Universidade de Aveiro Departamento de Química
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Molecular, realizada sob a orientação científica da Doutora Sónia Ferreira, Instituto de Educação e Cidadania, e do Professor Doutor Fernando Morgado, Professor associado com agregação do Departamento de Biologia da Universidade de Aveiro

Dedico este trabalho à minha noiva que com todo o seu inefável amor, carinho e incansável apoio tornou possível a realização desta obra.

Dedicação à minha mãe, *ad memoriam,* por todo o amor, carinho, apoio e valores que me legou. Este era um dos seus maiores sonhos agora conquistado.

o júri

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palavras-chave Microbiota, resistência a antibióticos, *Solea spp.*, pesca, aquacultura.

resumo

A ameaça emergente de doenças infecciosas originadas por estirpes de bactérias multi-resistentes está a causar grandes preocupações a nível mundial, especialmente devido ao aumento de infecções nosocomiais em unidades de saúde, colocando um grande perigo para os sistemas de saúde pública. Existem já evidências directas da transferência de genes de resistência de organismos marinhos para o ser humano através de vários mecanismos, tais como a Transferência Horizontal de Genes, com potenciais reservatórios e vectores ainda por determinar. Os efeitos da má utilização de antibióticos e outros compostos antimicrobianos no microbiota do tracto gastrointestinal (GIT) de espécies com algum grau de contacto com materiais e detritos humanos e que poderão constituir-se como vectores para uma variedade de estirpes de bacterias multi-resistentes, ainda permanecem desconhecidos. O presente estudo teve como objectivo principal determinar a existência de estirpes bacterianas resistentes a antibióticos em *Solea spp.* No contexto da saúde pública. Foram realizados testes de inibição de Kirby-Bauer em animais de duas aquaculturas e dois portos de pesca localizados a norte e a sul do rio Douro. Os resultados revelaram resistência para a penicilina em todos os locais de amostragem. Resistência à amoxicilina com ácido clavulânico foi verificada nos locais A, B, e no C com alguns resultados de nível intermédio. No local D a sensibilidade a este composto foi total. O nível de inibição foi intermédio para o trimetoprim-sulfametoxazol (SXT) e para a ciprofloxacina no local A. O local B e D revelaram sensibilidade para ambos e o C apresentou resistência ao SXT e inibição intermédia pela ciprofloxacina. Considera-se, assim, que o mau uso de antibióticos foi a causa mais provável da indução de resistências no microbiota do GIT da *Solea spp.* e que o potencial para ocorrer transferência de genes de resistência ao contexto humano é elevado.

keywords Microbiota, antibiotic resistance, *Solea spp.*, fishing, aquaculture.

abstract

Great concerns are developing worldwide over the emerging threat of infectious diseases caused by antibiotic-resistant strains of bacteria, especially for the rise of nosocomial infections in healthcare units, placing a major peril over public health systems. There are evidences of resistance genes being transferred from marine life to humans by several mechanisms such as Horizontal Gene Transfer (HGT), with potential reservoirs and vectors still to be determined. The effects caused by antibiotics and other antimicrobial compounds misusage on the gastrointestinal tract (GIT) microbiota of species with some degree of contact with human materials or wastes that could present themselves as vectors for a variety of resistant strains of bacteria still remain unknown. The main goal of this study was to determine the existence of antibiotic-resistant strains of bacteria in the *Solea spp.* in the public health context. Kirby-Bauer inhibition tests were performed in animals of two aquaculture industries and two fishing harbors located north and south of the Douro river. Results revealed a high resistance for penicillin in all sampling locations. Resistance to amoxicillin with clavulanic acid was obtained in sites A, B and C with few results of intermediate level. In location D the sensibility for this compound was total. The level of inhibition was intermediate for trimethoprim-sulfamethoxazole (SXT) and ciprofloxacin in site A. Sites B and D revealed sensitivity to both and location C presented resistance to SXT and intermediate resistance to ciprofloxacin. It was considered that antibiotics misusage was the most probable cause for inducing resistance in the GIT microbiota of the *Solea spp* and the potential for transfer of the genetic determinants of resistance to the human setting is high.

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1. Introduction

1. Introduction

The rising emergence of antibiotic-resistant strains of bacteria and the intrinsic increase in nosocomial or health care units-associated infections is one of the greatest dangers presently posed to human health and it is already one of the most important issues in public health discussions and policy making (3; 6-8; 26; 35; 49).

The width of the effect caused by the antibiotic-resistant strains of bacteria and their extensive impact on both morbidity and mortality, has taken the European Union to treat it as a special health issue listed in Annex 1 of the European Commission Decision 2000/96/EC of the 22nd of December, 1999 and also the United States government, for example, to consider multidrug-resistant microorganisms a significant threat to their public health and national security (3; 7; 8; 10; 16; 33; 59).

Estimates showed that roughly 160 newly Emerging Infectious Diseases (EID) caused by bacteria have gradually been identified over the last 70 years (49; 65). This EID increase is usually connected to certain agricultural practices, climate changes (affects the survival and distribution of disease vectors such as insects), and increases in human population densities, originating an augmented disease incidence (49; 65). Epidemics related to antibiotic resistance have already been described in a range of pathogens such as the global proliferation of drug resistance among common respiratory pathogens (*e.g. Streptococcus pneumoniae* and *Mycobacterium tuberculosis*) and episodes of epidemic increases in multidrug-resistance in gram-negative bacilli (8).

The specific issue concerning oceanic fish species is that the frequent contamination of coastal ecosystems with discarded antibiotics from clinical, industry and agricultural activities, through discharged effluents and runoffs, invariably results in the amplification of the selective pressures out of the parameters normally occurring in the ecosystems, therefore, creating favorable settings for the appearance of antibiotic-resistant strains of EID causing bacteria (3; 7).

The scientific emphasis, at the moment is being placed on bacterial resistance occurring in animal populations due to the past and the current wide use of antibiotics both in human and veterinary medicine, in food industry activities such as agriculture and animal production and also in certain industries of the biotechnology area (3; 13-17; 26; 34; 59).

Several studies have accomplished to isolate antibiotic resistant bacteria from specimens of the various animal groups such as mammals, birds, amphibians, fish, and insects (3; 19; 26). The presence of antibiotic-resistant strains of bacteria in animals is, in a great percentage, the result of scavenging activities or contact with human-associated materials (19; 26).

The proliferating antibiotic-resistant microorganisms have a high potential for developing infections in many aquatic species and some, as supported by strong epidemiological and molecular evidences, being capable of transferring their resistance genes to human pathogens through Horizontal Gene Transfer (HGT) via mobile genetic elements such as plasmids and transposons, thus endangering human health (3; 7; 15; 16; 17; 19; 26). Beiwen Zheng *et al.* (2011) address this particular case of gene transfer from marine animal populations to terrestrial communities (7). During their research, they have found that a particular carbohydrate active enzyme codifying gene was transferred from marine bacteria to human gut microbiota, through ingestion of sea organisms, thus providing the opportunity for the transfer of any particular resistance genes from marine to terrestrial populations due to the extensive amount of gene transfer that occurs in the midst of the human gut microbiota (7; 14; 27). While inside the original host, these resistance genes are normally involved in metabolic networks but once they disseminate, they acquire one single role that is to confer resistance (7).

The transfer of resistance genes, in most cases, is done through HGT via several mechanisms (61). Such mechanisms are, for example, the transduction, where the genetic material is exchanged between different bacteria, usually carried by a bacteriophage, the bacterial conjugation process, where genetic components are transferred directly from the donor to the recipient cell through direct contact between them using mobile genetic elements capable of moving from one genetic location to another, like transposons, or from cell to cell without

requiring the process of conjugation, like plasmids (45, 61). These are capable of transfer resistance genes by processes of recombination which can or cannot include some form of replication (45). These gene carrying platforms and the very high rate by which bacteria are capable of suffering mutations in their DNA have permitted bacterial strains to occupy new areas of operation and niches, namely hospitals or animal producing farms were antibiotics are routinely used in enormous amounts, by being capable of expressing some particular genes that confer some phenotypic capability of resistance to the microorganism in these environments presenting specific ecological conditions (45).

1.1. Antibiotics: chemistry, modes of action and mechanisms of resistance.

Antibiotics are chemical molecules produced either by microorganisms (bacteria and fungi) or be synthetically designed molecules (12).

The era of antibiotics started when they were first introduced in the 1930's, after the remarkable discovery of penicillin in September of 1928 by Alexander Fleming when he noticed the lysis of *Staphylococci colonies* by a substance produced by a *Penicillium spp.* mold (12; 51).

Later in 1940, researchers Chain and Florey, together with their associates, were the first group to be able to produce a considerable quantity of penicillin obtained from cultures of *Penicillium notatum* (51).

Ten years later, the clinical use of penicillin G was profusely extensive but its application in the clinical settings proved to have serious limitations, such as its relative instability in gastric acid, a high susceptibility to β-lactamases enzymes (also known as penicillases) and the fact that this antibiotic is somewhat inactive against Gram-negative bacteria. This inactivity is caused by the inability of the antibiotic to penetrate the Gram-negative cell wall, also to the lack of binding sites, the PBPs (Penicillin Binding Proteins) and finally, due to enzymatic inactivation. The need to improve the action of these molecules made the continuous research

on these antibiotics imperative which led, a few years later, to the isolation of the active moiety of the penicillin molecule, the 6-aminopenicillanic acid (51).

The 6-aminopenicillanic acid structure consists on a thiazolidine ring attached to a β-lactam ring which carries a secondary amino group (R-NH-), which makes a structure with a strong antibacterial activity. This in turn led to the development and design of semisynthetic penicillins in order to overcome the shortcomings of penicillin G (51).

The establishment of the cephalosporin family, a molecule sharing the β-lactam ring with penicillins resulted in an assortment of drugs with a varying capability to penetrate into different Gram-negative bacterial strains and to effectively oppose the action of numerous β-lactamases enzymes (51).

Since its discovery, antibiotics have been used to treat from mild to severe infectious diseases caused by bacteria and fungi, acting on them by blocking some essential process in microbial cells selectivity (12).

The production of antibiotics naturally results from the ability of a certain microorganism, in a pre-established habitat and a set of environmental conditions to affect the growth capabilities of other microorganisms in its surrounding area or to activate their elimination (12).

Today, the most well-known antibiotic producing group is the actinomycetes. In spite that antibiotics are able to have an antibacterial or antifungal activity there are no therapeutic agent highly effective both against bacteria and fungi at the same time. The reason for this is that both groups of microorganisms have different molecular and cellular targets. Another motive is the question of microbial cell penetration capability that varies amongst different classes of antibiotics (12).

The antibiotic mode of action is either bactericidal, where microorganisms are killed by the compound or bacteriostatic, in which, the microorganisms growth is rendered to a halt. In nowadays, most of the major classes of antibiotics in clinical use are naturally produced chemicals or semisynthetic derivatives. Yet, there are also synthetic antibiotics being used in the present day (12).

The three major classes of antibiotics used in this study were the β-lactams, the quinolones and the sulfonamides.

1.1.1. β-lactam antibiotics

1.1.1.1. Chemistry of β-lactams

The β-lactamic group of antibiotics is composed of drugs characterized by having a β-lactam center ring in their stoichiometric chemical structure (22).

Some classes have a chemical structure composed of a thiazolidine ring attached to a β-lactam ring which carries a secondary amino group (R-NH-), (51).

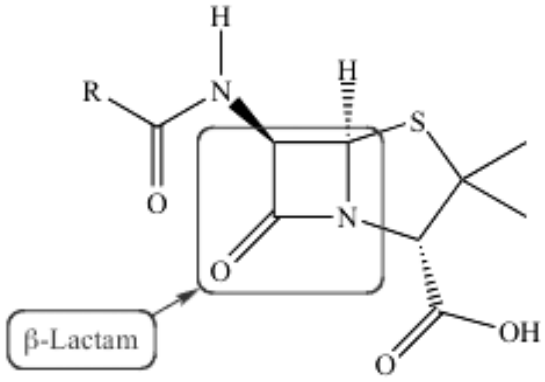


Figure 1: Stoichiometric structure of the β-lactam molecule (62).

The group is constituted by several classes, namely penicillins, cephalosporins, monobactams and also carbapenems, all having a bactericidal mode of action over microorganisms, exerting their action by inhibiting the synthesis of the bacterial cell walls. Bacterial cell walls are made of peptidoglycans which accounts for its rigidity (22). Peptidoglycans are biopolymers with both a D- and a L-amino acids, being the only biological structure to have D-aminoacids). The basic structure consists on a carbohydrate backbone of alternating units of N-acetyl glucosamine and N-acetyl muramic acid. The N-acetyl muramic acid residues make a cross-linkage with oligopeptides and the terminal peptide is usually D-alanine but other amino acids can became D-isomers and occupy the terminal position (70).

1.1.1.2. Mechanism of action of the β-lactam antibiotics

The β-lactam antibiotics work by inhibiting the Penicillin Binding Proteins (PBPs) trans- and carboxy-peptidases, the enzymes responsible for the cross-linkage of the glycopeptides polymer units that compose the bacterial cell wall, weakening it and allowing the lysis process to occur (22). The lysogenic bactericidal action of these drugs are exerted only on the cell walls of growing bacteria, because they are undergoing cell wall synthesis but this exact mechanism is still unknown (51).

In spite of the fact that β-lactams all share the same bactericidal mechanism of action, the PBPs of different strains of bacteria have different affinities to different classes of β-lactams, thus exhibiting various degrees of susceptibility (22). The different susceptibility of both Gram-negative and Gram-positive bacteria is due to differences in the PBPs receptor sites, to the relative amount of peptidoglycans present (the Gram-positive possess more than Gram-negative), to the capability of the drugs to penetrate the external cell membrane of Gram-negative bacteria and to the capacity to resist to different types of β-lactamase produced by different strains of bacteria (51).

The β-lactam antibiotics kill rate is lower when compared, for example, to aminoglycosides or fluoroquinolones. The bactericidal action starts after a lag period. It was found that β-lactams exhibit *in vitro* post-antibiotic effect against Gram-positive bacteria but not against Gram-negative bacteria, with the sole exception being carbapenems action on *Pseudomonas spp.* (51).

The efficiency of the β-lactams is dependent upon time and not of concentration, requiring that serum concentrations exceed the Minimal Inhibition Concentration (MIC – the lowest antibiotic concentration required to visibly inhibit the growth of a microorganism in a controlled test) of the pathogens for each dosage interval making the best administration of these drugs to be either with a frequent intake or by its continuous infusion into the organism (51).

1.1.1.3. Resistance to the β-lactam antibiotics

Resistance to the β-lactams is related intrinsically to the type and strain of bacteria. For example, in Gram-positive bacteria the phenomenon of resistance is achieved by the production of β-lactamases, enzymes that are capable of breaking the β-lactam ring of most penicillin compounds. These enzymes can, in some cases, be secreted extracellularly as inducible exoenzymes being mediated through plasmids (51).

The resistance shown by many Gram-negative bacteria to penicillin G results from the low permeability of their cell wall, the lack of PBPs and from the existence of a wide range of β-lactamase enzymes. Most Gram-negative bacteria express low levels of chromosomally mediated and species-specific β-lactamases within the periplasmic space which often contributes to the inherent resistance (51).

The β-lactamases work by hydrolyzing susceptible cephalosporins more rapidly than penicillin G but their hydrolysis capability is reduced on ampicillin, carbenicillin and β-lactamase-resistant penicillins (51).

The plasmid-mediated β-lactamases production is common amongst Gram-negative and opportunist pathogenic bacteria. The majority of these enzymes are penicillases instead of cephalosporinases and after being constitutively expressed they are secreted into the periplasmic space causing a high level of resistance to β-lactams (51).

The most profusely spreaded enzymes are those classified by their hydrolytic activity such as TEM-type β-lactamases that promptly hydrolyse penicillin G and ampicillin instead of methicillin, cloxacillin or carbenicillin. The OXA-type β-lactamases are the least widespread and they hydrolyze penicillinase-stable penicillins (oxacillin, cloxacillin and drugs related), (51).

It is considered that β-lactamases have probably evolved from PBPs as a sort of protective device in soil microorganisms highly exposed to β-lactams in their natural environments and spreaded by their production due to the activity of molds. Due to the large dissemination of transferable resistance, the production of β-lactamases by bacterial pathogens is now overreaching. Yet, a major milestone in the struggle against the effect of β-lactamases in β-lactam antibiotics was the

discovery of the broad-spectrum β-lactamases-inhibitory drugs such as clavulanic acid, sulbactam or still tazobactam. These particular drugs have a rather weak antibacterial activity but combined with penicillin G, ampicillin or amoxicillin they reveal an extraordinary synergism due to the characteristic irreversible binging of the β-lactamase enzymes of the resistant strains of bacteria. Still, other β-lactamase enzymes inhibitors, such as cefotaxime and carbapenems do have a very strong bactericidal activity (51).

1.1.2. Sulfonamides

1.1.2.1. Chemistry of sulfonamides

The role of sulfonamides as antimicrobial agents diminished greatly over the past 70 years, since the introduction of β-lactams and other antibiotics due to generalized acquired resistance. Yet there is still an important part to be played by this group of drugs. When they are combined with trimethoprim (trimethoprim-sulfamethoxazole or co-trimoxazole) or with ormetoprim the occurrence of resistance is vastly diminished, increasing its usefulness (51).

The sulfonamides derive from the sulfanilamide molecule, which contains the necessary structural design to have antibacterial activity. They only differ in having in the radical attached to the amino group (-SO_2NHR), or occasionally in the substituent on the amino group (-NH_2). The several derivatives vary in their physicochemical and pharmacokinetic properties as well as in the level of antimicrobial activity (51).

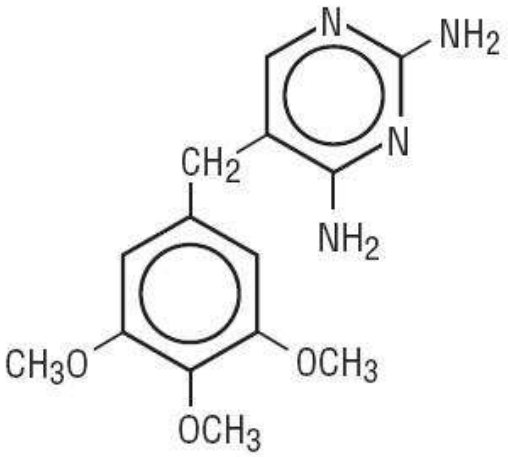


Figure 2: Stoichiometric structure of the sulfamethoxazole molecule (63).

Sulfonamides are rather insoluble being more soluble at an alkaline pH than in a solution with an acid pH value. Even in a mixture of sulfonamides, each component exhibits its own solubility. An excellent example of this is the trisulfapyrimidine preparation which results in the additive bacterial activity but in terms of solubility the agents behave independently. This mixture was specifically designed to offset the crystallization of sulfonamides in the acidic fluids inside the distal renal tubules and ureters (51).

Sulfonamides have sodium salts in their structure which are highly soluble in water and parenteral preparations are available for intravenous administration mode. These solutions produce a very alkaline reaction, with the exception of the sodium sulfacetamide, due to the fact that this compound is almost neutral, being available as an ophthalmic solution. In spite of this, there are sulfonamides molecules which are designed to have a low solubility in order to be gradually absorbed by the organisms. These molecules are used in the treatment of enteric infections (51).

1.1.2.3. Mechanism of action of sulphonamides

The sulfonamides mechanism of action consists in interfering with the biosynthesis of folic acid by bacteria. It inhibits the biosynthesis of folic acid by preventing the para-aminobenzoic acid (PABA) from being inserted into the folic acid molecule (pteroyglutamic) through direct competition with the PABA molecules for the active center of the enzyme dihydropteroate synthetase (51).

Thus, the bacteriostatic action of sulfonamides depends heavily on the difference between bacterial and mammalian cells in correlation to their source of folic acid because all susceptible microorganisms must produce folic acid, contrary to mammalian cells which use preformed folic acid (51).

Therefore, sulfonamides bacteriostatic activity can be reversed by bacteria through an excess of PABA (51).

Sulfonamides are broad-spectrum antimicrobial agents, which are able to inhibit bacteria and protozoans like toxoplasma and *coccidian*, but its antibacterial

action nowadays is greatly diminished by the cumulative resistance that has been developing over the last 60 years since it has been used in the clinical setting (51).

The composition of the medium and the bacterial inoculum concentration greatly alters the MIC of sulfonamides (51).

Due to this fact, tests performed *in vitro* can, at times, present a false result for resistance in a certain bacterium. But, using a suitable control with a thymidine-susceptible strain of *Enterococcus faecalis* this is generally avoided (51).

The best way to decrease the quantity of thymidine in the test medium when performing *in vitro* tests is to use Muller-Hinton agar medium containing lysed horse blood for it contains thymidine phosphorylase which will degrade the molecules present in the inoculum (51).

1.1.2.4. Resistance to sulphonamides

The phenomenon of resistance develops slowly but steadily by chromosomal mutations which results from impairment of drug penetration into the bacterial cell, from the production of a insensitive dihydropteroate enzyme or from the overproduction of PABA (51).

Other mechanisms of resistance mediated by plasmids and integrons, often encoded by the *sulI* and *sulII* genes which are in turn occasionally linked to other resistance genes like the trimethoprim *(dhfrI)* or the streptomycin *(aadA1a)*, are rather common, and in enteric bacteria is due to the impairment of drug penetration or the additional production of sulfonamide resistant dihydropteroate enzymes (51).

The disseminated resistance to sulfonamides in bacteria isolated from animals attests for the intensive use that these drugs over the course of decades resulting in a complete cross-resistance among this group of drugs (51).

1.1.3. Fluoroquinolones

The group of fluoroquinolones, designated as well as quinolones, 4-quinolones, quinolone carboxylic acids or by pyridine-β-carboxylic acids are a considerable sized and expanding group of synthetic antimicrobial molecules.

The primary compound of this group was nalidixic acid which was first depicted in 1962 and introduced for clinical practices in the year of 1963 but only being approved for medical therapeutic purposes in 1965 (51).

Because nalidixic acid revealed to have restricted clinical applications as a result of a low rate of absorption following oral administration, a moderate antibacterial activity, a high rate of protein binding capability (92%-97%) and a poor physiological tolerance, soon attempts were made to design an intravenous form of the nalidixic acid but without positive results (51).

Only from the middle 1960's to the early 1980's were various new quinolones approved to be used in a clinical setting (e.g. pipemidic acid, piromidic acid, oxolinic acid and flumaquine). In spite of the augmented antibacterial activity, these new drugs continued to have low absorption levels and physiological distribution capability (51).

Later, in the 1980's with the addition of a fluorine molecule was made to the 6-position of the basic chemical structure of the quinolone, together with the substitution of a piperazine on position 7, the antibacterial capabilities of these compounds were enhanced. This range of capabilities included the activity against microorganisms such as *Pseudomonas aeruginosa* and staphylococci, and at the same time it heightened the level of oral absorption and systemic distribution.

Norfloxacin was the first fluoroquinolone to be approved for clinical uses, seconded shortly after by ciprofloxacin (51).

The fluoroquinolones have a fast bactericidal activity in an appropriate drug concentration (Minimum Inhibitory Concentration - MIC ratios), showing a killing action dependent of concentration and possibly a prolonged post-antibiotic effect (PAE) *in vivo* in some bacteria. In spite of this, there is a huge potential for the rapid selection of resistance to these drugs by some pathogens which as turn out to be a shortcoming of this class of antibiotics (51). The way to circumvent this

issue is to select an appropriate dosage targeting a specific pathogen and the correct infectious disease process (51).

The classification of fluoroquinolones is based upon their chemical structure and biological activities (51).

Following an analysis of their chemical structure, the classification scheme depends on the number of rings associated with the pyridine-β-carboxylic acid nucleus. Accordingly, Group I is composed by the monocyclic derivatives and Group II, which comprises the largest amount of fluoroquinolones commercialized today, consists on bicyclic derivatives (51).

However, Group II is divided into two smaller subgroups based on substitutions in the 8th position of the fluoroquinolone nucleus (51).

The Group III is comprised of tricyclic derivatives, including marbofloxacin (51). Group IV includes quadricyclic molecules, of which only very few have been synthesized until the present day (51).

When the classification is based on the biological aspects of fluoroquinolones, then the categorization system comprises three groups with Group I being composed by quinolones that exhibit specific activity against Enterobacteriaceae (nalidixic acid and flumaquine) and could even be divided into molecules that are metabolized and those that are not such as oxolinic acid and pipemidic acid (51).

The Group II of this system is composed of molecules that have an extended spectrum of antibacterial activity, which includes the almost all of the fluoroquinolones, with the exception of a single one. Likewise, this group can be subdivided in two subgroups, subgroup IIA and IIB, depending on whether the molecules are metabolized or not. Group III has only one fluoroquinolone, which is pradofloxacin (51).

Nevertheless, grouping of fluoroquinolones can also be done based upon the physicochemical properties of these molecules. Research on new compounds now emphasizes the replacement of the fluorine atom in position 6 which may decrease the side effects, the metabolism and possible interactions with other drugs (51).

1.1.3.1. Chemistry of fluoroquinolones

Fluoroquinolones is a group composed of synthetic compounds (51).

The first 4-quinolone type compound (nalidixic acid) to be approved for clinical use lacked various characteristics associated with the fluoroquinolones, for example, having a nitrogen atom in the position 8 instead of a carbon atom (51).

Possessing a nitrogen atom located in position 1, the nalidixic acid exhibits two nitrogen atoms in its nucleus, turning it into a naphthyridone molecule instead of a quinolone molecule (51).

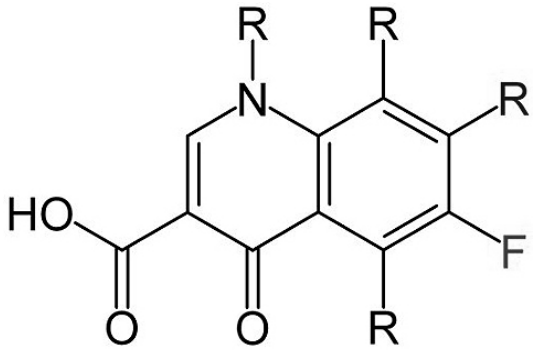


Figure 3: Stoichiometric structure of the fluoroquinolone molecule (64).

Contrary to other quinolones, nalidixic acid is not halogenated (51).

Following the discovery of nalidixic acid and its antibacterial activity, more than 10000 compounds have been developed from the bicyclic 4-quinolone matrix molecule. However, the nalidixic acid has a few clinical limitations, including a constricted spectrum of antibacterial activity, weak pharmacokinetic properties, toxic side effects and the capacity to induce a quick resistance capability in microorganisms. But changing the hydrogen atom in position 6 of the 4-quinolone molecule by a fluorine atom resulted in a rise of its activity against Gram-negative and Gram-positive bacteria alike (51).

1.1.3.2. Mechanism of action of fluoroquinolones

Quinolones produce their anti-bacterial action through the inhibition of bacterial DNA synthesis (9; 23) It promotes the cleavage of the DNA sequences in the DNA-enzyme complexes of type IV topoisomerase and DNA gyrase (9; 23).

These enzymes are composed of two pairs of subunits. The type IV topoisomerase enzyme is composed by *parC* (75 kDa) and *parE* (70 kDa) and it acts in the bacterial DNA structure by processing the removal of positive or negative supercoils and it can catenate and decatenate circular DNA molecules (23). The DNA gyrase also has the capability of both introducing and removing positive or negative supercoils in the DNA structure and is able also to catenate and decatenate closed circular DNA molecules (9; 23). These two enzymes produce, during their action, a breakage in both strands of DNA, followed by an ATP-dependent reaction were they insert a second DNA double helix through that breakage point, resealing it. This reaction is blocked by quinolones by trapping the enzymes in a drug-enzyme-DNA complex. This event produces a release of double-stranded DNA breaks which are lethal to the microorganisms (23).

The majority of bacteria have these pair of enzymes but only a few can survive having only a functional DNA gyrase. The DNA gyrase from Gram-negative bacteria is more susceptible to be inhibited by quinolones than is the type IV topoisomerase. In Gram-positive bacteria type IV topoisomerase is the prime target, leaving DNA gyrase less susceptible (23).

Similar to the other antibiotics, Quinolones possess a bactericidal capability dependent on its concentration. It becomes more evident as the antibiotic serum concentration reaches a level of 30 times the amount of MIC. However, for higher serum concentrations of antibiotic, RNA and protein synthesis are inhibited, decreasing its antimicrobial activity (9; 23).

1.1.3.3. Resistance to fluoroquinolones

The resistance manifested by bacteria to the action of fluoroquinolones results from target modification due to chromosomal mutations, by a decrease in the permeability of the bacterial cell membrane, through the mechanism of efflux pumps or by target protection (51).

The mutations that can induce a phenomenon of resistance will occur first in *gyrA* in Gram-negative bacteria and in *parC* in Gram-positive bacteria (23). Resistance is accomplished by amino acid replacements in the *gyrA* or *parC* subunits region known as “quinolone-resistance determining region” (QRDR), a region that occurs in the DNA-binding surface of the enzymes (23).

Following a first-step mutation in the DNA gyrase of a Gram-negative microorganism, supplementary mutations in *gyrA, gyrB* or on *parC* may produce an augment in bacterial resistance, but isolated they are ineffective in bacteria carrying wild type *gyrA*, due to the fact that the most susceptible target determines the level of susceptibility (23).

Nevertheless, the main mechanism by which bacteria are considered to be able to protect themselves from the bactericidal action of fluoroquinolones and antibiotic drugs in general is by creating obstructions to antibiotic entry inside the cell. This can be achieved by simply modifying the cell membrane composition or by increasing the efflux of the antibiotic to the extracellular environment (27; 42). Efflux pumps offer several mechanisms of resistance because they are able to pump a variety of toxins, like heavy metals and many other toxic molecules, to the external environment. Some pumps, through chromosomal modifications, are considered to play a primary role in environmental terms, for example, by providing tolerance to toxic compounds (27).

The microbiota of insect guts without any previous exposure to antibiotics exhibited efflux pumps with the inheriting capacity to provide resistance to antibiotics when transferred to *E. coli* (27; 42).

2. Objectives

2. Objectives

The objectives of this study, in accordance to the contextual analysis made in the introductory chapter, were:

1 – first, to determine the existence of antibiotic-resistant strains of bacteria both in animals from aquacultures and in animals from commercial fishing;

2 – and second, to determine the probable differences exiting at the microbiologic level between the different sampling sites, commercial fishing versus aquaculture and between the commercial harbors, north and south of the Douro river.

3. Materials and Methods

3. Materials and Methods

The reference data concerning the natural GIT microbiota of the Sole fish was determined by comparing wild animals with those produced in captivity using two different locations; the first being collected north of the Douro river, in Póvoa do Varzim, in an aquaculture operating a flow-through system. The second aquaculture is located south of river Douro, in the area of Aveiro and it uses a recirculating system which is completely closed to exterior environment when in full production mode. The animals obtained in aquaculture producing facilities were all from the same species, Senegalese sole, fed artificially and reared in two different systems of production.

The fish originated from commercial fishing where collected also north of the Douro river in the commercial harbor of Leixões and south of the Douro river respectively, in the commercial harbor of Aveiro.

The first lot of fish was acquired in the Leixões harbor followed by the aquaculture in Matosinhos area both in January 2011. The second lot was acquired in the aquaculture in the region of Aveiro and in the Aveiro harbor both on the end of March 2011. The animals were all adults (sizes of the animals) and the sampling size consisted of five live animals (n=5) per location amounting to a total experimental sample size of 20 (n_{total}=20), with a $n_{aquaculture}$=10 and $n_{ocean\ fishing}$=10 respectively.

Once the animals were acquired, they were immediately transported alive, inside small tanks containing water taken from their rearing facilities, to the laboratory in the Department of Biology at the University of Aveiro, where they were painlessly sacrificed by hypothermia and the fresh guts were removed aseptically in the laboratory.

3.1. Aquaculture systems

There were significant technical differences between the two aquaculture systems of production.

The first aquaculture industry is located north of river Douro and it operates a flow-through system where the sea water is pumped directly to the rearing tanks after being filtered in special filtering systems. Being a continuous flowing circulation system, the water from the rearing tanks flows out to the ocean having discarding the amounts of organic matter and wastes in solution resulting from the artificial feeding at the downstream end of the system (21).

The second aquaculture, located south of the Douro river in the area of Aveiro, uses a recirculating system in which the water flowing system is completely closed to exterior environment when in full production mode (21).

The fish are raised in long tanks called nurseries and then, at a stage near adulthood, they are transferred to wider and deeper tanks. In all these they carry out all their biological activities (21).

The optimal biophysical conditions of the water are maintained by a special filtering system were a primary filter at the exit of the tanks removes the particulate wastes (21). Then the water is conducted to a biological filter were wastes resulting from excretion are detoxified by bacteria (21). Toxic ammonia and nitrites (NO_2^-) are converted into nitrate (NO_3^-), a non-toxic form to the animals, and the water is inserted back into the rearing tanks (21). The amounts of water lost are very low (< 5%) of the each daily volume of water, so little new water is needed to be added (21). An air induction system maintains the oxygenation of the fish tanks and the filtering system in optimal levels (21).

3.3. Bacterial isolation methods

The animals were sacrificed by hypothermia. The process consisted on immersing the animals in ice for 30 min. after which they were dissected and for each gastrointestinal tract (GIT) the following protocol was applied:

- insertion into Falcon 50 mL tubes with Tryptone soya broth growth medium (TSB) from Sigma-Aldrich Chemie GmbH;

- incubation at 37ºC for 24h to guaranty the growth of the bacterial strains present in the biological samples;

- the samples were always stored at the end of each day at the temperature of 4ºC.

The next phase of the protocol, after the 24h period of incubation, commenced by:

- process of bacterial isolation - inoculation of 150 µL of sample in 3 Petri dishes containing MacConkey growth medium agar using previously standardized made plates from Biomérieux MacConkey agar 20 plaques kit. The sample was applied on the dish by scattered seeding in order to isolate the bacterial strains by depletion.

This step was done in order to make a first bacterial strain isolation procedure after the starting inoculation of the gastrointestinal biological extracts.

The conditions were replicated in order to refine the isolation procedure of the bacterial strains existing in the sample by using:

- Biomérieux MacConkey agar 20 Petri dishes kit growing at 37ºC over a 24h period.

Because there were signs of swarming (bacterial mobility) in some bacterial colonies, again we performed the culture of these motile colonies using:

- Biomérieux CLED (Cysteine Lactose-Electrolyte-Deficient) growth medium agar standardized plates (highly effective in preventing swarming phenomenons in the inoculated growth plates), (68).

3.3.1. Bacterial susceptibility testing

The next step on our work protocol consisted in performing the Kirby-Bauer growth inhibition testing using:

- trimethoprim-sulfamethoxazole (SXT) impregnated standardized testing discs;
- penicillin impregnated standardized testing discs;
- amoxicillin combined with clavulanic acid impregnated standardized testing discs (compound known as amoxiclav);
- ciprofloxacin impregnated standardized discs;

- the antibiotic discs were placed over the GIT bacterial isolates inoculates on Biomérieux Muller-Hinton (MHT) agar medium base plates.

The results were registered and treated statistically using Microsoft Excel 2007 producing the statistical correlation between the data acquired.

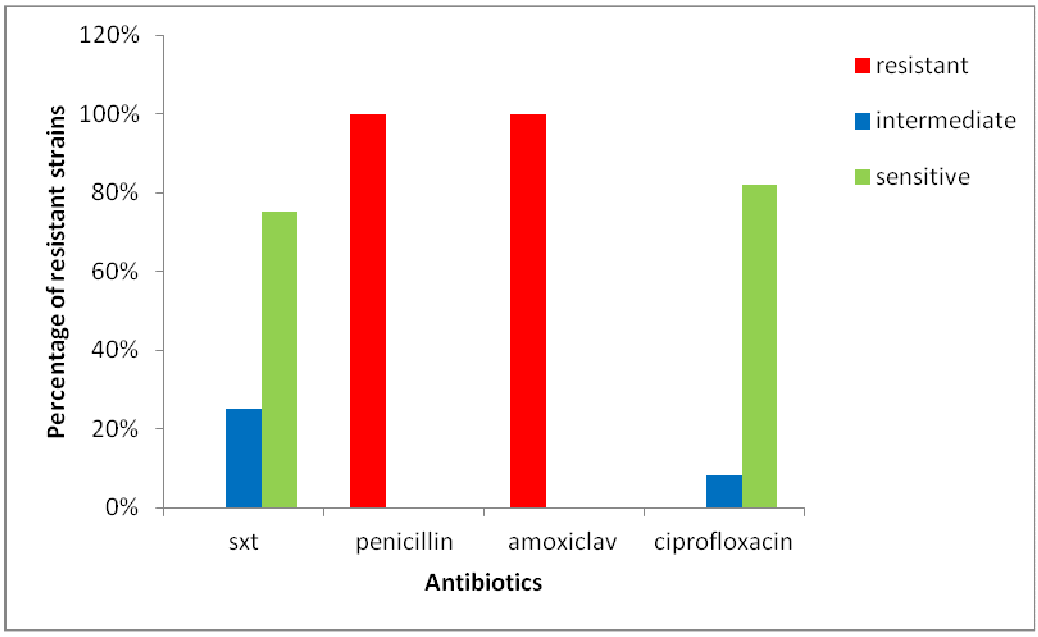
The graphics were made using an *n* value equal to the number of results *per* phenotype in the Kirby-Bauer inhibition testing *(n=number of phenotypes per site)* divided by the total number of results for each sampling site *($n_{(total}$* number *of Kirby-Bauer per sampling site)*:

Percentage of resistant strains per antibiotic = $n_{(per\ phenotype)}$ / $n_{(total\ number\ of\ Kirby\text{-}Bauer\ results\ per\ sampling\ site)}$.

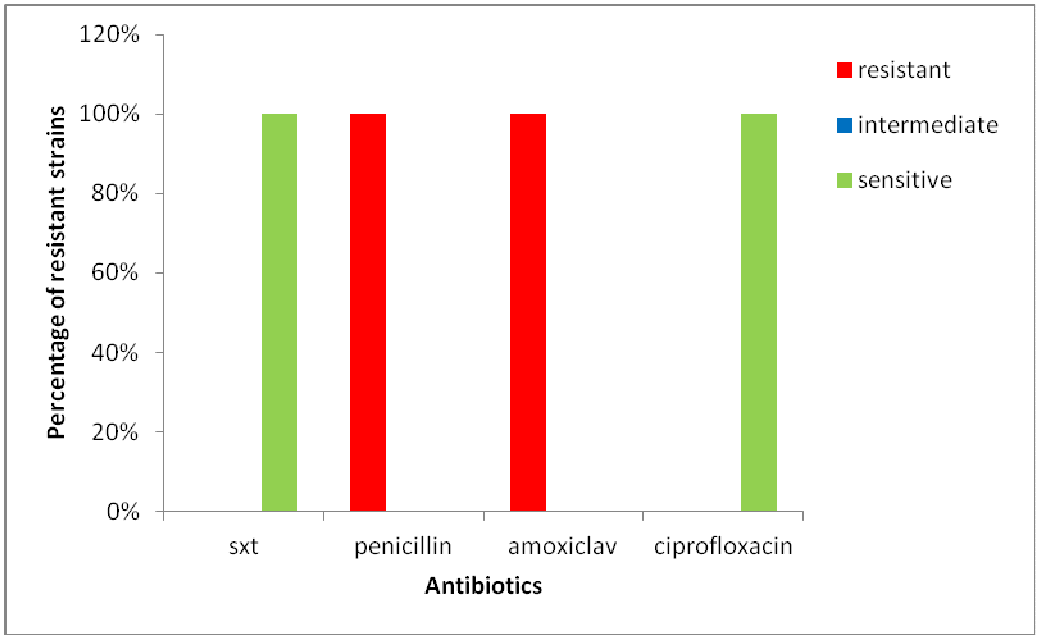
4. Results

4. Results

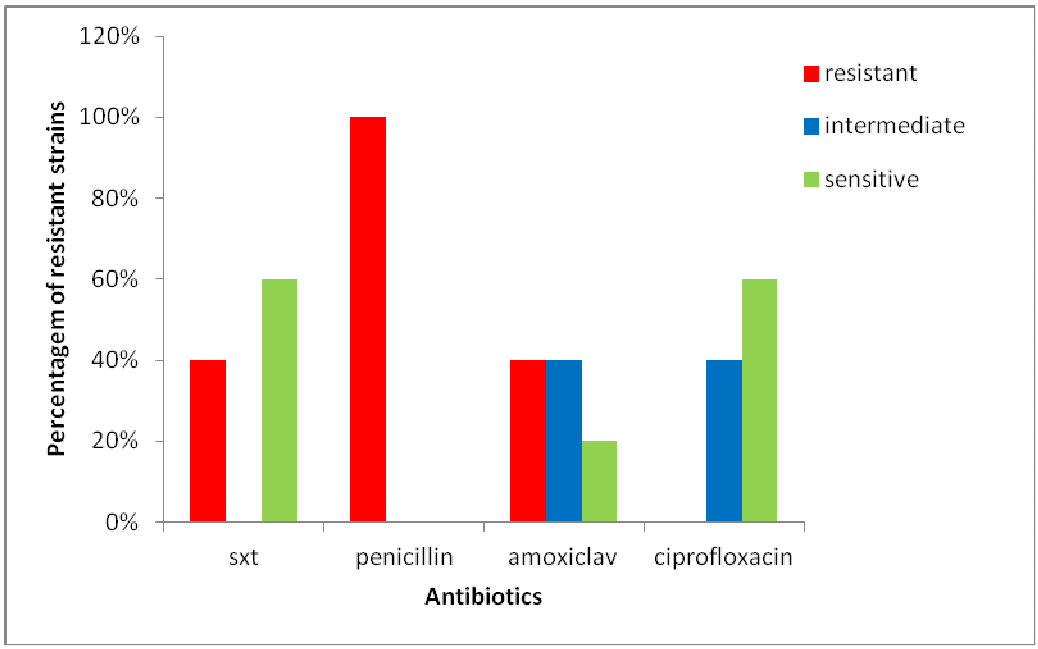
The results from the Kirby-Bauer inhibition testing produced the following graphics:



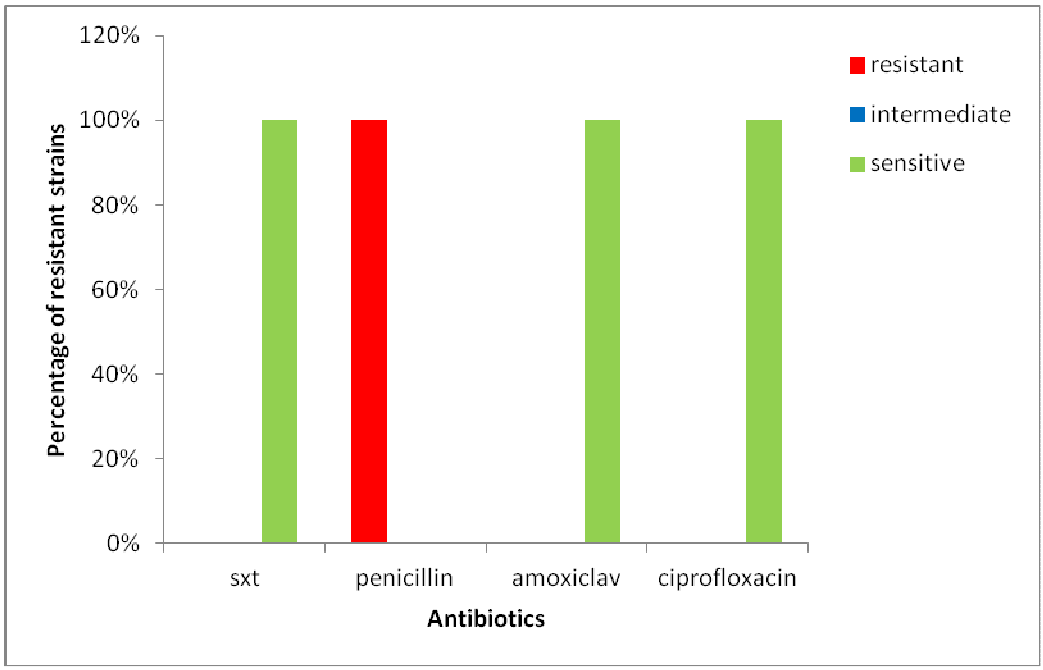
Graphic 1: Percentage of resistant strains – aquaculture of Aveiro.



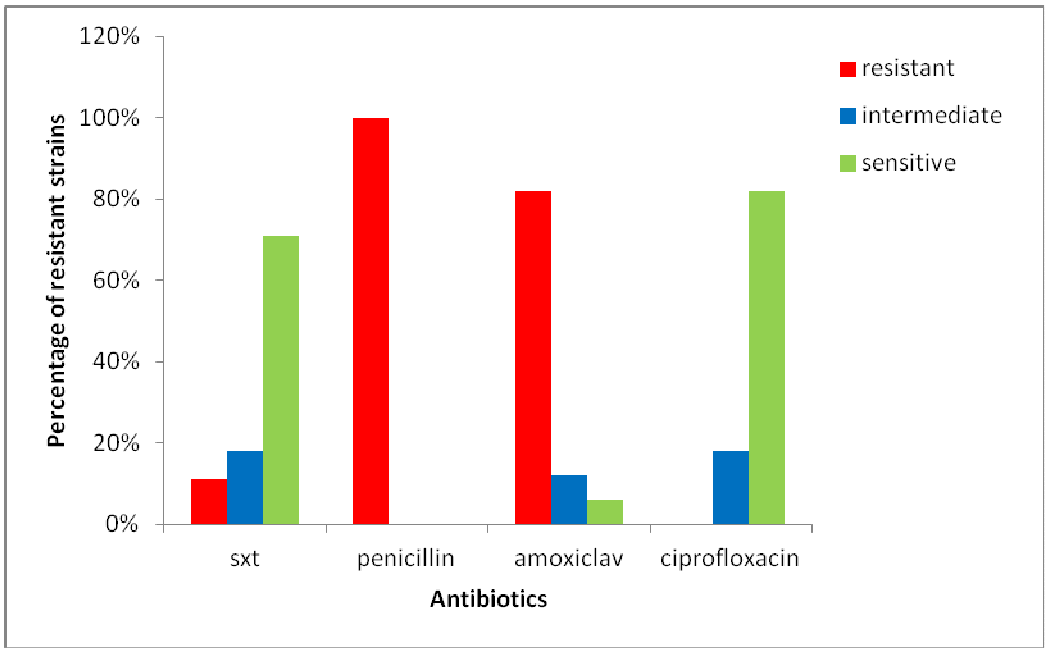
Graphic 2: Percentage of resistant strains – commercial harbor of Aveiro.



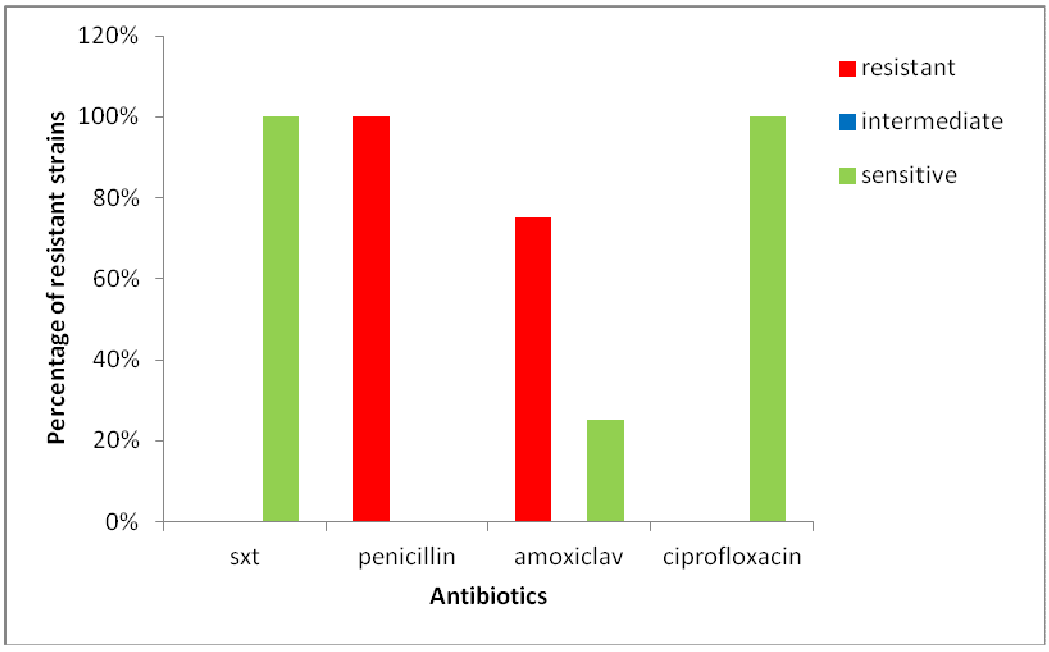
Graphic 3: Percentage of resistant strains – aquaculture of Póvoa do Varzim.



Graphic 4: Percentage of resistant strains – commercial harbor of Leixões.



Graphic 5: Total percentage of resistant strains in the two aquacultures.



Graphic 6: Total percentage of resistant strains in the two commercial harbors.

5. Discussion

5. Discussion

The careful analysis of the results quickly permits a rapid evaluation of the considerable differences in the percentages of resistant strains of bacteria between the animals collected by commercial fishing on the north and south of the Douro river.

Analyzing Graphic 2 it is visible the percentages of 100% of resistance phenotypes to both penicillin and ciprofloxacin with 100% of sensitive phenotypes to sxt and amoxiclav.

In graphic 4 the results are somewhat better, with only 100% of resistant phenotypes to penicillin and 100% sensitive phenotypes to all other three antibiotics, sxt, amoxiclav and ciprofloxacin.

The statistical analysis made to the aquaculture systems reveals several interesting results. The graphic 1 represents the aquaculture in Aveiro which uses a full recirculating system, which showed a full 100% of resistance phenotype by bacterial strains to penicillin and amoxiclav, 2% and 8 % of intermediate phenotype both to sxt and ciprofloxacin, together with 72% and 82% of sensitive phenotypes to sxt and ciprofloxacin respectively.

On the other hand, graphic 3, representing the aquaculture in Póvoa do Varzim which uses a flow-through system, revealed a similar but yet a bit slightly different pattern, by showing 100% of resistant phenotypes to penicillin and 40% of resistant phenotypes to sxt and amoxiclav. In terms of sensitive phenotypes it revealed 60% to sxt and ciprofloxacin and 20% to amoxiclav.

The differences between the percentages of resistant strains in commercial fishing and aquaculture production on fish are shown in graphics 5 and 6.

The results in graphic 5 represent the aquaculture production and they show the existence of a full 100%, 82% and 11% of resistant phenotypes to penicillin, amoxiclav and sxt respectively, 18% of intermediate phenotypes to sxt, ciprofloxacin and 12% of intermediate phenotypes to amoxiclav.

It also revealed the presence of 71%, 6% and 82% of sensitive phenotypes to sxt, amoxiclav and ciprofloxacin respectively.

The results in graphic 6 represent the total set of results for commercial fishing and they reveal a total of 100% and 75% of resistant phenotypes to penicillin and amoxiclav respectively and 100% of sensitive phenotypes both to sxt and ciprofloxacin and 25% of sensitive phenotypes to ciprofloxacin.

6. Conclusions

6. Conclusions

The differences registered in commercial fishing between north and south of the Douro river reveal that, in the case of the fewer percentages of resistant strains of bacteria obtained in the north, is most likely due to the dilution effect provided by the oceanic currents that run mainly from north to south along the entire Portuguese coast. This constant flux of oceanic water tends to dilute any concentration of pollutants (chemical or organic) that may come to be drained in effluents out to sea, therefore reducing the possibility of contact of the wild animals with any antibiotic compound.

The larger percentages of resistant phenotypes registered south of the Douro river are most likely to have their origin on the contamination by the industrial and hospital effluents from the Aveiro urban area. Here we should realize that Aveiro is a heavy industrialized region but we also must not forget the possible influence of the Douro river which may carry in its flow large amounts of contaminants from all the industrial and urban areas along its final stretch to the ocean (Porto urban area).

The industrial and hospital effluents are usually the largest sources of contaminants, carrying antibiotic molecules and chemicals in suspension which later precipitate onto the ocean bed out at sea due to physico-chemical conditions such as their molecular weights, pH values in the ocean waters, temperature and salinity values and even pressure.

The differences registered between the two aquaculture system are undoubtedly related to their systems of water circulation and production. In the case of the aquaculture using the flow-through system, its higher percentages of resistant and intermediate phenotypes are most likely to have been originated by external pollutant sources, since it uses a continuous water circulating system, pumping water from the sea directly into the rearing grounds. This increases the probabilities that the fish will come into contact with some external antibiotic and chemical contaminants inside the production facilities. The physical and biological environmental influences cannot be discarded. The possible influence of human activities, especially the overuse of antibiotics in health units and even inside the

family nucleuses that go out to sea in the drainage of human wastes and runoffs, spreaded by the ocean currents thus increasing the contact of even the GIT microbiota of these animals, sheltered inside closed rearing tanks with repeated doses of antibiotics and possibly with external resistant microorganisms that can transmit the resistance genes to the GIT microbiota of the Sole fish.

One other possibility is the use of antibiotics in the animals food supply since the industry of animal production is known to make the prophylactic use of antibiotics, even in minimal amounts according to the legal standards, but which could impose an artificial selective pressure to the GIT microbiota of the fish inside the tanks which, by living in close quarters, will increase even more the exchanges of genetic determinants amongst the population of the aquaculture, leading to increased percentages of resistance in the bacterial phenotypes.

The aquaculture in Aveiro uses a recirculating system, were the water flowing system is completely closed to the outside environment. This excludes de possibility of external influences, therefore, leaves us only with the probability of either two things, the possible use of antibiotics in the fish food supply which will directly influence the GIT microbiota of the animals, leading eventually to the appearance of resistant phenotypes with the prolonged exposure to these molecules. The second alternative is that there is a possibility of contamination of the water flowing system either by the use of some kind of biological filter unknown to this study or through the accumulation of microbial strains along the systems which in the right kind of environmental settings could have developed some degree of resistant phenotype later transmitting the genetic determinants to the fish GIT microbiota coming into contact with them by the recirculating water inside the system.

The differences between commercial fishing and aquaculture production regimes are well compared by graphics 5 and 6. The lesser percentages found in commercial fishing are probably the result of the pollutant dilution effect brought about by the ocean currents and physico-chemical conditions. The velocity, temperature, salinity, pH and even density of the oceanic waters vary constantly and that can exert a considerable clearing effect over any molecular concentration that may have been cast into the sea waters.

The larger percentages of resistant phenotypes found in aquacultures are probably the result of either the incorrect use of antibiotics in the animals fodder or perhaps even from the natural induction of resistant phenotypes occurring in the microbiota of the *Solea spp.* which, in association with HGT vectors (conjugation, transposition and transduction), creates an extremely high potential for the exchange of genes conferring resistance to neighboring phenotypically sensitive strains of bacteria.

Ultimately, these genetic and ecological processes create the possibility that the genes which code for the resistant phenotypes in the fish GIT microbiota may come to be inserted in the human gastrointestinal microbiota by the consumption of marine organisms, which may induce changes in the normal human GIT microbiota creating the conditions for the selection of antibiotic-resistant strains.

This study, therefore, recommends that a greater control must be made over the general use of antibiotics and that a greater number of microbiologic and genetic studies be made in order to monitor the possible development of the percentage of antibiotic-resistant strains of bacteria.

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