciprofloxacin, norfloxacin, ofloxacin, levofloxacin and moxifloxacin. Of these, ciprofloxacin represents not only the most used fluoroquinolone, but it is one of the most used antimicrobial agents in the world (Ola Sköld, 2011c). It is commonly used to treat primary and secondary pathologies, of systemic nature, such as septicemias or respiratory tract infections carried out by both gram-negative and gram-positive bacteria, also, ciprofloxacin is the only oral alternative for *Pseudomonas* aeruginosa infections. The mode of action is the same described for quinolones (Kocsis et al., 2016).

However, the efficiency of ciprofloxacin is limited to the presence of resistant strains, which also include some that are pathogens for the human. The resistance to this drug can come from a spontaneous mutation of the target of ciprofloxacin, the production of efflux pumps or even from a specific plasmid (Ola Sköld, 2011c).

3.6.1.3 Macrolides

Macrolides are a family of natural or semi-synthetic antibiotics chemically characterized by the presence of a macrolide lactonic nucleus to which an amino sugar is linked via a glycosidic bond. Macrolides can be divided according to origin into natural, obtained by fermentation from mycetes of the genus streptomyces and, semi-synthetic.

Erythromycin is one of the first six natural macrolides synthesized. Erythromycin is more effective against gram-positive bacteria. Most gram-negative bacteria are resistant to erythromycin thanks to their bacterial wall.

Erythromycin interferes with the ribosomal site during the amino acids transfer from amino-acyl soluble ribonucleic acid to protein (Griffith & Black, 1970).

3.6.1.4 Tetracycline

Tetracyclines constitute a family of amphoteric molecules with a common tetracycline nucleus composed of four hexa-atomic rings with several substitutions in position C5, C6 or C7.

These antibiotics are produced by different species of *Streptomyces* and can be divided into natural or semi-synthetic (Odore & Badino, 2009). Tetracyclines exert their bacteriostatic action by inhibiting protein synthesis as they bind with the 70S ribosome making it impossible for the amino acid that carries the tRNA molecule to occupy its site (Ola Sköld, 2011c). They are also able to chelate certain bivalent cations (like Ca^{++}) indirectly causing the inhibition of several bacterial enzyme systems. These antibiotics are effective on both gram-positive and gram-negative bacteria either aerobic or anaerobic.

Tetracyclines like tetracycline are used in almost every localized or systemic bacterial infection (Odore & Badino, 2009).

3.6.2 Sample preparation

Before performing MIC test, a pure colony from the same plate used for the 16s DNA extraction was picked and inoculated into a Muller-Hilton (MH) agar plate and incubated for 24 hours. Then a colony was directly taken from the plate and inoculated into a test tube containing 5 mL of sterile brain heart infusion (BHI) broth. The samples were then incubated at 37 °C for 24 to 48 hours until they reached a concentration of about 0,5 McFarland (±1 log), a standard method that correlates the turbidity of a bacterial suspension to the number of bacteria present in the sample (0,5 McFarland= $1,5x10^8$).

To determine the bacterial concentration, 1 mL of BHI broth was diluted in 9 mL of Ringer solution. Then, 10 µL of diluted BHI broth was pipetted into a microscope slide that contained a counting chamber (**Figure 10**).

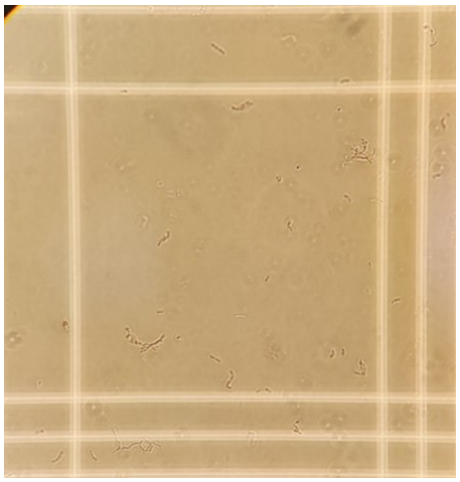

Figure 10: counting chamber of sample S8W.

The bacteria present in a random chamber were then counted under a microscope at a 40x magnification. The estimated number of bacteria was obtained with the following (**formula 2**):

$$[(n\ x\ 10)x\ 160]x\ 10^3$$

Where n is the number of bacteria counted (**Table 9**).

Table 9, counted and estimated number of bacteria present in the samples.

Sample	Bacteria	Number of bacteria counted	Estimated bacteria concentration (CFU/mL)
S1W	*Pseudomonas protegens*	31	$4{,}8x10^7$
S1FP	*Pseudomonas reinekei*	27	$4{,}32x10^7$
S2R	*Xanthomonas translucens pv. cerealis*	13	$2.08x10^7$
$S5R_G$	*Cohnella xylanilytica*	10	$1{,}6x10^7$
$S7FW^E$	*Pseudomonas chlororaphis subsp. aureofaciens*	28	$4{,}48x10^7$
$S7FW^C$	*Sphingopyxis alaskensis RB2256*	12	$1{,}92x10^7$
$S7FG_D$	*Caulobacter sp. SS14.14*	85	$1{,}44x10^7$
$S7FG_L$	*Phenylobacterium sp. V7*	12	$1{,}92x10^7$
S8W	*uncultured Acinetobacter sp.*	16	$2{,}56x10^7$
S8FW	*uncultured Acinetobacter sp.*	23	$3{,}68x10^7$
S8FG	*Brevundimonas sp. AbaT-2*	15	$2{,}4x10^7$
S10W	*Brevundimonas sp. AbaT-2*	25	$4x10^7$
S10G	*Stenotrophomonas maltophilia*	19	$30{,}4x10^7$
S10FG	*Stenotrophomonas sp.*	33	$5{,}28x10^7$
I1FG	*Stenotrophomonas maltophilia*	40	$6{,}4x10^7$
I2R	*Herbaspirillum huttiense*	127	$2{,}032x10^8$

After the count, a sterile swab was directly dipped into the BHI broth suspension, squeezed against the side of the tube to remove excess fluid, and then swabbed onto a MH agar plate. Particular attention was paid to ensure that the entire surface of the plate was evenly covered.

The plate was then rotated at 90° and swabbed again. Lastly, a 3rd layer was swabbed after a rotation of 45°. Afterwards, a gradient antibiotic strip either from ETEST© or Liofichem© was carefully placed into the plates by using sterile tweezers. The procedure was carried out two times for each antibiotic.

The plates were then incubated at 37 °C for 24 hours. After the incubation, the plates results were read by looking where the symmetrical inhibition ellipse that was eventually formed touched the strip (**Figure 11**). The final value was obtained by simply using the arithmetic mean (**Table 12**).

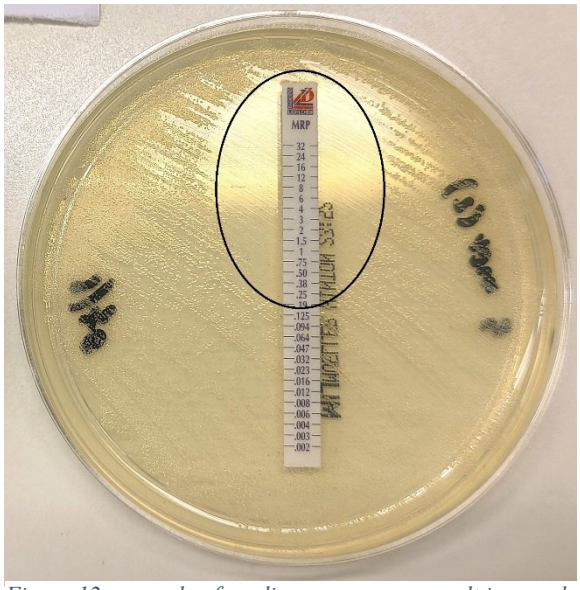

Figure 12: example of reading meropenem result in sample S8FW where the minimum inhibitory concentration is 0,19 mg/L.

Table 10: single MIC testing results for each antibiotic (top) and arithmetic mean (bottom) and where N.T. stands for "not tested".

Sample	Antibiotic (mg/L)																	
S1W	Ampicillin		Amoxicillin		Penicillin G		Cefepime		Cefotaxime		Meropenem		Ciprofloxacin		Erythromycin		Tetracycline	
	>256	>256	>256	>256	N.T.	N.T.	3	3	>256	>256	<0,002	<0,002	0,125	0,125	>256	>256	12	8
	>256		>256		N.T.		3		>256		<0,002		0,125		>256		10	
S1FP	Ampicillin		Amoxicillin		Penicillin G		Cefepime		Cefotaxime		Meropenem		Ciprofloxacin		Erythromycin		Tetracycline	
	>256	>256	>256	>256	N.T.	N.T.	3	3	>256	>256	<0,002	<0,002	0,74	0,64	>256	>256	4	3
	>256		>256		N.T.		3		>256		<0,002		0,69		>256		3,5	
S2R	Ampicillin		Amoxicillin		Penicillin G		Cefepime		Cefotaxime		Meropenem		Ciprofloxacin		Erythromycin		Tetracycline	
	3	8	>256	>256	N.T.	N.T.	0,5	0,75	4	4	<0,002	<0,002	0,5	0,75	2	2	1,9	1,9
	5,5		>256		N.T.		0,625		4		<0,002		0,625		2		1,9	
S5R_G	Ampicillin		Amoxicillin		Penicillin G		Cefepime		Cefotaxime		Meropenem		Ciprofloxacin		Erythromycin		Tetracycline	
	4	3	N.T.	N.T.	8	12	N.T.	N.T.	0	0	0,38	0,38	0,5	0,38	18	16	>256	>256
	3,5		N.T.		10		N.T.		0		0,38		0,44		17		>256	

sample	Antibiotic (mg/L)																	
S7F	Ampicillin		Amoxicillin		Penicillin G		Cefepime		Cefotaxime		Meropenem		Ciprofloxacin		Erythromycin		Tetracycline	
	>256	>256	>256	>256	>256	>256	6	6	>256	>256	>8	>8	0,125	0,125	>256	>256	8	12
W^E	>256		>256		>256		6		>256		>8		0,125		>256		10	
S7F	Ampicillin		Amoxicillin		Penicillin G		Cefepime		Cefotaxime		Meropenem		Ciprofloxacin		Erythromycin		Tetracycline	
	0,125	0,94	N.T.	N.T.	0,64	0,94	N.T.	N.T.	>256	>256	0,47	0,64	0,75	1	12	16	0,75	1
W^C	0,5325		N.T.		0,79		N.T.		>256		0,555		0,875		14		0,875	
S7F	Ampicillin		Amoxicillin		Penicillin G		Cefepime		Cefotaxime		Meropenem		Ciprofloxacin		Erythromycin		Tetracycline	
	0,94	0,94	N.T.	N.T.	0,64	0,64	N.T.	N.T.	0,5	0,5	0,094	0,125	0,75	0,75	1	1	0,25	0,38
G_D	0,94		N.T.		0,64		N.T.		0,5		0,1095		0,75		1		0,315	
S7F	Ampicillin		Amoxicillin		Penicillin G		Cefepime		Cefotaxime		Meropenem		Ciprofloxacin		Erythromycin		Tetracycline	
	>256	>256	N.T.	N.T.	>256	>256	N.T.	N.T.	>256	>256	3	2,5	>32	>32	8	12	0,2	0,5
G_L	>256		N.T.		>256		N.T.		>256		2,75		>32		10		0,375	

Sample	Antibiotic (mg/L)																	
S 8 W	Ampicillin		Amoxicillin		Penicillin G		Cefepime		Cefotaxime		Meropenem		Ciprofloxacin		Erythromycin		Tetracycline	
	0,5	0,75	>256	>256	6	8	8	6	1,5	1	0,12	0,64	0,5	0,38	1	0.75	>256	>256
	0,625		>256		7		7		1,25		0,38		0,44		0,875		>256	
S 8 F W	Ampicillin		Amoxicillin		Penicillin G		Cefepime		Cefotaxime		Meropenem		Ciprofloxacin		Erythromycin		Tetracycline	
	6,5	6	0,47	0,47	12	12	0,4	0,19	0,5	0,38	0,25	0,19	>32	>32	12	8	0,25	0,25
	6,25		0,47		12		0,295		0,44		0,22		>32		10		0,25	
S 8 F G	Ampicillin		Amoxicillin		Penicillin G		Cefepime		Cefotaxime		Meropenem		Ciprofloxacin		Erythromycin		Tetracycline	
	0,35	0,38	N.T.	N.T.	0,5	0,5	N.T.	N.T.	0,75	0,75	0,64	0,47	0,47	0,47	>256	>256	12	12
	0,364		N.T.		0,5		N.T.		0,75		0,555		0,47		>256		12	
S 10 W	Ampicillin		Amoxicillin		Penicillin G		Cefepime		Cefotaxime		Meropenem		Ciprofloxacin		Erythromycin		Tetracycline	
	1	1	0,5	0,5	2	6	0,38	0,38	0,75	1,5	0,19	0,38	1	1,5	>256	>256	2	6
	1		0,5		4		0,38		0,125		0,285		1,25		>256		4	

Sample	Antibiotic (mg/L)																	
S10G	Ampicillin		Amoxicillin		Penicillin G		Cefepime		Cefotaxime		Meropenem		Ciprofloxacin		Erythromycin		Tetracycline	
	>256	>256	64	64	>256	>256	1,5	2	24	16	0,125	0,2	0,5	0,5	>256	>256	3	3
	>256		64		>256		1,75		20		0,1625		0,5		>256		3	
S10FG	Ampicillin		Amoxicillin		Penicillin G		Cefepime		Cefotaxime		Meropenem		Ciprofloxacin		Erythromycin		Tetracycline	
	>256	>256	24	32	>256	>256	6	6	16	16	>32	>32	0,94	0,94	32	48	4	3
	>256		28		>256		6		16		>32		0,94		40		3,5	
I1FG	Ampicillin		Amoxicillin		Penicillin G		Cefepime		Cefotaxime		Meropenem		Ciprofloxacin		Erythromycin		Tetracycline	
	0,75	4	12	6	12	12	1,5	1,5	0,75	1	2	6	>32	>32	>256	>256	6	6
	2,25		9		12		1,5		0,875		4		>32		>256		6	
I2R	Ampicillin		Amoxicillin		Penicillin G		Cefepime		Cefotaxime		Meropenem		Ciprofloxacin		Erythromycin		Tetracycline	
	16	16	0,5	0,75	>256	>256	0,47	0,64	0,25	0,19	0,47	0,64	0,75	1	8	6	0,25	0,25
	16		0,625		>256		0,555		0,22		0,555		0,875		7		0,25	

3.7 Full genome sequencing

To identify the antibiotics resistant genes, four (S2R, S7FW^{E}, S8FW, and I1FG) of the 11 MIC tested samples were also subjected to a full genome sequencing.

The procedure was carried out from an external laboratory using Illumina sequencing technique.

The results have been processed with the online platform usegalaxy (Https://Usegalaxy.Eu/, 2022.).

Firstly, the sequences (".fasta" format) were run with *Trimmomatic* (version 0.38) a tool that cleans up the sequences to prepare them for the reading (Bolger et al., 2014). The two resulting sequences were then merged using the *Shovill* tool (version 1.10) (seemann, 2017). Lastly, using a tool named *Abricate* (version 1.0.1) the merged sequences were run through four different databases:

1) Comprehensive antibiotic resistance database (CARD)
2) Plasmid finder
3) Virulence factor database (VFDB)

CARD contains molecular references for antibiotic resistant genes. Plasmid finder, on the other hand is a database whose library contains antibiotic resistant plasmids.

Lastly, VFBD library contains virulence factors of pathogen bacteria (Carattoli et al., 2014; Chen et al., 2016; Feldgarden et al., 2019; Jia et al., 2017).

4 RESULTS AND DISCUSSION

4.1 Bacterial isolation

The first results showed that the samples that were subjected to filtration gave better results in terms of quantity and variety of colonies. The same can be said for the medium, as in water samples, CRE agar showed the presence of 14 colonies with colours other than white which is usually related to *Pseudomonas spp*. Moreover, the two white colonies (S8W and S8FW) were found to be a strain of *Acinetobacter*. Meanwhile on ESBL agar, only two samples (S1FP and S10FR) presented a non-white colour, anyway, both samples were later identified *Pseudomonas reinekei* and *Pseudomonas multiresinovorans* respectively thus presenting only strain of *Pseudomonas*. Comparable results were given by salad samples with ESBL agar presenting a lower number of colonies all of which were white and later identified as *Pseudomonas*. Meanwhile on CRE agar not only a larger number of bacteria grew, but also the only white colony were later identified again as a strain of *Acinetobacter*.

As previously stated, moulds were also found in the CRE agar filtered samples (S2F and S5F). These moulds presented a high resistance to this selective media, but since the project focuses exclusively on bacteria, no further investigations were carried out.

Regarding, the cross-resistance test, no cross-resistance was found in both Norwegian and Italian samples.

4.2 Presence of known antibiotic resistant genes

The reading of the PCR products via electrophoresis did not show any band at all, besides the control, in both Norwegian and Italian samples. Therefore, it can be concluded that the resistance comes from different genes that are not closely related to the ones present in the primer mix or the resistance could also be linked to one of the other systems of transferrable resistance, such as plasmids, transposons or integrons, previously described in chapter 1.2.2.

4.3 Bacterial identification

4.3.1 Pseudomonas spp.

Pseudomonas is a rod-shaped, gram-negative, aerobic, non-fermenting and ubiquitous bacterium commonly found in soil and water. Almost all strains are motile by means of a single or flagellum or peritrichous flagella.

Some strains of *Pseudomonas* such as *Pseudomonas aeruginosa* are known to be opportunistic pathogens for humans. Infections generally occur in immunocompromised patients rather than healthy subjects. *Pseudomonas* related infections generally take place in the lungs causing pneumonia that can ultimately result in death (Palleroni, 1993; Pang et al., 2019). The Center for Disease Control and Prevention (CDC) estimated that in the United States in 2017 out of a total of 32.600 infections of which, 2700 resulted in death (Centers for Disease Control and Prevention, 2021). The treatment via antibiotic of *Pseudomonas* infections is challenging due to its ability to avoid the action of several classes of antibiotics such as β-lactams, quinolones, and aminoglycosides (Pang et al., 2019). For these reasons the World Health Organization (WHO) listed carbapenem-resistant strains of *Pseudomonas* in the top priority (Priority 1: critical) list for the research and development of new antibiotics.

4.3.2 Caulobacter spp.

Caulobacter spp. is a rod-shaped, gram-negative strict aerobic bacterium.

It is commonly found in surface waters like ponds, streams, or seas but it can be also found in tap water or soil (Stove Poindexter', 1964).

In 2005, *Caulobacter* was associated with a nosocomial infection in a sixty-four-year-old patient. The clinical history of the man reported hypertension, smoking, and a chronic renal insufficiency. The man was hospitalized with abdominal pain as the only symptom. After a 21-days cycle of intraperitoneal gentamicin, the man made a full recovery (Justesen et al., 2007).

Thus, the presence of *Caulobacter* in water samples is not surprising and its role as an opportunistic pathogen for humans is not very relevant. Although, the presence of three antibiotic-resistant strains, coming from three completely different sources (S1FG, S5R, $S7FG_D$) might be worthy of attention.

4.3.3 Xanthomonas spp.

Xanthomonas spp. is a rod-shaped, gram-negative, strict aerobic, motile by a single polar flagellum bacterium.

Different strains of *Xanthomonas spp.,* including *Xanthomonas translucens pv. cerealis*, are known to be a plant pathogen.

Xanthomonas translucens pv. cerealis is the leading cause of bacterial leaf streak (BLS) in small grains cereals like wheat, rye or barley that are grown in warm and humid climates (Bragard et al., 1997).

Despite the importance of *Xanthomonas* a plant pathogen, the same cannot be said for humans since in scientific literature no cases of human diseases have ever been reported.

4.3.4 Cohnella xylanilytica

Cohnella xylanilytica is a rod-shaped, gram positive, facultatively aerobic (usually the other strains are obligated aerobic), motile by peritrichous flagella and spore-forming (Khianngam et al., 2010). it was firstly isolated from soil in Thailand, and it is considered a novel specie as it differs from all the other species of the genus for both its physiological and biochemical characteristics and DNA-DNA hybridization.

No further studies were carried out on this specific strain and as for the main specie *Cohnella thermotolerans*, no cases of human diseases related to the presence of this bacterium have ever been reported in scientific literature.

It is worth noticing though that its ability to form spores could play an important role in the transfer of its antibiotic-resistance to other bacteria in food products even if they were subjected to a heat treatment such as pasteurization.

4.3.5 Sphingopyxis alaskensis

Sphingopyxis alaskensis is a rod-shaped, gram negative, obligated aerobic, ultramicrobacteria that was firstly found in the see water of Alaska (Cavicchioli et al., 2003).

S. alaskensis is naturally resistant to many chemical agents like PH stress, hydrogen peroxide and ethanol and physic agents like sonication, heat, and UV (Eguchi et al., 1996; Joux et al., 1999).

Cavicchioli *et al* (2003), report that *Sphingopyxis alaskensis* is inherently resistant to some antibiotics like ampicillin, gentamycin, streptomycin, and tetracycline, making it a multi-drug resistant bacterium.

In conclusion, the presence of *Sphingopyxis alaskensis* in water sources might be worrisome due to both its high resistance to antibiotics and its inherent resistance to chemical and physical agents.

4.3.6 Phenylobacterium spp

Phenylobacterium spp is a rod-shaped, gram negative, aerobic, immobile, non-spore forming bacteria that belongs to the family of *Caulobacteriaceae* (Baek et al., 2019).

This bacterium is commonly found in fresh water, soil, and human blood (Abraham et al., 2008).

A recent study conducted by Li et al., (2021), tested four different antibiotics: sulfamethoxazole, chlortetracycline, ciprofloxacin, and amoxicillin. The results show a high resistance to ciprofloxacin.

As for *Cohnella xylanitica* and *Sphingopyxis alaskensis*, in scientific literature there are no reported cases of human disease.

4.3.7 Acinetobacter spp.

Acinetobacter spp. is a coccobacillus, gram-negative, aerobic non fermenting, and ubiquitous bacterium (Visca et al., 2011).

Great attention has been paid to this bacterium as the cases associated with nosocomial infections are rapidly increasing. As for Pseudomonas, the concern is so high that the WHO listed carbapenem-resistant *Acinetobacter baumannii* in the highest priority of the list for research and development of new antibiotics (Tacconelli et al., 2018).

baumannii infections usually involve the respiratory system, causing pneumonia in intensive care unit (ICU) patients. More rarely it can cause catheter-related infections and endocarditis. The reason why carbapenem-resistant *A. baumannii* infections are worrisome is due to the lack of treatment options because of a lack of data on them (Bartal et al., 2022).

However, contrary to common belief, *A. baumannii* is very rarely isolated outside the hospital environment. The main species present in the environment or in food samples like vegetables, RTE salads, meat, or milk, are *A. calcoaceticus*, *A. lowolfi*, *A. junii*, and *A. johnsonii* (Atrouni et al., 2016).

Even tough, *A. baumannii* plays a major role when it comes to nosocomial infections, there are some reported cases of Pneumonia associated with *A. calcoaceticus* (Mostachio et al., 2012).

In conclusion, even though the presence of *Acinetobacter* in water samples is rather common, it should be taken into consideration as a possible source of infection in debilitated subjects and moreover if it is a carbapenem-resistant strain, since that resistance might be transferred to other bacteria.

4.3.8 Brevundimonas spp.

Brevundimonas is a bacillus, gram-negative, aerobic, non-fermenting and motile bacterium largely found in water and soil (Liu et al., 2021).

Many strains of this bacteria have been associated with nosocomial infections, with the most common strains being *Brevundimonas diminuta and Brevundimonas vesicularis*.

Some strains have also been found to be resistant to ampicillin, ciprofloxacin and more importantly to carbapenems like meropenem and imipenem (Ryan & Pembroke, 2018).
Brevundimonas spp. infections commonly cause bacteriemia and in severe cases it can cause meningitis. Usually, the course is benign but there are reported cases of death due to *Brevundimonas* infections (Papaefstathiou et al., 2005; Zhang et al., 2012).
To conclude, the role of *Brevundimonas* as an opportunistic pathogen should be taken into consideration especially when it comes to immunocompromises patients.

4.3.9 Herbaspirillum spp.

Herbaspirillum is a bacillus, gram-negative, aerobic, non-fermenting bacteria commonly found in the environment in both soil and water sources.
Even though this bacterium plays a role in plant biological growth as it fixates the nitrogen, recently it started a sort of transition in favor to human where it acts like an opportunistic pathogen bacterium.
There are cases in literature of cystic fibrosis caused by *Herbaspirillum huttiense* in both immunocompromised and non-immunocompromised patients. Regardless of the health status of the subject, all the cases resolve without complications after antibiotic treatment (Dhital et al., 2020).

4.3.10 Stenotrophomonas spp.

Stenotrophomonas is a rod-shaped, gram-negative, obligatory aerobe, motile by means of polar flagella.
Initially, these bacteria were firstly named *Pseudomonas maltophila*; later, thanks to rRNA cistron analysis, were named *Xanthomonas maltophila.* Recently, with DNA-rRNA hybridization technology, the *Stenotrophomonas* genus was found. Up to date, this genus consists in four different species where only *Stenotrophomonas maltophila* is known to be an opportunistic pathogen for human beings (Looney et al., 2009).
Even though *Stenotrophomonas maltophila* is not considered a highly virulent pathogen, its importance is growing as more precise methods of identification are becoming available.
This bacterium was often found in hospital environments as a contaminant of different medical devices such as catheters, which are thought to be the main vehicle to urinary infections, disinfectants, and sterile water.
Stenotrophomonas maltophila infections are more common in severely immunocompromised patients that were subjected to broad-spectrum antimicrobials, including carbapenems,

cephalosporins, and quinolone. The infection usually causes pneumonia that can be followed by blood-stream infection.

Clinical outbreak of *S. maltophila* pneumonia does not show specific symptoms, most of the patients present with fever, cough, and dyspnea, that make it difficult to early identify a *S. maltophila* infection (Brooke, 2012).

4.4 Antibiotic susceptibility testing

To be considered multidrug resistant, a bacterium should show a phenotypic resistance to at least three different classes of antibiotics.

To establish the MIC breaking points (**Table 11**), the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guide was used. (*"The European Committee on Antimicrobial Susceptibility Testing. Breakpoint Tables for Interpretation of MICs and Zone Diameter. Version 12.0*, 2022).

Table 11 MIC breakpoints for Enterobacteriaceae, Pseudomonas non aeruginosa and Acinetobacter. Where N.W.T stands for "not worth testing" and IE for "insufficient evidence". (Adapted from "The European Committee on Antimicrobial Susceptibility Testing. Breakpoint Tables for Interpretation of MICs and Zone Diameter. Version 12.0, 2022).

Antibiotic	*Pseudomonas non aeruginosa*		*Acinetobacter*		Non-species related	
	MIC breakpoints (mg/L)		MIC breakpoints (mg/L)		MIC breakpoints (mg/L)	
	Nonresistant ≤	Resistant >	Nonresistant ≤	Resistant >	Nonresistant ≤	Resistant >
Ampicillin	N.W.T.	N.W.T.	N.W.T.	N.W.T.	2	8
Amoxicillin	N.W.T.	N.W.T.	N.W.T.	N.W.T.	2	8
Penicillin G[1]	N.W.T.	N.W.T.	N.W.T.	N.W.T.	2	8
Cefepime	0,001	8	N.W.T.	N.W.T.	4	8
Cefotaxime	N.W.T.	N.W.T.	N.W.T.	N.W.T.	1	2
Meropenem	0,001	4	2	8	2	8

[1] No MIC breakpoints were found in literature, so the same breakpoints for ampicillin and amoxicillin were observed.

Antibiotic	*Pseudomonas non aeruginosa*		*Acinetobacter*		Non-species related	
	MIC breakpoints (mg/L)		MIC breakpoints (mg/L)		MIC breakpoints (mg/L)	
	Nonresistant ≤	Resistant >	Nonresistant ≤	Resistant >	Nonresistant ≤	Resistant >
Ciprofloxacin	0,002	0,5	0,002	2	0,25	0,5
Erythromycin	N.W.T.	N.W.T.	N.W.T.	N.W.T.	IE	IE
Tetracycline	N.W.T.	N.W.T.	N.W.T.	N.W.T.	IE	IE

4.4.1 Pseudomonas non aeruginosa

Three out of 16 samples fell within the *Pseudomonas non aeruginosa* family (S1W, S1FP, $S7FW^{E}$). As expected, all the strains were found to be multi-drug resistant. Almost every strain was significantly resistant to the antibiotics that, according to the table, were not worth testing with the exception being sample S1FP which was not significantly resistant to tetracycline but was found be highly resistant to ciprofloxacin. Also, sample $S7FW^{E}$ was found to be completely resistant to carbapenem.

When compared, no significant difference in terms of levels of resistance between the strain sample in Norway and the ones sampled in Italy was found, with the exception being again sample $S7FW^{E}$ which is the only strain highly resistant to carbapenems and its presence in the environment of a gene that confers resistance to carbapenem is rather worrisome.

4.4.2 Acinetobacter

Regarding Acinetobacter, neither of the two samples were found to be resistant to carbapenem thus, reinforcing the hypothesis that the resistance to this class of antibiotics is still almost entirely limited to the hospital environments. Of the two samples only sample S8FW turned out to be multidrug-resistant. Also, the two samples gave contrasting results with different classes of antibiotics. For example, sample S8W presented at least some sort of resistance to cefepime and tetracycline, meanwhile sample S8FW was found to be much more resistant to ciprofloxacin and erythromycin.

These inconsistencies might be a sign of a strain-specific difference suggesting that different strains might have developed different mechanisms to avoid the antibiotic. However, these

differences are interesting only when ciprofloxacin or meropenem are considered, since the other classes of antibiotics are not worth testing according to the European MIC testing guide.

4.4.3 Non-species related

The remaining 11 samples fell into the non-species related category. Out of these, six samples (S2R, S7FW^{C}, S7FG$_{L}$, S10G, S10FG, and I1FG) [2] were found to be multidrug-resistant.

Penicillins still show some effectiveness, among the samples, with the exception being samples S2R, S7FG$_{L}$, S10G, S10FG, I1FG, and I2R. It should be noted though that samples S10G, S10W, S10FG, I1FG, and I2R are not technically *Pseudomonas* but they do belong to the same phylum: *Pseudomonadota* or *proteobacteria*. So, if the results were to be compared with the limits in the *Pseudomonas non aeruginosa* group, they would all be considered completely resistant to penicillins. Also, sample I1FG would be considered completely resistant to meropenem.

As for S2R, it was found to be significantly resistant not only to Penicillins, but also to cefotaxime and ciprofloxacin.

About meropenem, sample S10FG seems to be completely resistant meanwhile sample I1FG shows some sort of resistance. These results are no surprise since both samples are a strain of *Stenotrophomonas maltophila* which is known to be multidrug-resistant, and cases of resistance to meropenems are reported in literature.

Another point to note is that resistance to ciprofloxacin is very common, with only three samples (S5RG, S8FG, and S10G) being only partially resistant to this drug. These results should be considered since ciprofloxacin is one of the only drugs (the other one being meropenem), that is always considered worth testing.

Lastly, the results show that erythromycin turned out to be the least effective antibiotic[3] with eight samples out of 10 presenting a high resistant in the non-species related category and 12 out of 16 in general.

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[2] Even though sample S5RG is resistant to three antibiotics (penicillin G, erythromycin, and tetracycline), is not considered to be multidrug-resistant since penicillin G is a first-generation cephalosporin therefore, its role as antibiotic is not as important as ampicillin or amoxicillin.

[3] It should be considered that the limits considered are completely arbitrary. Therefore, its relevance is not as valid as for other classes of antibiotics.

4.5 Full genome sequence analysis

4.5.1 Xanthomonas translucent pv. cerealis

The research in the two databases for antibiotic resistance (CARD, and plasmid finder) for the *Xanthomonas translucent pv. cerealis* (S2R) shows the presence of only one gene for antibiotic resistance in CARD database. The gene found is smeB, a gene that codes for an inner membrane multidrug exporter of efflux complex smeABC. This gene is responsible for the resistance to several antibiotics: aminoglycosides, cephalosporins, cephamycin and penam (the primary skeleton that defines the penicillin subclasses).

As expected, the results from the MIC testing for ampicillin, cefotaxime and ciprofloxacin show a high resistance 5,5 mg/L and 4 mg/L respectively and 0,625 mg/L.

It is also worth noticing that the gene shows a relatively low identity percentage of 80,34% compared to the gene present in CARD database (Jia et al., 2017). This may mean that the gene found in this specific strain is a new gene never described before. This could also mean that the reason for the absence of plasmids responsible for the resistance could be due to both small size and high variability of these genes, since even a small change in the genomic sequence leads to a high percentage of differentiation with the one present in the database.

As for the virulence factors, the search in the VFBB shows the presence of two genes, pilT and PilG. Both genes encode for a type IV pili, a twitching mobility protein that is commonly found in *Pseudomonas aeruginosa PA01* (Chen et al., 2016).

In conclusion, as expected by the presence of the smeB gene, which should grant resistance to at least four different classes of antibiotics, this strain can be considered multidrug resistant since it does present resistance to three classes of antibiotics: penicillins (ampicillin and amoxicillin), cefotaxime and ciprofloxacin.

As for the virulence factors, as described earlier in chapter 4.6.3., no cases of human disease were ever associated with this specie therefore, despite the presence of two difference virulence factors, the role of pathogen or even opportunistic pathogen for strain of *Xanthomonas is unlikely.*

4.5.2 Pseudomonas chlororaphis subsp. aureofaciens

As previously described in chapter 4.3.1, *Pseudomonas spp*. is known to be resistant to different classes of antibiotics and the strain tested ($S7FW^{E}$) is no exception as it can be considered as multi-drug resistant since it was found to be resistant to almost all the antibiotics tested including carbapenem with the only exceptions being cefepime and ciprofloxacin.

In total, 10 different genes associated with antibiotic resistance were found in CARD database. Meanwhile, no correspondence was observed in the Plasmid finder database (**appendix 2**).

9 out of 10 genes were either inner or outer membrane pumps or similar efflux proteins which gave resistance to many classes of antibiotics including tetracyclines, fluoroquinolones, and more importantly carbapenems. Meanwhile, only one gene, arnA, allowed an intrinsic resistance to polymyxin via the degradation of the peptide.

As for *Xanthomonas chlororaphis subs. aureofaciens*, these 10 genes present a relatively low percentage of identity compared to the ones in the CARD database, with the highest being 88.5% (Jia et al., 2017). Therefore, as for *Xanthomonas* the complete lack of plasmid that confers resistance to antibiotics might be due to new plasmids that are too different to the ones present in the database.

Out of the 10 genes, two CpxR and MexB, deserve particular attention as they are responsible for the resistance to carbapenems and, according to the MIC test results, this strain of *Pseudomonas* is completely resistant to meropenem (8 mg/L) and as widely described, carbapenems, and more especially meropenem, represent the last beach of antibiotic treatments. CpxR is a gene involved in the activation of expression of RND efflux pump MExAB-OprM and its presence enhances the expression of and the resistance of said pump, which in this case is coded by the other gene MexB which codes for an inner membrane efflux pump. Besides carbapenem this pump can also effectively remove several other antibiotics with the most important being cephalosporin, cephamycin, diaminopyrimidine, fluoroquinolone, macrolide, and tetracycline.

As for the virulence factors, the search in the VFBB database found a total of 40 different virulence factors. All of them are closely related to the ones found in *Pseudomonas aeruginosa PAO1* (**Appendix 3**). A closer look at the results reveals that there are three major families of genes that code for a certain virulence factor such as the fli family which is responsible for the production of virulence factors linked to the flagellum, the pil family which codes for some twitching mobility protein and the clp/his family which is responsible for producing a type VI secretion system. Said system was thought to be a classic virulence factor, but later it was discovered that this system serves as an inter-bacterial competition device thus, enhancing the possibility of survival by delivering toxic antibacterial effector into a rival cell (Coulthurst, 2019). As for the antibiotic resistant genes, the percentage of identity was relatively low, the highest being 89.53%. So, as for the antibiotic-resistant genes described above, all of 40 genes, may be considered as new virulence factors (Chen et al., 2016).

To conclude, the presence of this strain of *Pseudomonas* is worth attention for both its high resistance to antibiotics, particularly to carbapenem, and the large number of virulence factors, which could make it a suitable opportunistic pathogen.

4.5.3 Acinetobacter johnsonii

As for *Acinetobacter* (S8FW), thanks to the full sequence of the genome it was possible to identify the specie, since the results given by the 16s DNA failed the task. To identify the specie, the "config" file resulted from the trimmomatic program was run on the PubMLST platform (*Https://Pubmlst.Org/Bigsdb?Db=pubmlst_rmlst_seqdef_kiosk*, 2022). The research identified this bacterium as *Acinetobacter johnsonii* (Bolger et al., 2014; Jolley et al., 2018). Whereas the search in the CARD database shows the presence of the gene OXA-334, which is a β-lactamase of the OXA-211 family. This β-lactamase is responsible for the resistance to carbapenems and cephalosporins. However, as seen in the results of MIC test in chapter 4.4.2, the phenotypic resistance to both cephalosporins and carbapenems is not that high. Therefore, it can be concluded that this β-lactamase is not very efficient at hydrolysing these antibiotics. Whereas the search in the Plasmid Finder and VFBB databases did not show the presence of known plasmid or virulence factor whatsoever.

4.5.4 Stenotrophomonas maltophila

The research in the databases for *Stenotrophomonas maltophila* (I1FG) did not give any result for antibiotic resistance genes, plasmids, or virulence factors. The complete absence of antibiotic resistance genes or plasmids is rather strange since, the isolated strain was earlier found to be multi-drug resistant and even present a higher resistance to meropenem when compared to the strain of *Acinetobacter* covered in the previous chapter.

A possible explanation for these odd results could be a fallacious DNA sequencing. Such theory is supported by the fact that the search in the pubMLST platform did not give any result either which is also weird since the 16s DNA was able to identify the bacteria. The issue might be a wrong quantification of DNA present in the sample which could have led to a fallacious extraction.

5 CONCLUSIONS

The ability of bacteria to develop new ways to avoid the efficacy of modern antibiotics combined with a low number of alternatives for antibiotics like β-lactams or even the lack of alternatives when it comes to antibiotics like carbapenems, represent a major threat for human health.

This project aimed at assessing the presence of ARB in water samples and ready to eat salads and at testing their resistance to the most used antibiotics to understand how resistant these strains really are.

Results showed the presence of 10 different species of ARB of which, three samples (S1W, S1FP, and $S7FW^{E}$) were part of the *Pseudomonas non aeruginosa* group, one sample (S8FW) was part of the *Acinetobacter* family, and five samples (S2R, $S7FW^{c}$, $S7FG_{L}$, S10G, S10FG and I1FG) were part of the other non-species related group.

Full genome sequencing was carried out on sample $S7FW^{E}$, a strain of *Pseudomonas chloraphis subs. aureofaciens,* which was found to the be highly resistant to every penicillin tested (>256 mg/L), cefotaxime (>256 mg/L) and meropenem (8 mg/L). Its resistance to antibiotics was mainly due to efflux pumps, of which the one coded from CpxR and MexB are responsible for its resistance to carbapenem. All the AR genes show a relatively low percentage of identity with the genes present in the CARD database; this means that they are likely going to be genes never described before. This bacterium also presents 40 different virulence factors which could make it a suitable opportunistic pathogen.

As for the *Acinetobacter* group, samples S8W and S8FW[4], showed strain-specific differences in terms of AR. Specifically, sample S8W was highly resistant to tetracycline (>256 mg/L) and Penicillin G (7 mg/L), meanwhile sample S8FW, which can be considered multi-drug resistant, showed a high resistance to penicillins like ampicillin (6,25 mg/L) and penicillin G, ciprofloxacin (>32 mg/L), erythromycin (10 mg/L), (12 mg/L). The full genome sequencing of sample S8FW showed the presence of the gene OXA-334 which codes for a β-lactamase.

Full genome sequencing also indicated that sample S8FW is a strain of *Acinetobacter johnsonii*, a strain of *Acinetobacter* widely found in the environment and unlike *Acinetobacter baumanii* or any other pathogenic strains it does not show any virulence factor.

[4] A strain of *Acinetobacter* was also present in sample I3FW but due to time limitations it was not undergone MIC testing.

Regarding *Xanthomonas* translucent pv. cerealis, the full genome sequencing showed the presence of smeB gene codes for an efflux pump which does confer a total to three classes of antibiotics (penicillins, cefotaxime, and ciprofloxacin). This bacterium also presents two virulence factors, but since no cases of human disease have ever been reported in scientific literature, its role as pathogen or even as opportunistic pathogen is unlikely.

Only two out of three samples of ready to eat salads underwent the MIC test. Sample I1FG, a strain of *stenotrophomonas maltophila,* was found to be multi-drug resistant. Specifically, this strain showed a strong resistance to penicillins like amoxicillin (9 mg/L) and penicillin G (12 mg/L), ciprofloxacin (> 32 mg/L) and erythromycin (>256 mg/L). This bacterium was also subjected to full genome sequencing, and the results did not show the presence of any antibiotic resistance gene or plasmid thus, it can be assumed that the full genome sequencing was fallacious.

Even though the significance of these results is limited due to the low number of samples analyzed, results are consistent with the findings of similar studies stating that antibiotic resistance is widespread in both the environment and food products.

None of the strain were found to be strictly pathogenic for humans, however the presence of antibiotic resistant bacteria in the environment is worrisome since the resistance to antibiotics like carbapenems, which are considered the ultimate hope when it comes to antibiotics, might be spread across pathogenic bacteria. Further studies on ARB are necessary but what is urgently needed is a new mindset on the part of citizens and the food supply chain regarding the use of antibiotics.

Appendix 1: Primers mix with relative target genes and control used in the amplification of samples. adapted from (Finton et al., 2020)

Primer mix	Target gene	Primer sequence	Amplicon Size (bp)	Function	Study
ESBL-1	blaCTX-m2-F	F-CGTTAACGGCACGATGAC	404	CTX-M-group2 class A extended-spectrum β-lactamase (ESBL)	(Dallenne et al., 2010)
	blaCTX-m2-R	R-CGATATCGTTGGTGGTTCCAT			
	blaOXA-F	F-GGCACCAGATTCAACTTTCAAG	564	blaOXA carbapenemase	(Dallenne et al., 2010)
	blaOXA-R	R-GACCCCAAGTTTCCTGTAAGTG			
	blaSHV-F	F-AGCCGCTTGAGCAAATTAAAC	713	SHV Extended-Spectrum β-lactamase (ESBL)	(Dallenne et al., 2010)
	blaSHV-R	R-ATCCCGCAGATAAATCACCAC			
ESBL-2	blaCTX-M9-F	F-TCAAGCCTGCCGATCTGGT	561	CTX-M-group9 class A extended-spectrum β-lactamases (ESBL)	(Dallenne et al., 2010)
	blaCTX-M9-R	R-TGATTCTCGCCGCTGAAG			
	blaCTX-M1-F	F-TTAGGAARTGTGCCGCTGYA	688	CTX-M-group1 class A extended-spectrum β-lactamases (ESBL)	(Dallenne et al., 2010)
	blaCTX-M1-R	R-CGATATCGTTGGTGGTRCCAT			
	blaTEM-F	F-CATTTCCGTGTCGCCCTTATTC	800	TEM extended-spectrum β-lactamase (ESBL)	(Dallenne et al., 2010)
	blaTEM-R	R-CGTTCATCCATAGTTGCCTGAC			
ESBL-3	blaNDM-F	F-TGGCCCGCTCAAGGTATTTT	157	New Dehli Metallo-β-lactamase, carbapenemase	(Finton et al., 2020)
	blaNDM-R	R-GTAGTGCTCAGTGTCGGCAT			
	blaVIM-F	F-ATAGAGCTCAGTGTGTCGGCAT	564	Verona Integron-mediated Metallo-β-lactamase, carbapemase	(Finton et al, 2020)
	blaVIM-R	R-TTATTGGTCTATTTGACCGCGT			
	blaKPC-F	F-TCCGTTACGGCAAAAATGCG	460	*K. pneumoniae* carbapenemase	(Finton et al, 2020)

Primer mix	Target gene	Primer sequence	Amplicon Size (bp)	Function	Study
	blaKPC-R	R-GCATAGTCATTTGCCGTGCC			
ESBL-4	blaCMY-F	F-GCATCTCCCAGCCTAATCCC	188	Resistance to 3rd-generation cephalosporins	(Finton et al, 2020)
	blaCMY-R	R-TTCTCCGGGACAACTTGACG			
	blaOXA-48-F	F-GCTTGATCGCCCTCGATT	281	blaOXA-48 carbapenemase	(Dallenne et al., 2010)
	blaOXA-48-R	IR-GATTTGCTCCGTGGCCGAAA			
	blaIMP-F	F-ACAGGGGGAATAGAGTGGCT	939	Imipenemase (IMP) is a metallo-β-lactamase that confers resistance to almost all β-lactams	(Finton et al, 2020)
	blaIMP-R	R-AGCCTGTTCCCATGTACGTT			
Control	rpoB-F	F-CAGGTCGTCACACGGTAACAAG	512	Positive for Enterobacteriaceae DNA	Universal primers
	rpoB-F	R-GTGGTTCAGTTTCAGCATGTAC			
	16s-F	F-AGAGTTTGATCMTGGCTCAG	1505	Positive for bacterial DNA	Universal primers
	16s-R	R-GYTACCTTGTTACGACTT			

Appendix 2: antibiotic resistance genes type and associated resistance in sample S7FW^{E} (adapted from Jia et al., 2017)

Gene	% Identity	Product	Resistance
OpmH	80,37	outer membrane efflux protein required for triclosan-specific efflux pump function	Triclosan
TriC	81,63	resistance nodulation cell division transporter that is a part of TriABC-OpmH a triclosan-specific efflux protein	Triclosan
MexF	88,5	multidrug inner membrane transporter of the MexEF-OprN complex. mexF corresponds to 2 loci in *Pseudomonas aeruginosa* PAO1 (gene name: mexF/mexB) and 4 loci in Pseudomonas aeruginosa LESB58 (gene name: mexD/mexB)	Diaminopyrimidine, fluoroquinolone, phenicol
mexK	83,26	inner membrane resistance-nodulation-cell division (RND) transporter in the MexJK multidrug efflux protein	Macrolide, tetracycline, triclosan
CpxR	82,43	gene involved in activation of expression of RND efflux pump MexAB-OprM in *P. aeruginosa*. CpxR is required to enhance mexAB-oprM expression and drug resistance in the absence of repressor MexR	Aminocoumarin, aminoglycoside, carbapenem, cephalosporin, cephamycin, diaminopyrimidine, fluoroquinolone, macrolide, monobactam, penam, penem, peptide, phenicol, sulfonamide, tetracycline
mexW	82,44	RND-type membrane protein of the efflux complex MexVW-OprM	acridine_dye, fluoroquinolone, macrolide, phenicol, tetracycline

Gene	% Identity	Product	Resistance
arnA	81,3	modifies lipid A with 4-amino-4-deoxy-L-arabinose (Ara4N) which allows gram-negative bacteria to resist the antimicrobial activity of cationic antimicrobial peptides and antibiotics such as polymyxin. arnA is found in *E. coli* and *P. aeruginosa*	peptide
MexD	87,49	multidrug inner membrane transporter of the MexCD-OprJ complex	Aminocoumarin, aminoglycoside, cephalosporin, diaminopyrimidine, fluoroquinolone, macrolide, penam, phenicol, tetracycline
OprJ	80,45	outer membrane channel component of the MexCD-OprJ multidrug efflux complex	Aminocoumarin, aminoglycoside, cephalosporin, diaminopyrimidine, fluoroquinolone, macrolide, penam, phenicol, tetracycline
MexB	81,10	inner membrane multidrug exporter of the efflux complex MexAB-OprM	Aminocoumarin, carbapenem, cephalosporin, cephamycin, diaminopyrimidine, fluoroquinolone, macrolide, monobactam, penam, penem, peptide, phenicol, sulfonamide, tetracycline

appendix 3: Virulence factors Pseudomonas chloraphis subs. aureofaciens (adapted from Chen et al., 2016).

Gene	% identity	Product
pvdA	81.33	L-ornithine N5-oxygenase
pvdM	80.31	dipeptidase precursor
motA	80.59	flagellar motor protein (Deoxyhexose linking sugar 209 Da capping structure)
waaF	81.35	heptosyltransferase I
algW	80.70	protein Alginate regulation
algA	83.05	phosphomannose isomerase / guanosine 5'-diphospho-D-mannose pyrophosphorylase
algI	82.05	alginate o-acetyltransferase
alg8	84.72	alginate-c5-mannuronan-epimerase
phzB1	81.49	phenazine biosynthesis protein
motC	80.89	flagellar motor protein (Deoxyhexose linking sugar 209 Da capping structure)
fliA	80.70	flagellar biosynthesis sigma factor FliA (Deoxyhexose linking sugar 209 Da capping structure)
fleN	83.83	flagellar synthesis regulator
flhA	82.66	flagellar biosynthesis protein
fliR	80.16	flagellar biosynthetic protein
fliQ	80.60	flagellar biosynthetic protein
fliP	85.92	flagellar biosynthetic protein
fliN	80.29	flagellar motor switch protein

Gene	% identity	Product
fliM	87.45	flagellar motor switch protein
fliI	82.84	flagellum-specific ATP synthase
fliG	86.06	flagellar motor switch protein G
fliE	80.37	flagellar hook-basal body complex protein
fleQ	80.58	transcriptional regulator
flgI	86.19	flagellar P-ring protein precursor
flgH	84.63	flagellar L-ring protein precursor
flgG	86.61	flagellar basal-body rod protein
flgC	83.33	flagellar basal-body rod protein
pilJ	80.67	twitching motility protein
pilH	85.79	twitching motility protein
pilG	85.57	twitching motility protein
pilT	82.36	twitching motility protein
algB	82.50	two-component response regulator
algU	81.96	alginate biosynthesis protein AlgZ/FimS
mbtH-like	85.78	MbtH-like protein from the pyoverdine cluster
pvdH	83.25	diaminobutyrate-2-oxoglutarate aminotransferase
pvdS	83.82	extracytoplasmic-function sigma-70 factor

Gene	% identity	Product
clpV1	85.22	type VI secretion system AAA+ family ATPase
hsiG1	81.43	type VI secretion system hcp secretion island protein
hcp1	83.92	type VI secretion system substrate
hsiC1/vipB	89.53	type VI secretion system tubule-forming protein
hsiB1/vipA	88.07	type VI secretion system tubule-forming protein
pvdA	81.33	L-ornithine N5-oxygenase

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Times goes by but people don't change, so here we are again, another thesis another acknowledgment stripped down to the essentials as my communication skills did not improve significantly over the past years, even though this time I can always blame the English language for that.

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