DOMINGOS IMBUNDÉ

GENOTYPING AND SUSCEPTIBILITY ASSESSMENT OF BACTERIA OF THE ORDER *ENTEROBACTERIALES* ISOLATED FROM PATIENTS SUFFERING FROM FECAL INCONTINENCE

Dissertação para a obtenção do grau de Mestre em Ciências Biomédicas

UNIVERSIDADE DO ALGARVE

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Mestrado em Ciências Biomédicas - Mecanismos de Doenças

Trabalho efetuado sob a orientação de: Professora Doutora Leonor Faleiro

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Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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(Domingos Imbundé)

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ABSTRACT

The human intestinal microbiota is composed of large number of microorganisms that reside in our intestine. This community is mainly constituted by a great diversity of bacteria, fungi and bacteriophages. Amidst them, a large proportion of bacteria that live in the gastrointestinal tract are bacteria that belong to the order of *Enterobacteriales*. When the community of the members of the order *Enterobacteriales* unbalanced, they can lead to intestinal dysbiosis that can impact the health status of the host. The alteration in the bacterial community of human intestine can be associate to many factors, for instance unproper use of antibiotics, which change the intestinal bacteriome equilibrium. The results can include inflamed intestine, faecal incontinence (FI). The goal of the current study was to isolate and identify members of the order *Enterobacteriales* followed by their genotyping BOX-PCR and determine their antibiotic susceptibility. For this purpose, 25 faecal samples of women over 60 years that suffered from faecal incontinence that attended in hospitals in the Algarve region.

From these 25 samples analyzed, 72 isolates were obtained, which identification was performed using the Remel RapID ONE System. *Escherichia Coli, Shigella sp., Klebsiella pneumoniae,* and *E. coli 0157:H7* were the more prevalent among the samples. Regarding the BOX-PCR profile the bacterial isolates showed a large diversity. Moreover, 80 to 94,7% of similarity was observed between 27 clusters, unveiling high degree of enterobacterial diversity among the isolate. It is important to highlight that only 3 faecal samples showed to carry specific strains.

The antibiotic susceptibility was evaluated by using Kirby-Bauer disk diffusion method. The resistance profiles were observed for amoxicillin (AMC), with 72, 20% among the 72 isolates. the Multidrug Resistance profile (MDR) was more frequent for penicillins than other antibiotic class with 79% of MDR.

The multidrug resistance observed among these samples will challenge the treatment of the patients. The BOX-PCR technique showed to good distinction between the bacterial genus and species, unfortunately it was no able to discriminate variants in the same species. Thus, more effective genomic studies are required.

Key words: Faecal incontinence, intestinal microbiota, *Enterobacterales*, antibiotic resistance

Resumo

A microbiota intestinal, é grupo de microorganismo que reside no intestino humano. Entre eles podem-se encontrar bacterias, bacteriófagos, e alguns fungos. As bacterias que residem no trato gastrointestinal são designados de enterobacteria, pois constitue a ordem de *Enterobacterales*. Os membros da ordem *E*nterobacterales, são gram-negativas cuja a forma é de bastonete, são aeróbio ou anaeróbios facultativos, esta comunidade mutualista de bacterias está adaptada ao ambiente do intestino do hospedeiro para sobreviver.

Algumas bactérias como *Bifidobacterium spp.* (do filo Actinomycettota) são produtores de algumas substâncias como neurotransmissores que atua no Sistema Nervoso Central (SNC). As bactérias comensais são essenciais na produção das vitaminas, no Auxílio a digestão, e, conferem proteção contra as bactérias patogénicas. Enquanto as bactérias oportunistas, como *Escherichia coli* e *Klebsiellas pneumoniae* não afetam pessoas saudáveis, mas causam infeções em pacientes imuno-comprometidas.

A sequência BOX, é uma das sequências da repetição extragénica palindrómica que está dispersa ao longo do DNA bacterianos seja de bactérias gram-negativa, assim como gram-positivas. Esta sequência tem sido utilizada para estabelecer a relação de similaridade entre os membros da ordem *Enterobacterales.*

Quando ocorrer um desequilíbrio da microbiota intestinal, seja em quantidade, ou na diversidade entre os membros da ordem *Enterobacterales*, este é designado por disbiose. A disbiose, pode resultar do uso de antibióticos, dietas desequilibradas, e estado de stresses contínuo. No estado da disbiose, podem ocorrer reações imunológicas que desencadeiam muitas patologias, tais como a inflamação do intestino, cancro do colon e do reto. Portanto, é essencial o estudo da microbiota intestinal para estabelecimento do seu perfil e avaliar a sua responsabilidade em quadros patológicos. Em particular, o caso das mulheres que sofrem de incontinência fecal, é importe compreensão das interações entre os pathobiontes e o hospedeiro.

A incontinência fecal (IF), é uma alteração da fisiologia do esfíncter anal, permitindo a passagem da massa fecal sem a consciência do paciente. A disfunção de esfíncter anal nas mulheres, resulta na rotura de musculo anal durante parto, levando à destruição do nervo pudendo responsável na transmissão da sensação na zona anal e do reto. Entretanto, sem essa sensação o resultado é a evacuação inconsciente que denominada de incontinência fecal. A

massa fecal tem uma permanência curta no colon, o que pode resultar no desenvolvimento da resistência a antibiótico caso o doente esteja sob antibioterapia.

A resistência aos antibióticos, resulta da capacidade das bactérias possuírem mecanismos que anulam a ação do antibiótico. Esses mecanismos, podem ser intrínsecos ou adquirido. A transferência horizontal de genes (THG) está muito envolvida no desenvolvimento de resistências. A TGH pode ocorrer através do processo de conjugação, transformação ou pela transdução.

Presente estudo teve como principal objetivo isolar membros da ordem *Enterobacterales* de pacientes que sofrem IF e proceder à sua caracterização e determinação da sua suscetibilidade aos antibióticos e estabelecer a sua relação filogenética, através de BOX-PCR.

Neste estudo foram recolhidas 25 amostras fecais proveniente das mulheres com idade igual ou superior a 60 anos da região do Algarve que sofrem da incontinência fecal.

O isolamento de bactérias entéricas nestas amostras, foi realizado em meio de cultura MacConkey. As colónias isoladas, foram submetidas à coloração do Gram. Os isolados com características de gram-negativas, foram selecionados para identificação através da galeria Remel RapID^{TM} ONE. No total foram identificados 72 isolados através deste sistema. Os isolados mais frequentes foram as bactérias *Escherichia. coli (44%)*, *Shigella sp. (11%)*, *Klebsiella pneumoniae (10%)*, e *E. coli 0157:H7(9%).* Entre as 25 amostras fecais analisadas, as IF25, IF20, IF18 são as que apresentam maior diversidade de bactérias entéricas.

O teste de suscetibilidade aos antibióticos foi realizado utilizando o método da difusão de Kirby-Bauer em meio de Muller Hinton agar. Este método baseia-se no procedimento segundo as normas do EUCAST (Version 14.0, 2024. http://www.eucast.org.). Foram utilizados antibióticos das classes de penicilinas, aminoglicosídeos, fluoroquinolonas, Carbapenem, Cefalosporina da 3º geração, Sulfonamidas e Tetraciclinas.

Das 72 isolados submetido ao teste de suscetibilidade, foram observados diferentes perfis da resistência. Dentre os quais, o perfil da resistência à amoxicilina (AMC) foi o mais frequente, incluindo 52% da resistência de entre todos os isolados, seguido da resistência à amicacina (AK) 28% e ampicilina (AMP) 24%.

Quanto a multirresistência (MDR), foi mais observada na classe das penicilinas com 72,2%, seguida pelo amiglucosideos (AK) 38,8%.

Quanto às relações filogenéticas, foi utilizado o método de BOX-PCR. A extração do DNA dos isolados bacterianos foi realizada com o kit Bioline DNA extraction. O DNA foi quantificado no Nanodrop e a sua integridade verificada em gel de agarose à 1% (p/v). A amplificação foi feita, utilizando o primer BOX-A1R, e posteriormente os fragmentos da amplificação foram visualizados em gel de agarose. O gel foi corado com brometo de etídio durante 30 minutos e observado sob luz Ultra Violeta.

As imagens dos géis agarose foram analisadas no programa Gelcompar II para estabelecer relação filogenética dos isolados utilizando o teste de similaridade de Dice.

Com este método foram identificados 4 agrupamentos principais. Dentro deles, foram observados 27 sub-grupos com 80% a 94,7% de grau da similaridade.

Para concluir, neste estudo foram identificados diferentes perfis da resistência aos antibióticos entre os 72 isolados analisados. Em três amostras (IF2, IF10, IF23) foi apenas possível isolar uma espécie. As resistências observadas alertam-nos para a necessária continuidade no seguimento destes perfis de resistência nestes pacientes.

Em relação aos resultados da análise de relações filogenéticas por BOX-PCR, o resultado permitiu agrupar os isolados pelo género e espécie, mas não pela sua origem. Pelo que outras abordagens genómicas poderão ser testadas

Palavras-chave: Incontinência fecal, microbiota intestinal, *Enterobacterales*, resistência aos antibióticos.

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ADON: Adonitol

AK: Amikacin

AMC: Amoxicillin

AMP: Ampicillin

CAZ: Ceftazidime

CFU: colony-forming units

CNS: Central nervous system

DNA: Deoxyribonucleic Acid

dNTP: Deoxynucleoside triphosphate

BOX-PCR: repetitive intergenic consensus sequence- polymerase chain reaction

et al. - et alia (and others)

EUCAST: European Committee on Antimicrobial Susceptibility Testing

FI: Faecal Incontinence

g: grams

GABA: gamma-aminobutyric acid

CN: Gentamicin

ID: Identity

i.e., that is

IMP: Imipenem

IND: Tryptophane

MDR: Multidrug-resistant

MHA: Mueller Hinton Agar

MRP: Meropenem

MXF: Moxifloxacin

PBS: Phosphate-buffered saline

PRL: Piperacillin

PRO: Proline-β-naphthylamide

PYR: Pyrrolidonyl-β-naphthylamide

RP: Resistance profile

SXT: Trimethoprim-sulfamethoxazole

TSB: Tryptic Soy Broth

TSA: Tryptic Soy Agar

TZP: Piperacillin-tazobactam

TGC: Tigecycline

TAE: Tris-acetate–EDTA

UPGMA: Unweighted Pair Group Method using Arithmetic Averages

UV: Ultraviolet

WGS: whole genome sequencing

1. INTRODUCTION

Human beings are born normally free of microorganisms. During birth, we acquire different type of microorganisms that later join and colonize different parts, according to the environment that favours them to thrive in our body as shown in figure 1. During breastfeeding, meals also lead us to acquire different sorts of microorganisms. All these microorganisms come together to form intestinal microbiota, from which the member of the *Enterobacterales* order emerges (Farmer et al., 1985). The *Enterobacterale* order are recognized in many clinical contexts due to enteritis diseases (Farmer et al., 1985). *Escherichia coli*, *Klebsilla pneumoniae*, are example of some enterobacterias pathogen (Belotserkovsky & Sansonetti, 2018). In the other hand, they are easy to be transmitted, and they possess excellent mechanism in antibiotic resistance (Dandachi et al., 2016). They reside in a long system which contain different zone known as gastrointestinal (GI) tract where occure digestive process as shown in figure 2. The GI tract is 250-400m^2 length. This long surface connects, the host with environment. The interaction and exchanges that happen among bacteria and our body is made by GI connection. The colon is formed by ascending, transverse, descending, sigmoid, rectum and appendix caecum. This tract harbors diverse and dynamic community of microbiome, consisting basically in bacteria, fungi, virome (Tojo et al., 2014). This community of bacteria mediate energy metabolism (Zafar & Saier, 2021). The Large intestine is a region responsible for absorption of digestive fluid, vitamins production, and it is where the fermentation of undigested food by gut microbiota occurs (Jandhyala et al., 2015)(Greenwood-Van Meerveld et al., 2017). Within it, many exchange take place like mutualism, symbiosis and interaction among microorganisms (Litvak et al., 2019)(Hou et al., 2022).

Antimicrobial resistance is a menace that led us concern about infectious disease. The capability that Enterobacterias or other microorganisms owns to support and growth amidst exposition of substance which used to treat infection they caused, is known antimicrobial resistance (Harbottle et al., 2006). This adaptation that microorganisms do, lead them to be resistance to many antimicrobials for instance: antibiotic resistance, ant-retroviral resistance, anti-fungal resistance, and anti-parasite resistance. This study will focus on determining antibiotic susceptibility and establish genetic relationship of the member of *Enterobacterales* order.

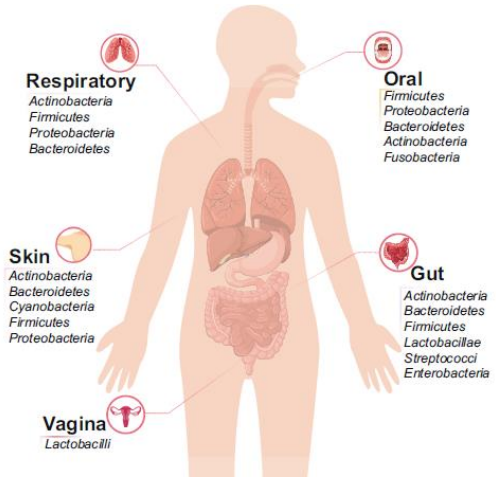

Fig. 1 Human microbiota localization. Image adapted (Hout et al. 2022)

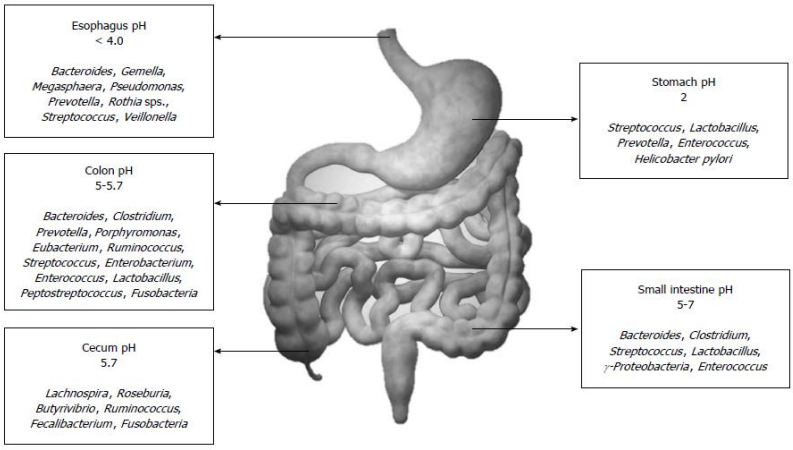

Fig.2 Anatomy of the gastrointestinal tract. Image adapted (Jandhyala et al., 2015)

1.1. Microbiota

1.2. Intestinal microbiota

It is not only microbes but all their genes, and genomes (Quigley, 2017). The gut microbiota it is a large community of microbes that dwell in the gastrointestinal tract and contribute to maintenance and fortify our immune system. Beyond strengthen our defense system, they are responsible in metabolic functions. Some research unveils their deep impact on brain development, function of central nervous system (CNS), neurogenesis, and its great

connection with enteric and central nervous systems through the “gut-brain-axis” (Strandwitz, 2018). In them, symbiotic harmony develops inside the host´s intestine, where bacteria and host can take advantage of it. So, the host provides a propitious environment for bacterias to develop, and microbiota contributes significantly to preservation of intestinal integrity, vitamin production, short-chain fatty acids production and defense against pathogens (Banaszak et al., 2023). Additionally, scientific study proved that intestinal microbiota could synthetize metabolites with high neuroactive function (neurotransmitters) for instance: norepinephrine, tryptamine, serotonin, dopamine, and γ-aminobutyric acid (GABA) (Otaru et al., 2021), the short-chain fatty acids (SCFAs) (Louis & Flint, 2017). Another essential compound synthetize by microbiome is vitamin k2 (menaquinone), which help in blood clot process and bone maintenance (Conly & Stein, 1992) (NODA et al., 1994). However, the living of microbiome is due to varied chemical environment that occurs in the colon structure (Berrilli et al., 2012). Each intestinal section has diversity microbial community and density as shown in figure 2. The mutual relation among the bacterias has originated around thousands of years ago, through their strong liaison. (Thursby & Juge, 2017; Zafar & Saier, 2021). There are 10^{12} - 10^{14} microbiome present in our intestine in a steady and dynamic and relation. Some studies brought us some knowledge regarding mycobiome (fungi) that they might play an essential function in nutrient metabolism. The mycobiome constitute roughly 0.1% of gastro-intestinal microorganisms in symbiose with bacterias (Li et al., 2018).

The onset of many intestinal diseases can be promoted by reduction of microbiome. The phageomes are considered intestinal microbiota (Ma et al., 2018). The phageomes are grouped in two categories: First, consists of lytic phages, which reproduce within bacterial host cell and later destroy it, releasing great viral particles (Hobbs & Abedon, 2016). The second one, a lysogenic, this can use both lytic and lysogenic cycles to reproduce.

The lysogenic cycle, the phage normally integrates its genetic material into the host cell´s DNA, begetting replication alongside the host, without generating virions. However, due to environment variation, the lysogenic phages can change in lytic cycle, begetting destruction of the host cell, leading to bacteria death (Echols, n.d.). Therefore, with this destruction, the phages can alter intestinal microbiota, which can cause intestinal permeability and chronic inflammation (Tetz et al., 2017). In this sense, the phages have an interesting role in regulate intestinal bacteria and later lead to intestinal dysbiosis (Dalmasso et al., 2014).

1.3. Intestinal dysbiosis

During the very beging of life, we had contact with microbes, and they initiate to colonize our intestines. These bacterias help us in various ways, some are mutualists, giving us support to digest food and protect us alongside pathogens. These bacteria live in harmonious association with us, obtaining shelter without harming us, and promoting mutualism (*Commensal Bacteria and Immunity of the Gastrointestinal*, n.d.). Some benigns bacteria are involved in bacteriocins production, the substance which acting as antibiotic to another different bacterias; food digestion, vitamins protuction, and strengthen the immune system (Heilbronner et al., 2021) (Lynch & Pedersen, 2016). Despite the good relations that bacteria show off, there are opportunistic one, that might cause disease in some people with weak immune like *Escherichia coli* (Denamur et al., 2021) *and Klebsiella pneumoniae* (Karampatakis et al., 2023).

When emerge an imbalance among the microbiota dwelling in intestinal environment, is called dysbiosis. The unbalance can occur due to many processes for instance the lytic process of phage, some bacitracin production done by some the same of the member of the *enterobacterales* order or by long used of antibiotic. All these as said previously, can changes composition, diversity, and function of the enterobacteria, causing alteration of the host´s immune response, and physiological process (Petersen & Round, 2014). The unbalance many times it involves changes in abundance of specific bacteria level, and decrease beneficial one (Gaucher et al., 2019) or increase pathogenic one (Cigarran Guldris et al., 2017).

The signs of gut dysbiosis related to reduction in microbial diversity, is characterized through losses of benign species and unbalance among them. Apart of the species reduction bacteria related to dysbiosis, the alteration in nutrient metabolism, and immune regulation can be affected by dysbiosis, as well and interpreted as a negative effect (Landman & Quévrain, 2016) (Hooks & O'Malley, 2017).

1.4. The cause of dysbiosis

The intestinal dysbiosis can arise from various factors depending on the situation. According to some researchers, have revealed that prolonged use of broad-spectrum antibiotics can eliminate both beneficial bacteria and pathogenic (Becattini et al., 2016) (Duan et al., 2022) (Gnatzy et al., 2023). Aging also is contributing factor (Malik et al., 2023). Diet it is essential for all living being. However, an unbalanced diet can reduce beneficia bacteria

while increasing the pathogenic ones (David et al., 2014), (Cuevas-Sierra et al., 2019). High consumption of processed is also related (Sugihara et al., 2019).

Excessive stress can affect intestine goblet cells resulting in decreased motility and mucin secretion. Mucins, when dissolved in intestinal fluid, it becomes mucus. Mucus forms biofilms, and when the goblet cells lose their capability, the intestine environment is altered, and directly impacting the composition of microbiota (Bansil & Turner, 2018) (stress and the gut, pathophysiology, clinical consequences, n.d.). Smoking it is another factor that can alter the composition of the intestinal microbiota, playing pro-tumourigenic role by altering intestinal metabolites and compromising the intestinal barrier function (Bai et al., 2022).

Intestinal parasites, including protozoa and helminthes, might secrete the molecules which can alter the gut environment, and jeopardizing the normal intestinal microbiota (Naveed & Abdullah, 2021).

1.5. Aging and dysbiosis

As we age, various body functions gradually decline, often accompanied by changes in health conditions, lifestyle, and dietary. Beyond the perceptible declines in mobility and cognitive abilities, there are also some changes in gut microbiota composition and activities (S. Kim & Jazwinski, 2018). Mucin, a sort of glycoprotein synthetized by goblet cells which displays an important function by protecting and lubricating hollow surfaces of the body essentially the gastrointestinal, respiratory, and reproductive tracts (Bansil & Turner, 2018) (Hansson, 2020). This Propriety can be impacted negatively by dysbiosis preventing mucin production.

Moreover, the aging is contributing that affect the gastrointestinal motility (Saffrey, 2013), the muscle (wave-like contractions), the enteric nervous system (alteration in neurotransmitter levels) (Nguyen et al., 2022), and might alter intestinal physiology, and some changes in esophageal motility, neurons of the myenteric, and sphincters (Soenen et al., 2016). All these elements decrease intestine function causing alteration in intestinal biofilms.

1.1.1. The consequence of intestinal dysbiosis

when the relationship between host and bacteriome is disrupted, it can lead to a condition known as dysbiosis. The dysbiosis frequently it causes a rise in intestinal permeability and weaking the protective mucus defenses (Ohtani & Hara, 2021), disrupting homeostasis, and

develop various inflammatory disease, irritable bowel, metabolic disease, obesity, and allergies (Banaszak et al., 2023).

1.1.2. Symptoms

Symptoms related to intestinal unbalance can vary from person to person. Normally, abnormality include the digestive problem such as bloating, gas, diarrhea, or constipation (the hard stools), discomfort, tiredness, mood disturbances or mental health problem, the skin disease such like eczema, the food intolerance, even allergies teased by food (Di Rosa et al., 2023)(Iancu et al., 2023)(Banaszak et al., 2023).

1.1.3. Diagnosis and treatment

Diagnosis of the intestinal dysbiosis, can involve the comprehensive evaluation of medical history of patient, the symptoms, and analyses of the patient stool to identify the composition and diversity of intestinal microbiota (Y. Yang et al., 2021).

The strategies of treatment and management to intestinal dysbiosis, tend to restore a healthy equilibrium of the intestinal microbiota and can include use of probiotics, dietary changes, managing stress, avoiding the trigger foods, diminish the use of antibiotic.

1.6. Faecal incontinence

The interaction between the anal sphincter and pudendal nerves work to control rectal muscle, preventing involuntary loss of rectal contents (faeces or gas). However, this process depends on faecal consistency and the characterist of the rectal reservoir. When this process disfunctions, delaying evacuation at appropriate time may become difficult. The structure of the anus is 2 to 4 cm in the length and consists in internal of external sphincter, with 0.3-0.5 cm in thickness as shown in figure 3 (Rao, 2004)(Kumar & Emmanuel, 2017).

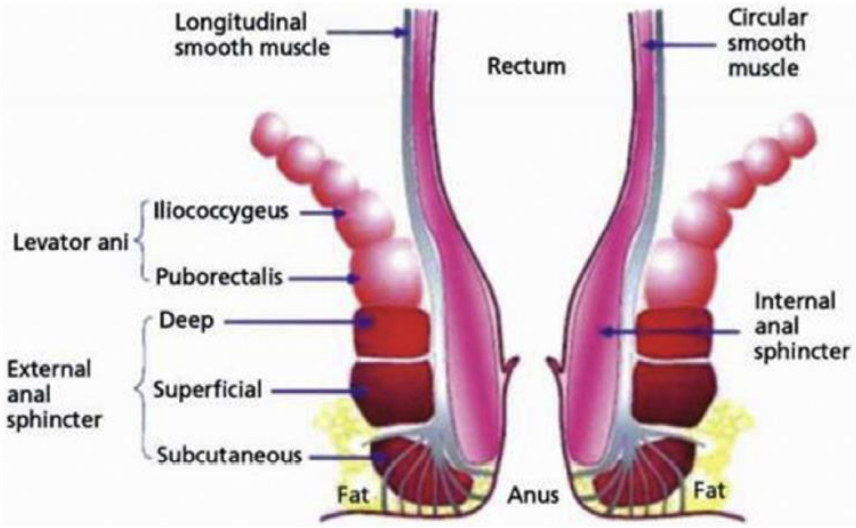

Fig. 3. The anatomy of anal sphincter. The image adapted (kumar and emmanuel, 2017)

1.1.4. Causes and consequences faecal incontinence

Due to the patient´s shame in revealing their suffering, some anomalies linked to or congenital malformations, the degenerative conditions, and the neurological disorders, are issue related to faecal incontinence occurrence (Bharucha et al., 2015).

Faecal incontinence is classified into passive incontinence which result in involuntary evacuation without being aware; The urge incontinence, this happen, even with active attempts to retain contents; The faecal seepage, it is faecal leakage that happen in people with a normal continence depend on faecal consistency and situation (Ruiz & Kaiser, 2017).

Demoralization among patients that suffering faecal incontinence led to psychological challenges that increase familial burden. Affected patients may experience social restriction due to low self-esteem, and altered perception of their bodies, which can have negative psychosocial effects and undermine their daily lives, placing additional on caregivers (Managing Passive Incontinence and Incomplete Evacuation, 2013).

1.1.5. Risk factor for Faecal Incontinent in Older Patients

Given their advanced age, patients may suffer from immobility, diarrhea, faecal constipation, rectal prolapse, the anal sphincter injury or change in anorectal sensation. With aging, the enteric nervous system and release of neurotransmitters may gradually decrease, and myenteric ganglia, also can be affected and resulting in alteration of intestinal motility

(María et al., 2018). Due to sequelae caused by childbirth, the faecal incontinence may originate from nerve damage, rectal prolapse, or anal sphincter tears. All these conditions can affect woman´s quality of life (Bharucha et al., 2015).

1.1.6. Diagnosis

Due to the patient´s shame on revealing their suffering, the clinical observation is neecessary to obtain the pelvis with electromyography to ascertain the truth, and physical examination must be accompanied macroscopically to verify if there is some sphincter injury (Assmann et al., 2022) (Reza et al., 2024).

1.1.7. Treatment

Sometimes, the treatment is based on intestinal training to develop standard evacuation. To achieve this, adequate ingestion is necessary, and some habits is needed to be quit for instance the coffee consumption. This is to lessen the stool consistency and neuronal stimulation (Assmann et al., 2022) (Reza et al., 2024).

1.1.8. *Enterobacterales* order

The *Enterobacteria* known *as Enterobacterale,* are a group of gram-negative bacteria found in the human being intestine and forming bacteriome. They display an important contribution in maintaining the equilibrium of intestinal bacteriome (Farmer et al., 1985). However, some of them suck as *Escherichia coli* can be commensal and pathogenic. *E. coli* is a non-pathogenic form known as commensal strain which form the normal intestinal bacteriome, as mentioned above, and helps prevent the growth of pathogenic bacteria. Its pathogenic form is responsible for food poisoning, gastroenteritis and the and the production of colibactin (Tenaillon et al., 2010a) (Secher et al., 2013)(Pennington, 2010)(S. C. Yang et al., 2017)

The *Shigella* is a genus of bacteria discovered by Japanese physician Kiyoshi Shiga. Since its discovery, it has become great concern in medical field due to bacillary dysentery in the last century and continues to pose a challenge in African country with low healthcare resources and sanitary knowledge. This *genus* include *Shigella* consist in *S. dysenteriae, S. flexneri, S. boydii, and S. sonnei* (Lampel et al., 2018).

The *genus Klebsiella* is responsible for many of nosocomial infection. Its transmission among patients in hospitals primarily occurs through hospital staff (Farmer et al., 1985; Podschun & Ullmann, 1998)

1.1.9. Faecal incontinence related to resistance of antibiotic

Faecal incontinence is defined as failure to contain faecal material and can involve decontrolled passage for at least three or more months. In the contrast, anal incontinence presents challenges regarding to managing the release of faecal waste and gas (Rao et al., 2016). These complex situations are influenced by many factors, including muscle function, nerve pudendal control, and all gastrointestinal tract (Kumar & Emmanuel, 2017).

The main issue that can lead to faecal incontinence is a rupture of anal muscle responsible to hold unwanted passage of faecal waste during vaginal labour. This rupture is known as anal sphincter tear or laceration. In addition, the anal sphincter laceration is more common in primogest resulting in damage of pudendal nerve as shown in figure 4 (*Anal-Sphincter Disruption during Vaginal Delivery*, n.d.; Stiens et al., 1997; Urbankova et al., 2019) .

Beyond the childbirth, other factors can contribute to faecal incontinence in women, especially the anal sex. Some women adore anal sex, to please their partners to mantain their relationship. This practice led to some risk, such as fissure and rectal haemorrhage, and the issue related to intestinal problem and even disease spread (Faustino, 2021). The tear can happen in anus because of anal tissue which is fragile than vaginal one.

Ultimately, faecal incontinence in women can impose significant burden on their daily lives discouraging them to participate in social events. This short duration may not allow the antibiotic to reach their target effectively and complete its action.

1.1.10. Antimicrobial resistance in *enterobacterales* order

1.1.11. Antibiotic

Meanwhile, any molecule synthesized by microbe and possesses the capacity to prevent growth or kill other microbes while being relatively harmless to human being cells is described as an antibiotic (Clardy et al., 2009).

Antimicrobials, on the other hand, are a group of substances used used to treat infection caused by microbe. These substances are categorized according to their capacity to destroy microbes, for instance antibiotics, antivirals, antifungals, antiparasitic. They are synthetized by certain microorganisms such as *Fungi of the genus penicillium* and *Actinomycetes*. The

antibiotics discovery of antibiotics marked excellent breakthrough in the medical field, thanks to the miraculous work of British bacteriologist Alexander Fleming in 1928! (*Antimicrobial Action*, n.d.; Hutchings et al., 2019). This remarkable progress occurred when the Alexander´s staphylococcus plate bacteria was unintentionally contaminated by *fungi penicillium*.

Nowadays, the group of bacteria residing in soil is known to be producer of large number of metabolites called antibiotics, which are used both medical and farm agricultural fields (Bhatti et al., 2017; Clardy et al., 2009; van der Meij et al., 2017). When bacteria overcome the mechanisms of antibiotic action, they become resistant to those antibiotics, making infections caused by these microbes difficult to be treat.

1.1.12. Susceptibility test

It is important to determine the susceptibility of bacteria to ensure adequate treatment for the patient. The most common methods to unveil antibiotic resistance is based on Kirby-Bauer disk diffusion method. This method allows us identify bacteria that are susceptible, those that show increased exposure, and those that are resistant. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) provides guidelines recommended for use in Europe (Version 13.1, 2024).

Drawing on this information, it is possible to choose appropriate therapy to treat patients. Bcteria resistant to more than one group of antimicrobials, are considered multi-drug-resistant (MDR).

This study focuses on the members of the *Enterobacterales* order. The high risk of developing antimicrobial resistance is more pronounced within this group, making them a significant concern in the medical field (Dandachi et al., 2016). Many antimicrobial agents are required realize the Kirby-Bauer disk diffusion method, which can either inhibit bacterial growth (bacteriostatic), or cause their death (bactericidal) (Nemeth et al., 2015)

Antibiotic targets can prevent cell wall synthesis, protein synthesis, nuclei acid synthesis, and antimetabolite effects, depriving bacteria from its essential metabolism of living (Ullah & Ali, 2017)(Pancu et al., 2021)

Classes of the antibiotics for instance: β-lactam, penicillins, cephalosporin and carbapenems are effective inhibitors of the cell wall synthesis due to the β-lactam ring they possess. This process allow instability within the cell to prevent the cell growth by blocking connection between the peptides. This process leads to instability within the cell, because the selective permeability is no longer exist (Pancu et al., 2021).

The penicillin class is effective against many gram-negative bacteria; however, ineffective against β-lactamases producers (Ullah & Ali, 2017). Those broad-spectrum antibiotics that are considered to and neutralize the β-lactamases enzyme producers, allowing bacteria destruction to occure (Pancu et al., 2021).

Additionally, disease caused by gram-positive or gram-negative bacteria can be treated with carbapenems, which have broad-spectrum of activity against many bacterias, both aerobic and anaerobic one (Ullah & Ali, 2017)(Pancu et al., 2021).

Regarding protein synthesis inhibitors in bacteria, tetracyclines are chosen, for their capability to block RNAt entry in 30S or 50Ss. In this case, the polypeptide chain cannot be synthesized (Ullah & Ali, 2017).

As for nucleic acid inhibitors concern, fluoroquinolones are selected to hinder activity of topoisomerase and DNA gyrase. When these two enzymes are blocked, DNA replication remain inactive, inhibiting bacterial cell division (Ullah & Ali, 2017).

About antimetabolites, the inhibition in synthesis of dihydrofolic and tetrahydrofolic acids synthesis prevent purine formation. If there are not purines, DNA synthesis, cannot occur, leading to bacterial destruction (Ullah & Ali, 2017).

1.1.13. Historical context of antibiotic resistance

1.1.14. Antibiotic resistance in the world

The antibiotic resistance in United Stated is responsible for two million case of disease and twenty-three thousand demises, (Antibiotic Resistance Threats in the United States, 2019, 2019). In European Union, multiresistant infections are responsible for twenty-five deaths due to these infections (Machowska & Lundborg, 2019). In west Africa countries, where there is a lack of well-trained professionals and adequate health infrastructure combined with improper behaviour, the results are expected to worsen (Ouedraogo et al., 2017) .

1.1.15. Resistance

The incorrect use of antibiotic can result in antibiotic resistance. Th antibiotic resistance is a mechanism developed by bacteria to circumvent the effect of antibiotics used to treat them. This process has begun before discovery of antibiotics and it has increased due to improper use of antibiotic(Perry et al., 2016). The strategies employed by bacterias, when they

encounter the adverse environment created by antibiotic enable them to thrive despite their presence (Baran et al., 2023). The antibiotics resistance poses a significant a menace to humanity by jeopardise the effective treatments. When this occure, the disease may take longer time to treat, increasing healthcare costs and exacerbating the risk of spreading infection in the community, especially considering the lack of new antibiotic production (Lee Ventola, 2015) . The world health organization concerned about the rapid emerge of antibiotic resistance, which arise from country to country due to improper behaviour in various field (Christaki et al., 2020).

1.1.16. The cause of antibiotic resistance

The practice of self-medication without medical guidance, obtained via internet or purchased from sources where the acquiring antibiotics is easy, is acknowledged as hindrance halting the proliferation of antibiotic resistance and ensuring public health, according to European enquiry (Safety, n.d.).

1.1.17. Mechanisms of Antibiotic Resistance

1.1.18. How can bacteria become resistant to antibiotic?

To escape the lethal action of a class of antibiotic, bacteria must possess pathways to overcome these obstacles. This challenge has led them to develop many mechanisms to fight-back and become multi-resistance. The single bacterial cell may use a range of mechanisms to neutralize the effects of an antibiotic and develop mutation in its genes which encode the target site. In this way, they can express efflux pumps to expel the drug from the cell allowing the bacteria survive (Christaki et al., 2020).

1.1.19. Type of Antibiotic Resistance

The bacterias can demonstrate antibiotic resistance I two forms: intrinsic and acquired one. However, it is known that both types, harbors irreversible phenotype.

The antibiotic resistance that bacteria possess before getting contact with antibiotic is considered intrinsic, and the one which it begot after haven contact with antibiotic is known as acquired resistance.

1.1.20. The Intrinsic resistance

Is proper feature that all bacterium species possesses before getting contact with antibiotic, this includes the glycopeptide resistance displayed by Gram-negative bacteria. Due to this characterist they reveal outer membrane impermeable through mechanism involving biochemical pathways that allow them to survive in the presence of antibiotics as shown in figure 4 (Blair et al., 2015; Cox & Wright, 2013) .

1.1.21. The Acquired resistance

Acquired resistance occure after previous contact with antibiotics in sensitive bacteria. Therefore, the newly mutated genetic material can be shared with other bacteria through horizontal gene transfer. This transfer of genetic material can occur, through three essential mechanisms such as *Transduction, Conjugation, Transformation* (Fernández et al., 2011; Lee, 2019)(Munita & Arias, 2016).

Additionally, certain conditions may act as cues to initiate acquire resistance. This may be due to environmental signals (e.g. stress, growth state, pH, concentrations of ions, nutrient conditions, sub-inhibitory levels of antibiotics) that help bacteria to recognize when to begin production mutations (Fernández et al., 2011)

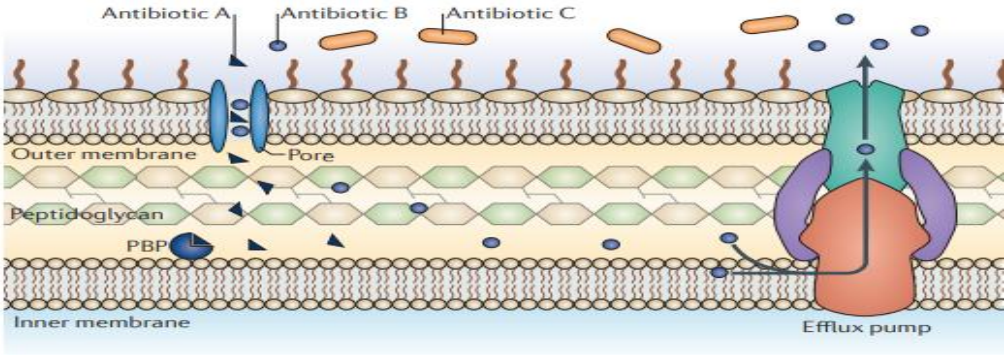

Fig. 4 The figure demonstrate details of intrinsic resistance mechanisms. adapted image (Blair et al., 2015)

Having antibiotic without following the physician´s guidance, is a reckless practice that undermine the antibiotic control (Llor & Cots, 2009). A regular approach based on 35 community studies conducted in all country of the world, from 1970 until 2009, has showed 19 up to 100% of individuals engaged in self-medication in north of Europe and America (Morgan et al., 2011).

In Portugal, according to a case study held in matosinhos, unveil that 3.4% of self-medication in local community succeed in treatment by antibiotic without medical consent. Also, in another study in Portugal, has revealed that Portuguese people tend to use more leftover antibiotic and has the highest rate of antimicrobial resistance compared to other European countries (Ramalhinho et al., 2014).

In Africa, the problem is more severe than Europe due to the lack of physician, inadequate hospitals, counterfeit of antibiotic lifestyle factors accompanied by socio-cultural behavior all of which exacerbate the emergence of antibiotic resistance (Ouedraogo et al., 2017).

In Guinea-Bissau, this threat is not well known among the population due to lack of information. Through a culture of sharing belongings, people exchange medicines within their communities; Pharmacist in village sells medications without proper licenses, and some pharmacist, lacking sufficient knowledge in pharmaceuticals field, don't own proper conditions for storing and sell the medicines. In both village and cities, individuals often discard antibiotic in common trash or on the ground, exposing them to other bacteria. Additionally, many health centers or hospitals, lack incinerator to incinerate some expired. The significant issue stems from a lack of commitment on the part of government, the ministry of health and National Health Institute (INASA) to improve sanitary conditions, strengthen the diagnostic quality, and ensure drug control laboratory at the National Public Health Laboratory (LNSP) operate effectively.

Pathogenic bacteria that showed resistance to most of antibiotic used to treat disease, have been identified in the well water of Guinea-Bissau. This problem not only increase the proliferation of antibiotic-resistant genes through well water consumption but may lead endemic multidrug-resistant infections (Machado & Bordalo, 2014). Furthermore, a study conducted at LNSP in Bissau revealed that all sources of water available for consumption in Guinea-Bissau were contaminated (Bancessi et al., 2020). From 2014 to 2020, no action was taken to address the lack of potable water. This study reinforces the idea that conditions in Guinea-Bissau still exist for the spread of antibiotic resistance Bacteria, which can impose to a significant health and economic burden on poor communities.

The ecosystem can be affected by rising temperature promoted by climate change. When the temperature rise above normal, it can cause land drought and force to relocate the safe areas. Additionally, an increase in biting insect can occur due to a reduction in their predators, leading to a rise in infectious disease (Burnham, 2021). Furthermore, water scarcity will pose significant challenges due to many factors such as deforestation and environment pollution, all which decreases microbial diversity due destruction of natural barriers that

prevent the spread of antibiotic resistance (Rzymski et al., 2024). In the other hand, improper disposal of pharmaceutical waste can result in high concentration of antibiotics found in African and European rivers, with 50µl/L detected in African rivers compared to 10µl/L for European ones (Eduardo-Correia et al., 2020) is another problem in ecosystem destruction and antibiotic resistance spread.

Bacteria collect DNA debris, which sometimes include genes that carry information about antibiotic resistance. This method of acquiring gene element from deceased bacterial cells suport the Frederich Griffith discovery in 1928, attributing the transformation as a factor in propagation of resistance (Riffith, n.d.).

Concerning all these factors, antibiotics-resistant bacteria could be the responsible of 700,000 deaths yearly, and estimated suggesting even more death in the future (Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations, n.d.)

1.1.22. Gene transfer among bacterias

Genes, as we know are hereditary complex found in living being organisms and are responsible for transmitting hereditary identity to the descendants. Therefore, when a given bacterium, beget new bacteria, it means, the descendant will possess the same genetical information as their ancestors. In addition to being transmitted through conjugation, transformation and transduction are also other forms responsible for spreading antibiotic resistance genes am bacteria. (Manson et al., 2010). When bacteria encounter debris in the environment from other dead bacteria that contain genes related to antibiotic resistance, this may lead to transformation. This process is expected to be slow because bacterial debris is dispersed in the environment. Given these conditions, the process may not work due to various factors (Munita & Arias, 2016; Rodríguez-Beltrán et al., 2021). When a bacteriophage infects bacteria, it can incorparote plasmid DNA into its genome and subsequently infect another bacterium. The bacteriophage acts as a vector, fransfering resistant genes from resistant bacteria to non-resistant ones. Once the plasmid DNA enters the new host, it is rearranged into its original form. Additionally, bacteriophages are responsible for gene transfer between related bacteria (Hobbs & Abedon, 2016; Rodríguez-Beltrán et al., 2021). Conjugation is another mechanism, which requires attachment between two different bacterial cells which allows transferring genetic material known as reproduction process. During this

process antibiotic resistance genes can be shared among bacteria, resulting in spread of antibiotic resistance (Rodríguez-Beltrán et al., 2021).

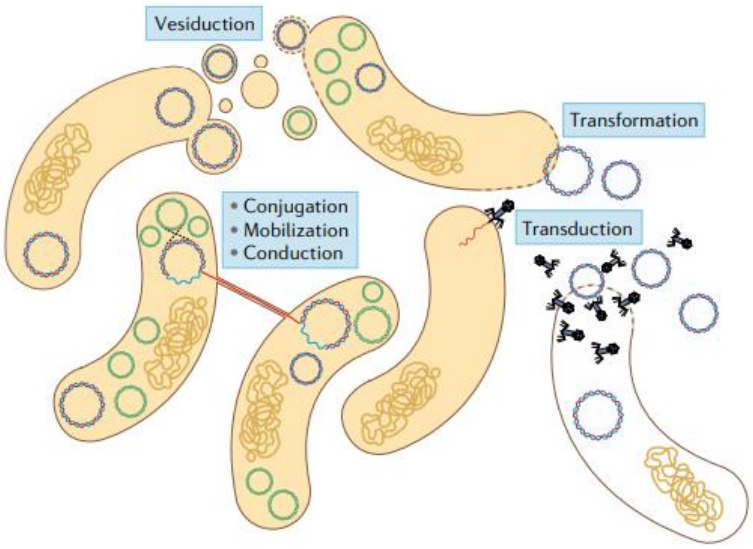

Fig. 5 -Schematic representation of the different forms of gene transfer between bacteria. A: Transformation, B: Transduction and: Conjugation adapted image (Rodríguez-Beltrán et al. 2021)

1.7. GENETIC MUTATIONS

1.1.23. Mutation

Mutations are error that can occur during DNA replication, where the nucleotide triplets can be affected due to reason such as insertion or deletion of nucleotides, promoting carcinogenesis. Consequently, the cell cycle can be halted when checkpoints detect these errors. However, this phenomenon can go undetected and be transmitted to descendant bacterial cells. However, mutations can happen without any exogenous factors, although they can also be influenced by chemicals, ionizing radiation, or ultraviolet light. The blockage of the cell cycle during mitosis, is related to nucleotide dimerization, which deform DNA structure. As a result, replication enzymes such as DNA topoisomerase and Girase may fail to function due to the alterations that occur (Cuevas-Ramos et al., 2010). Apart from this, the transposons are another issue, by being able to jump within genome. This jumping behaviour can disrupt genes and cause irregularities, resulting in mutations that increase genetic plasticity, allowing bacteria to acquire virulence factors and become more pathogen (Khedkar et al., 2022; Kimball, 1978).

1.1.24. Plasmids

Plasmids are tiny molecules that replicate independently of chromosomal DNA. Gene can be transferred from chromosome to plasmids within bacterial genome. These elements acts like complete genome by harbouring an origin of replication (ori), virulence factors, resistance genes, and metabolic factor that enable production of enzymes. Furthermore, plasmids contain elements that autonomously control the initiation and termination of replication such as promotor and terminator, as shown in figure 6. While bacteria do not depend on plasmids for survival, they become essential for adapting to certain environmental change (Bennett, 2008). Plasmids containing antibiotic resistance genes are referred to as *plasmids R*, and they may be in all antibiotic-resistance bacteria (Harbottle et al., 2006; Poirel et al., 2018).

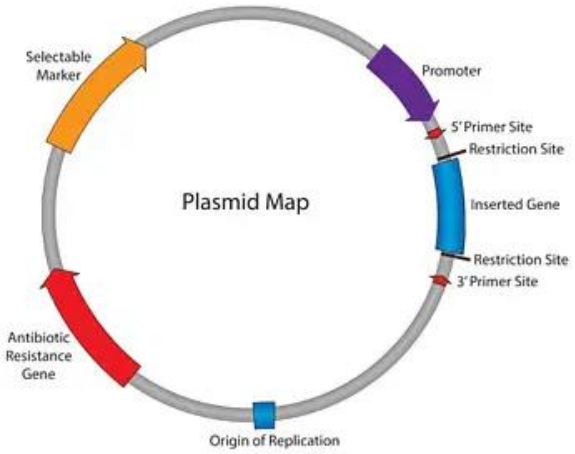

Figure 6 Schematic representation of plasmid adapted image at http://www.plasmids:properties, types, and function. Microbe online.

1.1.25. Insertion sequence (ISs)

Insertion sequence are tiny elements smaller than 2.5kb in genome length, that can be passed on among bacteria through reproduction by via transposase enzyme, which guide connection of sequence with transposons. Beyond reorganizing genome, the role of transposase promotes the spread of resistance and increases disease severity (Mugnier et al., 2009)

1.1.26. Transposons

Transposons constitute movable gene that jump aleatory among specific genome sites. Because of this random jumping, they may insert into regulatory regions or coding sequence, which can lead to loss of protein function and result in new mutations. There are two classes of these DNA sequences: retrotransposons which belong to class I, and are most frequently encountered in eukaryotes, and transposons which pertain class II. The latter can be found in the DNA genomes of both eukaryotes and prokaryotes

1.1.27. Retrotransposons

Retrotransposons are DNA templates derived from double strand RNA, that can be reverse-transcribed back into double strand DNS and be inserted into genome. This process is known as “copy and paste”.

1.1.28. Transposase

Without DNA intermediate, the transposon cannot transform into a DNA template, meaning that transposition cannot occur due to lack of enzyme transposase. This enzyme work by recognizing prime sequence at the 3´ and 5´ ends of transposons, cutting out the transposable fragment, and reinsert it elsewhere, a process known “cut and paste”. Transposons can jump from plasmids to chromosomal DNA and from plasmids to plasmid, which indicates that that antibiotic resistance genes can spread out among bacteria through transposons. When bacterias are antibiotic resistant, their transposons are also resistant, making them carriers of transposable vectors. (Babakhani & Oloomi, 2018).

1.1.29. Integrons

Integrons are system capable to capturing, reorganizing, integrating, and expressing exogenous DNA genes incorporated into promoters. Their purpose is to accumulate the resistance in cells and serve as source of it, contributing to adaptative characteriss. Integrons are composed of the integrase enzyme(intl), attachments site (attl), and promotor (Pc) (Gillings, 2014).

The integrase is encoded by integrase gene, which enhance recombination at the connection sites located in gene cassettes, allowing the promotor to activate the expression of gene cassettes once they are integrated into integrons. Integrons are two essential group: resistance integrons and superintegrons, resistance integrons are responsible for carrying genes that code for antibiotic resistance found in plasmids or chromosomes (Fluit & Schmitz, 2004; Richard et al., 2022)

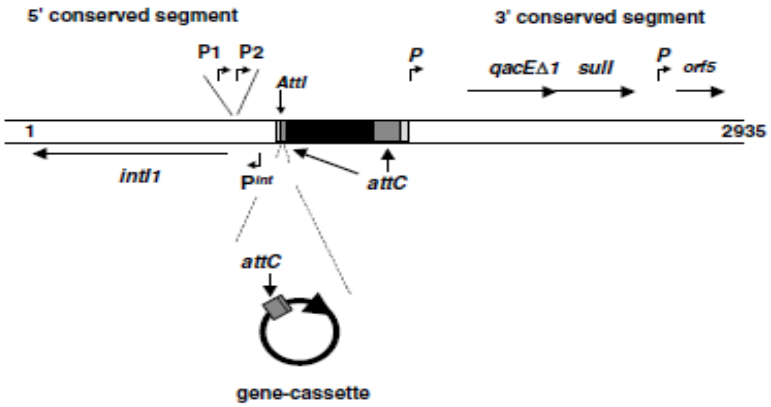

Fig.7 schematic representation of a class 1 integrons P1, promoter of transcription of gene cassettes; P2, it is not always active; *int*, integrase gene. Adaptive image (Fluit and Schmitz, 2004)

1.1.30. Genotyping of member of the *Enterobacterales* order

Before molecular technique were developed, bacterial genotyping was performed through phenotypical comparisons based on morphology, serotype, biotype, phage typing, or susceptibility to one or more antibiotic(Foxman et al., 2005). Nowady, genotyping displayed extraordinary role and can be classified into DNA analysis and data analysis. This procedure involves analyze of genetic variations through various molecular biology techniques, yielding substantial information regarding genetic variation and differences among species of microorganism based on genomic level characterist (Foxman et al., 2005)

DNA analysis is performed based using PCR-Methods that amplifies necessary sequences found in specific sites of *enterobacterial* genome, which harbours repetitive intergenic consensus (ERIC) sequences of 124 –127 bp well conserved state, as well BOX elements with 154 bp (Ishii & Sadowsky, 2009). Data analysis is based on bioinformatics tools and statistical software. These two tools, help to analyze and compare individual genetic profiles

of many microorganisms, particular member of *Enterobacterial*es order (Agapito, 2019). Repetitive extragenic palindromic are DNA sequences that disperse along the bacterial genome. These dispersed bacterial characteristics are clustered into three main types of repetition of different sizes: the REP sequence comprises 35-40 base pairs, the intergenic consensus (ERIC) sequences ranges from 124-127 base pairs, and BOX sequence contains 154 base pairs. (Versalovic1 et al., n.d.) (Matos et al., 2021).

1.1.31. BOX-PCR

The BOX-A1R primers are complementary to the conservative sequences present in the genomes of many bacterial species (Brusetti et al., 2008). These BOX elements can be found both in gram-negative and gram-positive bacteria. Thus, it is possible to generate multiple amplicons by employing specific primers designed to target these repetitive sequences. The size of these amplicons will differ from one bacterial species to another based on their genomic repetitive elements. The pattern of amplicons will represent genomic DNA of each isolate or strain, which can be analyzed through gel electrophoresis. Finally, the gel electrophoresis outcome will undergo to genecompar II analysis to determine similarity among isolate (Hiett & Seal, 2009; Jena et al., 2021)

2. THE OBJECTIVE

The principal objective of this study was, to isolate and characterize the members of the *enterobacterales* order from women that suffer from faecal incontinence in Algarve region. For this, our specific aims are:

1. Isolate and identify the member of the *Enterobacterales* order from faecal samples.
2. Determine the antibiotic susceptibility of the bacterial isolates.
3. Establish the genetic relationship of the bacterial isolates by BOX-PCR.

3. MATERIAL AND METHODS

3.1. Equipment and Reagents

1. Autoclave Uniclave 88 AJC (Lisbon, Portugal)
2. Analytical scale AE 200, Mettler (USA)
3. Analytical scale XS-410, Fisher Scientific (Portugal)
4. Multiplaces dry heating bath, Selecta (Spain)
5. Bio48 Laminar Flow Chamber, Faster (Italy)
6. Mini-V / PCR camera, Telstar (Spain)
7. Mikro 22R Centrifuge, Hettich Zentrifugen (UK)
8. Ultra-low temperature freezer -80ºC U725, Innova New Brunswick Scientific (USA)
9. Electrophoresis Power Supply - EPS 301 (USA)
10. Binder Incubator (Germany)
11. Kodak EDAS 290 (USA)
12. ATC 2000 Microscope, Leica (Portugal)
13. Agarose gel trough for PCR, Pharmacia Biotech GNA100, (USA)
14. Thermocycler T-Gradient (Biometra, Germany)
15. Thermocycler T-personal (Biometra, Germany)
16. Thermocycler T1 (Biometra, Germany)
17. Vortex L46, Labinco (The Netherlands)
18. Centrifugal tube (50ml) with graduation and flat caps, VWR, USA
19. Petri-DISH (90mm)), ABDOS, Belgium
20. Spatular, deltalab, Spain
21. Cotton Swabs, aptaca, Italy
22. Pipetas, VWR, USA (10 µL, 20 µL, 100 µL, 1000µL)
23. Macfarland Scale, Biomérieux, France
24. Pipettes of 5ml, 1ml, 200ml and their tips
25. pH meter GLP21 (Crison Instruments, Spain)
26. Eppendorf tube
27. Marker

3.2. Culture media

All culture medias have been prepared based on instructions of manufacture

1. VRBG agar (39 grams/l), Biokar, France
2. Mueller Hinton Broth (21 grams/l), Biokar, France
3. MacConkey agar (50 grams/l), Biokar, France
4. Trypto-Cadein soy Broth (30 grams/l), Biokar, France
5. Tryptic Soy agar (40grams/l), Biolife, Italy
6. Salmonella, Shigella agar (63 grams/l), Oxoid, England
7. Bacteriological agar (15 grams/l), Biokar, France
8. General Purpose Agar, Lonza, Denmark
9. Isolate II Genomic DNA kit, Bioline, England
10. Wizard^{R} Genomic DNA purification kit, Promega, USA

3.2.1. For Remel RapIDTMONE System

1. TSA plates (Biolife)
2. Cotton swabs
3. Bunsen burner
4. McFarland #2 turbidity standard or equivalent
5. Remel RapIDTM ONE System kit (Thermo Fisher Scientific, USA)
6. RapID Inoculation Fluid (CaCl2 0,05% + KCl 0,6%) (Thermo Fisher Scientific, USA)
7. 1000 μL pipette and pipette tips (VWR, USA)
8. Non-CO2 incubator (Rayda)
9. RapID ONE Reagent (Thermo Fisher Scientific, USA)
10. RapID Spot Indole Reagent (Thermo Fisher Scientific, USA)

3.2.2. Materials used in DNA extration

1. Falcon tubes with 10 ml of TSB medium
2. Nitrile gloves (Newmark)
3. Shaking water bath (Julabo SW20, Germany)
4. Nanodrop (Thermo Fisher Scientific, USA)
5. Bioline extraction kit (Bioline, USA) with:

- Lysis Buffer GL
- Proteinase K
- Lysis Buffer G3
- Wash Buffer GW1
- Wash Buffer GW2
- Elution Buffer G
- ISOLATE II Genomic DNA Spin Column

6. 1000/200/100 µl pipettes and pipette tips (VWR, USA)
7. Mikro 22centrifuge (Hettich zentrifugen, Germany)
8. Dry block heater (JPSELECTA, Spain)
9. Vortex mixer (Heidolph Instrument REAX 2000, Germany)
10. 100% ethanol
11. 2mL Collection Tube
12. 1.5 mL microcentrifuge tube
13. 1% agarose gel (Lonza Copenhagen, Denmark)
14. TAE buffer1X
15. Sybr-safe DNA gel stain
16. Electrophoresis power supply EPS301 and electrophoresis gel tray (Amersham Pharmacia Biotech, United Kingdom)
17. UVillumination (Gene Flash)

3.3. Solutions

- TSB media
- Alcohol 70%
- Phosphate Buffered Saline (PBS) - 8 g /L NaCl, 0.2 g / L KCl, 1.44 g / L Na2HPO4, 0.24 g / L KH2PO4
- Tris-Acetate-EDTA buffer (TAE) 50x - 242 g / L Tris base (Sigma), 57.1 mL Glacial acetic acid (Panreac), 100 mL 0.5 M EDTA pH 8.
- Kcl potassium chloride 0,6% and Calcium chloride CaCl2 0,05%
- MilliQ water.

3.4. BIOLOGICAL SAMPLES

The 25 samples used in this study, have been provided (0.5 grams of faecal) by lab 2.21 of ABC.RI center of university of Algarve.

Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
IF1	IF2	IF3	IF4	IF5	IF6	IF7	IF8	IF9	IF10	IF11
IF12	IF13	IF14	IF15	IF16	IF17	IF18	IF19	IF20	IF21	IF22
IF23	IF24	IF25								

Table 1. The samples collected (n=25) to this study.

3.4.1. Primers

The primers used in this test have been supplied by lab 2.21 of ABC-RI.

Primers	5'-3' sequence	Reference	Melting temperature (ºC)	Amplicon (bp)
BOXA1R	CTACGGCAAGGCGACGCTGACG	Versalovic *et al.*, 1991)	76.5	

Table 2. the primers BOXA1R used in this study.

3.4.2. Preparation of Culture Medium

The main procedure for preparing the medias is as follows:

Using a scale (Fisher Scientific XS-410, USA), the required amount of medium is weighed, and the necessary volume of distilled water is added to obtain the final volume needed. The mix is then thoroughly mixed using a magnetic stirrer (Agimatic) to homogenize the solute.

Afterward, a microwave (Panasonic, Japan) is used to melt and dissolve agar and sediment.

Finally, the medium undergoes autoclaving in UNICLAVE 88 (AJC, Portugal) for 20 minutes at 121ºc. MHA (Biokar Diagnostics, France), and VRBG agar media (Biokar, France)

after autoclaving, the media was poured onto Petri dishes and is labeled and allowed to cool at room temperature. For the PBS solution (NaCl-PanReac; and Na_2HPO_4-Merckl; KH_2PO_4-Montplet and Esteban), the salts are dissolved in 800 ml of distilled water, then mixing with stirrer. Next, the pH meter GLP21 (Crison Instruments, Spain) to adjust pH slightly neutral (≃ 7,4), which is necessary for it to function as an isotonic solution.

3.5. METHOD

3.5.1. The attendees of this study

The study involved 25 elderly women with faecal incontinence, aged 60 living in the Algarve region.

3.5.2. Isolation of Member of the *Enterobacterales* Order

After weighing 0.5 grams of faecal samples in 15 mL falcon tube, it was quickly diluted in TBS media, to homogenize the content. Then, series of dilution were prepared in six falcon tube. A 0.5ml portion of the homogenized dilution from the falcon tube one was transferred to tube two mixing thoroughly. This procedure was repeated from falcon tube two to falcon tube six to achieve dilutions from 10^{-1} to 10^{-6}. The alphabet letters A, B, C, D, E, etc., were used for data registration.

Next, 100µL was withdrawn from 10^{-3} and 10^{-4} dilutions, which are less concentrated, and placed on no-selected medium (Mac conkey plate), to obtain dispersed colonies, facilitating colony counting. Each dilution (C and D) was duplicated. The 100µL withdrawn was spread on MacConkey plates to achieve well-dispersed bacterial colonies. The plates were incubated overnight at 37º C in non-CO2 incubator.

The following day, the colonies were characterized based on their shape, morphology, and colour, identifying big, pink and lactose fermenter colonies. Th colonies were counted using loupe and counter. 136 colonies were counted per Mac-Conkey plate. The normal range is 30 to 300 colonies per plate within 100µl of diluted content.

After counting, one identified colony was inoculated onto a non-selective Tryptic Soy Agar (TSA) plate and incubated for 18hours to 24 hours at 37ºc, in non-CO2 incubator. Following incubation, the plate was observed under a magnifying glass to check the shape and morphology of the strains.

After incubation, the plate was observed in magnifying glass to check shape and morphology of the strains.

Below is a list of the dilution samples used in this experiment.

Sample	Isolate	Dilution
IF1	D1	10^{-4}
	D2	10^{-4}
	E1	10^{-5}
IF2	A1	10^{-3}
	A2	10^{-3}
	A4	10^{-3}
	B1	10^{-4}
	B2	10^{-4}
	C3	10^{-3}
IF3	D1	10^{-4}
	D4	10^{-4}
	D7	10^{-4}
	D10	10^{-4}
	D11	10^{-4}
IF4	D1	10^{-4}
	D4	10^{-4}
	D7	10^{-4}
IF5	E1	10^{-5}
IF6	D1	10^{-4}
IF6	D4	10^{-4}
IF6	D6	10^{-4}
IF7	D1	10^{-4}
IF7	D2	10^{-4}
IF8	D1	10^{-4}
IF8	D4	10^{-4}
IF8	D8	10^{-4}
IF9	F1	10^{-6}
IF9	F4	10^{-6}
IF9	G1	10^{-7}
IF10	C1	10^{-3}
IF10	C4	10^{-3}
IF11	D1	10^{-4}
IF12	F1	10^{-6}
IF12	F4	10^{-6}
IF12	F7	10^{-6}
IF12	F8	10^{-6}
IF13	E1	10^{-5}
IF13	E2	10^{-5}
Sample	**Isolate**	**Dilution**
IF13	E5	10^{-5}
IF14	E1	10^{-5}
IF15	D1	10^{-4}
IF16	D2	10^{-4}
IF16	D4	10^{-4}
IF16	D5	10^{-4}
IF16	D6	10^{-4}
IF17	F3	10^{-6}
IF18	C3	10^{-3}
IF18	C5	10^{-3}
IF18	C6	10^{-3}
IF18	C12	10^{-3}
IF19	C1	10^{-3}
IF19	C4	10^{-3}
IF19	C7	10^{-3}
IF20	E1	10^{-5}
IF20	E4	10^{-5}
IF20	E7	10^{-5}
IF20	F3	10^{-6}
IF21	E2	10^{-5}
IF21	E4	10^{-5}
IF22	D1	10^{-4}
IF22	D2	10^{-5}
IF23	F1	10^{-6}
IF23	F3	10^{-6}
IF23	F5	10^{-6}
IF23	F7	10^{-6}
IF24	D4	10^{-4}
IF24	D7	10^{-4}
IF24	D10	10^{-4}
IF24	D15	10^{-4}
IF25	D10	10^{-4}
IF25	D1	10^{-4}
IF25	D4	10^{-4}
IF25	D7	10^{-4}

Table 3. The isolates collection (n=72) utilized in this study

3.5.3. Gram procedure

This procedure is commonly used in bacteriology to study bacterial morphology and the gram group to which they belong. A drop of PBS solution was used to make a colony smear, which was left to dry at room temperature. Next, the smear was fixed by passing the slide indirectly through the flame of a Bunsen burner three times. Then, the smear was covered with crystal violet solution for 1 minute. Afterward, the crystal violet was poured out, and the smear was rinsed with distilled water before being covered with Lugol´s solution for 1

minute. The Llugol´s solution was then poured out, and the smear was rinsed with distilled water. Following this, it was covered with acetone alcohol or 96% alcohol for 1 minute, rinsed well with distilled water, and then covered with safranin or diluted fuchsin solution for 30 seconds. Finally, the safranin or fuchsin solution was poured out, the smear was rinsed with distilled water, left to dry at room temperature, and observed under a binocular microscope to identify the Gram type of the bacteria.

In this procedure, crystal violet solution stains those bacterias that have affinity with it. And Lugol solution helps to fix Crystal of violet in bacteria walls. And Acetone alcohol, discolours those bacterias that have no affinity with Crystal violet. Finally, the bacteria that discolour with alcohol will take colour of safranin or fuchsin and become red or pink colour of this dye.

In this procedure, the crystal violet solution stains bacteria that have an affinity for it. Lugol´s solution helps fic the crystal violet in the bacterial cell walls. Acetone alcohol decolorizes those bacteria that do not have an affinity for crystal violet. Finally, the bacteria that are decolorized by alcohol will absorb the safranin or fuchsin solution, resulting in a red or pin color.

After thoroughly checking under microscope and performing Gram staining, a pure colony confirmed to be Gram-negative was inoculated into a falcon tube containing 10mL of tryptic soy broth (TSB) medium an incubated at 37ºc overnight. The next day, turbidity was observed in the tube compared to TSB control tube. Afterward, 1.5 mL of the content was centrifuged at 5000 rpm for 5 minutes to obtain a bacterial pellet. The pellet was mixed with 200 µL of 25% TSB glycerol and thoroughly homogenized to preserve the bacteria. Finally, it was stored in a freezer at - 80ºC.

3.5.4. The identification of the islolates with Remel RapID^{TM} ONE System

Initially, 72 samples were recovered from the freezer and allow them to thaw before being inoculated in tryptic Soy Agar medium (TSA) using a sterile inoculation loop. They were then incubated at 37ºc overnight in non-CO2. Incubator.

After incubation, some of these colonies isolated, were swabbed and suspended into 2mL of the fluid inoculation (CaCl2 0,05% plus KCl 0,6%) to achieve the necessary turbidity of McFarland 2. This measure helps to avoid undesirable results that might arise from

insufficient or excessive turbidity in the suspension used. Next, the gallery was peeled back carefully, and the wells were loaded by transferring the entire dilution from the tube to the top right-hand corner of the gallery at 45 degrees through 1000 µL pipette. The port was then carefully resealed to avoid air trapping, which can obstruct the flow of fluid. The gallery was placed on the bench and tilted slightly from rear six times to achieve proper distribution of the content as recommended. The gallery was identified and incubated at 35 - 37º C during for hours in non-CO_2 incubator.

After the incubation period, the gallery was opened carefully, and two droplets of RapID ONE reagent were added to cavities 15 (PRO), 16 (GGT) and 17 (PYR). The reading test was conducted from left to right across the gallery, starting from URE in cavity one the cavity to 18 (ADON). Additionally, two drop of RapID Spot Indole reagent were added in cavity 18 (ADON/IND) and allow to stand for 10 seconds to react properly (IND).

The test score was recorded by noting the test codes representing each cavity of the gallery used for this test, through comparison of the colours observed in the reaction cavity using appropriate report form provided by manufacturer as shown in figure8. The results of cavities reaction were interpreted and annotated according to the provided guidelines. Finally, the microcode obtained from the reading gallery, were entered on the website (Eric Remel: *htt://www.remel.com/eric/*) to obtain identification of the *Enterobacterales* strains. This procedure was applied to all 71 remaining isolates.

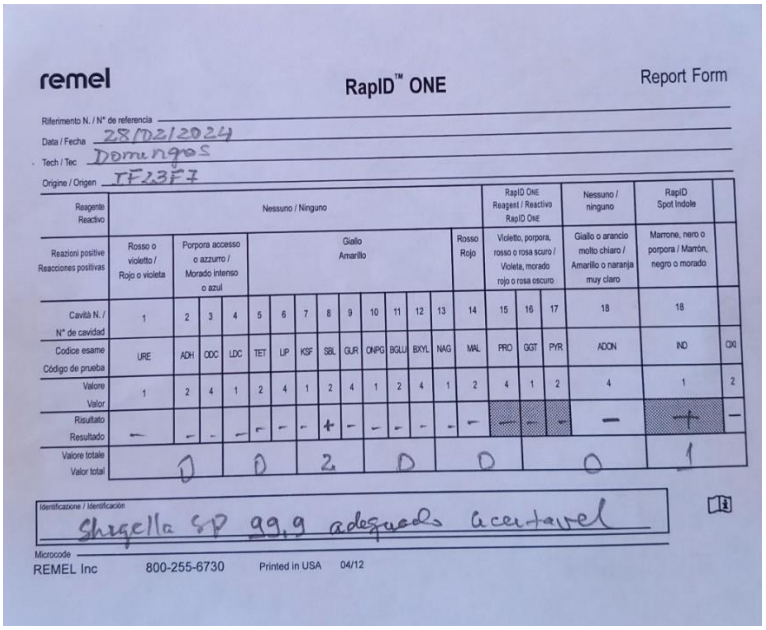

Figure 8. Illustration of the remel reading score to obtain the exact number that possesses a strain.

3.5.5. Antimicrobial susceptibility test based on Kirby–Bauer disk diffusion method

This test was conducted on all 72 isolates. The falcon tube containing 4mL of phosphate-buffer saline (PBS), was used to dilute 2 to 4 colonies selected from the TSA plate with aid of a sterile loop. The turbidity of the isolate suspension was measured using the McFarland scale 0.5 and adjust 0.5 turbidity concentration. The McFarland beforehand, was verified by its standard.

Afterward, a sterile cotton swab, soaked into the suspension, was used to spread the suspension across the surface of a Mueller Hinton Agar (MHA) plate. The Mueller Hinton medium is recommended for Kirby-Bauer method of antimicrobial susceptibility testing. The spreading was done in four steps by rotating the MAH plate 90 degrees, while evenly distribution the suspension. Each strain´s suspension was prepared in duplicated to minimize the margin of reading error.

After spreading, antibiotic disks were placed on the surface of the plates using a disk dispenser. The duplicated were left for 15 minutes in incubator at 37 degrees allow the disks to adhere to the surface, after which the plate were inverted, with the lid facing down. The plates were incubated at 37ºC for 18 to 24 hours.

After incubation, strains susceptible to any antibiotic disk would not grow in the area surrounding the disk, while strain resistant an antibiotic would grow up to the disk. The inhibition zone is the area where the antibiotic´s spectrum of action prevents bacterial growth. The area where the strain grows uninhibited by the antibiotic is considered the resistant zone. The results were measured using a ruler to determine the diameter of the inhibition zone.

Once all antibiotic disks were measured, the zone diameters were compared and interpreted according to EUCAST standards (Version 14.0, 2024. http://www.eucast.org) to assess the susceptibility of the strains and determine if they were resistant to any of the antibiotics used.

3.5.6. DNA extraction

DNA extraction is a process used to obtain DNA from cell or other living being. In this study, the procedure was to obtain pure DNA from the strains being used to study phylogenetic tree of isolates from the stool sample.

For the extraction and purification of genomic DNA from the isolated strain, the identified, strains were thawed from the storage and inoculated into tryptic Soy Agar (TSA), followed by incubation at 37ºC for 18 to 24 hours to allow recovery. After this, one to two colonies from the fresh incubation were inoculated into 10 mL of TSB medium in 50 mL falcon tube and incubated for 18 hours to 24 hours at 37ºC in a shaking warm water at 120 rpm.

Following incubation, turbidity was observed in the falcon tube, indicating successful strain growth. Subsequently, 1 mL of inoculum, corresponding to approximately 10^7 cells, was transferred to pre-labeled Eppendorf tube and centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded, and the sediment was resuspended in 200 µL of Lysis Buffer GL, proteinase K 25 µL, and 200 µL of Lysis Buffer G3 were added, and the mixture was incubated at 70ºC for 10 to 15 minutes in dry block heater to facilitate cell lysis.

The extraction procedure was then carried out following the DNA extration protocol provided by the manufacturer (Bioline extraction ki).

After heating, a brief vortexing was performed. Then, 210 µL of 100% ethanol was added, and the mixture was vortexed vigorously to allow DNA bind to proteinase K. Then, ISOLATE II Genome DNA Spin Column was then used to purify DNA from the cell debris through centrifugation for 1 minutes at 11,000 X g. The liquid collected in the collection tube was discarded. DNA purification continued with a wash sequence using Wash Buffer. First 500 µL of GW1 was added, followed by centrifugation. then 600 µL of Wash Buffer was added. Each washing step was followed by centrifugation at 11,000 X g, with filtrates being discarded after each step. To ensure through washing the samples were centrifuged for an additional minute without anu washing solution to remove any residual solution or ethanol. one minute without any addition of solution to remove any rest of washing solution or ethanol. The DNA spin Column was then transferred to a 1.5 mL microcentrifugation tube. Preheated Elution Buffer (G at 70ºC) was added, and the sample was left to incubate at room temperature. After 1 minute of centrifugation, purified DNA was obtained

The purified DNA was stored at -20ºC until needed. To verify the integrity of the genome DNA, the purified samples, were subjected to electrophoresis in 1% (w/v) agarose gel with Tris-Acetate-EDTA (TAE) Buffer 1x for 30 to 40 minutes at 120V. After electrophoresis, the agarose gel was exposed to UV light to visualize DNA fragment and assess their quality.

3.5.7. PCR amplification of the box elements from *Enterobacteral* isolates

The BOX element genome, of the isolate to be amplified through BOX-PCR, with primers BOX-A1R to identify and describe members of the *Enterobacterial* order that share the same repetitive elements. The final volume of reaction was 25 µL for each reaction. The PCR reaction compositions were prepared based on the specific requirements, as shown in table 4.

The T- personal thermocycler was used to run the PCR reactions according to the required conditions, which are outlined in the table below.

The PCR reaction was made through T personal thermocycler with necessary programming conditions.

The amplicons from all isolates were subjected to agarose gel electrophoresis in 1x of Tris-Acetate-EDTA (TAE) Buffer for 6 hours at 100V and 100mA. Afterward, the gel was stained for 30 minutes with Sybr-safe DNA stain in dim environment. During staining, the agarose gel was gently shaken every 15 minutes. Finally, the gel was visualized under UV illumination (Gene Flash). The alignment of the bands was performed using GeneCompar II programme to generate phylogenetic tree for the member of *Enterobacterales* order isolate.

Description and composition of PCR reaction with its respective conditions of amplifications

Reagent	Volume per Sample µL	Conditions	
		Temperature	Time
Buffler	2.5	94 ºC	5 min
$MgCL_2$	1	95 ºC	45s
DNTPS`S	1	50 ºC	1 min
Primers BOX-AIR	1	72 ºC	7 min
Taq	0.125	72 ºC	5 min
H_2O MiliQ	18.375		
DNA	1.0		

Table 4. the composition of PCR reaction.

4. RESULTS

4.1. Isolation of the member of *Enterobacteral* order

The aim of this study was to describe the members of the *enterobacterales* order in elderly women in Algarve region who are suffering from faecal incontinence. For this, 25 stool samples were obtained from laboratory 2.21 of Microbiology at the University of Algarve. The isolation of the member of *Enterobacterales* order was performed by solation analyzing 74 to 98 colonies based on their morphology characteristic. The characterist of Mc conkey agar plate, as shown in Figure 9, were examined. Various colonies were documented based on size, color, shape, fermentation, and colony margin.

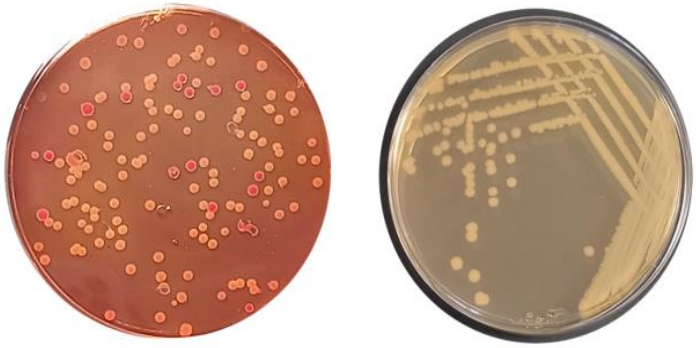

Figure 9. Representation of isolate in Mc conkey from stool sample (10^{-4}) on the left; and on the right, is pointed out the *E. coli* in TSA medium isolated from yellow colony on Mc conkey medium.

4.1.1. Gram results

This method is based on physical and chemical principles. The physical principle relates to the behavior of bacteria during staining, where they absorb and retain components of the staining solution, allowing bacteria to obtain color according to their wall cell. In figure (figure 10), can see bacterias colored with red color. These bacteria are gram-negative that are essential for his study.

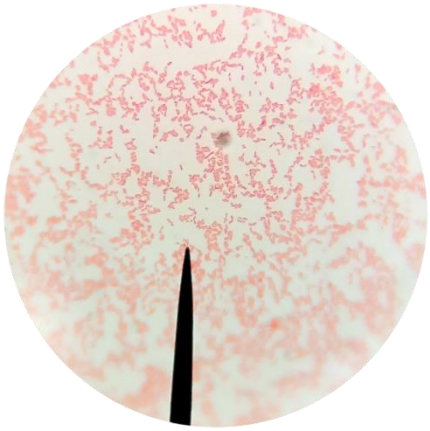

Figure 10. the sample IF25D7 shown gram-negative bacteria stained in red.

4.1.2. The members of the *Enterobacteral* order identified by Remel RapID ONE system

The Remel RapID ONE system was made according to instruction of protocol issued by manufacturer, where 72 isolates from 25 stool samples were identified based in the different color of the reaction as shown in the table 5. The microcode obtained from the reading gallery, were entered on the website (Eric Remel: *htt://www.remel.com/eric/*) to obtain identification of the *Enterobacterales* strains. This procedure was applied to all 71 remaining isolates.

Figure 11. Demonstration of *Enterobacterales* identification through Remel RapID ONE after addition of reagents as shown in figure below.

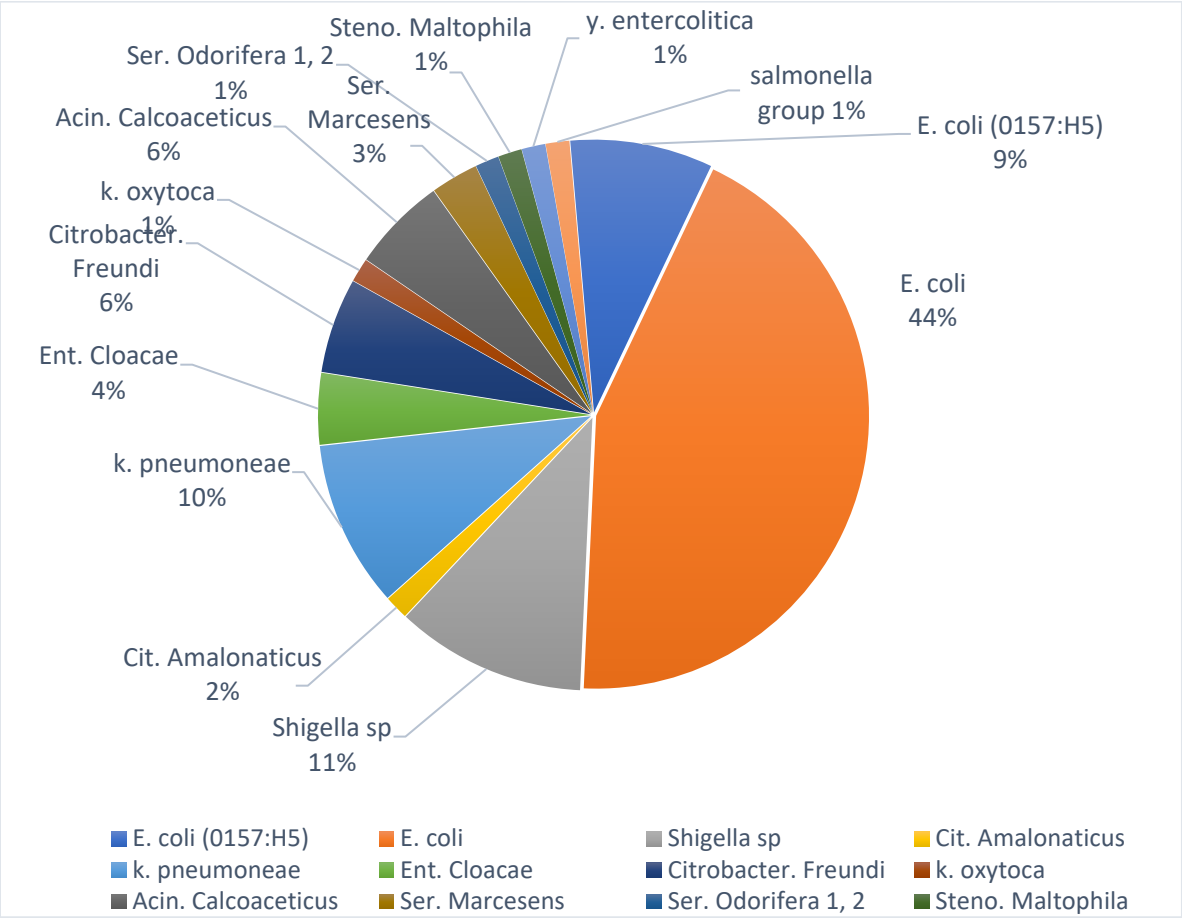

Figure 12. The table below, illustrates the members of the *Enterobacterales* order identified through Remel RapID ONE from 72 strain isolated, as you can check *Escherichia coli*, *E. coli (0157:H7)* with more representation followed *Klebsiella Pneumoniae*, *Shigella sp*, *Seratia Marcenses*, *Acin. Calcoaceticus*, *Steno. Maltophilia*, *Cit. Freundi*, and *Cedicea davisae*

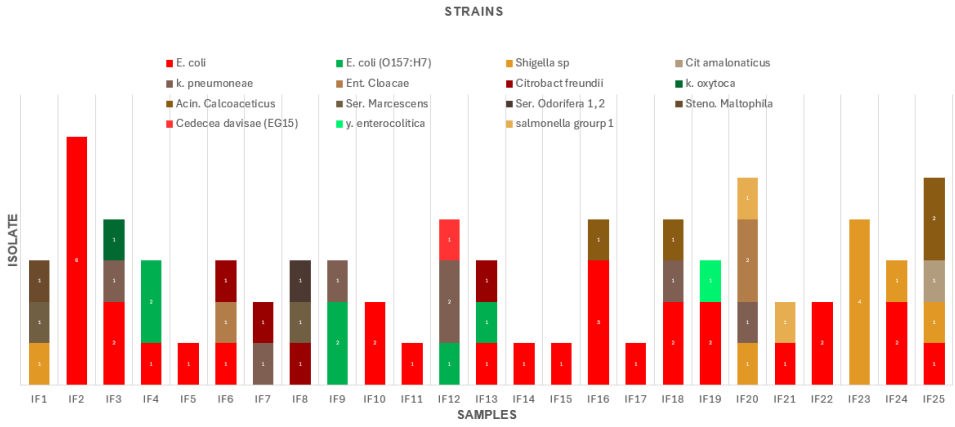

Figure 13. The isolates (n=72) from each sample are constituted of different bacterial species.

The analyzes was done and each sample displayed different isolate. Just The IF2, IF5, IF10, IF11, IF14, IF15, IF17, and IF22, had the same strains of *E. coli*. IF23 only has *shigella sp* as shown figure 13. IF8 have *Serratia marcescens*, *Ser. ordifera* and *Citrobacter freundii*. IF25 showed different strain such as *Shigella sp*, *Citrobacter Amalonaticus* and *acinobacter Calcoaceticus*. The large diversification of bacterial species was observed in IF8, IF25 compared to IF2, IF5, IF10, IF11, IF14, IF15, IF17, IF22. More than 60% (n=15/25) of the samples have two different bacterial species in each sample studied. Diversification among these isolates, led to significant comprehension based on the composition of the member of the *Enterobacterales* order, which indicate more clues to understanding the characteristics of each of the isolates.

	E. coli (O157:H7)	*E.coli*	*Shigella sp*	*Cit amalonaticus*	*k. pneumoneae*	*Ent. Cloacae*	*Citrobact freundii*	*k. oxytoca*	*Acin. Calcoaceticus*	*Ser. Marcescens*	*Ser. Odorifera 1, 2*	*Steno. Maltophilia*	*Cedecea davisae (EG15)*	*Y. enterocolitica*	*salmonella group 1*	total
IF1	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	3
IF2	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	6
IF3	0	2	0	0	1	0	0	1	0	0	0	0	0	0	0	4
IF4	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	3
IF5	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
IF6	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	3
IF7	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	2
IF8		0	0	0	0	0	1	0	0	1	1	0	0	0	0	3
IF9	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	3
IF10	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	2
IF11	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
IF12	1	0	0	0	2	0	0	0	0	0	0	0	1	0	0	4
IF13	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	3
IF14	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
IF15	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
IF16	0	3	0	0	0	0	0	0	1	0	0	0	0	0	0	4
IF17	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
IF18	0	2	0	0	1	0	0	0	1	0	0	0	0	0	0	4
IF19	0	2	0	0	0	0	0	0	0	0	0	0	0	1	0	3
IF20	0	0	1	0	1	2	0	0	0	0	0	0	0	0	0	4
IF21	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	2
IF22	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	2
IF23	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	4
IF24	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	3
IF25	0	1	1	1	0	0	0	0	2	0	0	0	0	0	0	5
Total	6	31	8	1	7	3	4	1	4	2	1	1	1	1	1	72
percentag	8,3%	43,1%	11,1%	1,4%	9,7%	4,2%	5,6%	1,4%	5,6%	2,8%	1,4%	1,4%	1,4%	1,4%	1,4%	100%

Table 5. illustrate the members of the Enterobacteral detected per samples (n=25)

4.1.3. Antimicrobial susceptibility test results

The outcome of 72-strain in antimicrobial susceptibility test, obtained by using susceptibility test, based on Kirby Bauer method according to EUCAST (Version 14.0, 2024. http://www.eucast.org.) were shown in figure 14.

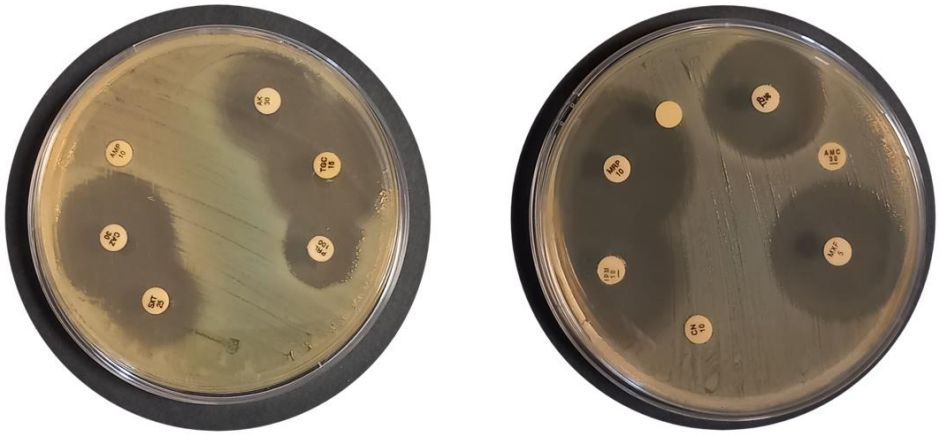

Figure 14. The antimicrobial susceptibility test of IF24D10, shows a halo of inhibition and resistant antibiotics disk. On the left plate, with antibiotics, AMP, AK, TGC, PRL, SXT, CAZ. On the right with TZP, AMC, CN, MRP, MXF, IPM and BLANC disk.

Remel INFO	>20, <20		>19, <19		>14, <14		>20, <20		>17, <17		>18, <18		>22, <22		>22, <16		>22, <22		>22, <19		>14, <11		>18, <18	
	Penicillins								Aminoglycosides				fluoroquinolone		Carbapenems				-ges Cephalosp		Sulfonamides		Tetracyclines	
	TZP (30;6µg)		AMC (20;10µg)		AMP (10µg)		PRL (100µg)		CN (10µg)		AK (30µg)		MXF (5 µg)		MRP (10 µg)		IMP (10 µg)		CAZ (30µg)		SXT (25µg)		TGC (15µg)	
	Halo	R/S	Halo	R/S	Halo	R/S	Halo	R/S	Halo	R/S	Halo	R/S	Halo	R/S	Halo	R/S	Halo	R/S	Halo	R/S	Halo	R/S	Halo	R/S
IF1 D1	24	S	17	R	0	R	8	R	16,5	R	18	S	24	S	23	S	23	S	14	R	22	S	24	S
IF1 D2	24	S	15,5	R	15,5	S	25	S	16,5	R	18	S	25	S	26	S	25	S	24	S	26,5	S	21	S
IF1 E1	23	S	0	R	0	R	26	S	18	S	25	S	11,5	R	27,5	S	32	S	23	S	0	R	10	R
IF2 A1	20	S	0	R	19	S	25	S	18	S	18	S	26	S	30	S	26	S	24	S	25	S	23	S
IF2 A2	20	S	18	R	3	R	20	S	7	R	0	R	20	R	11	R	22	S	21	I	21	S	17	R
IF2 A4	13	R	0	R	16	S	20	S	12	R	12	R	22	S	22	S	24	S	20	I	21	S	14	R
IF2 B1	17	R	0	R	17	S	16	R	13	R	12	R	20	R	20	I	22	S	17	R	18	S	17	R
IF2 B2	16	R	17	R	12	R	18	R	10	R	13	R	19	R	19	I	19	R	20	I	22	S	10	R
IF2 C1	16	R	16	R	17	S	20	S	10	R	13	R	18	R	22	S	22	S	21	I	13	S	12	R
IF3 D1	26	S	0	R	20	S	30	S	21	S	22	S	28	S	32	S	30	S	30	S	19	S	22	S
IF3 D11	28	S	0	R	16	S	30	S	21	S	23	S	30	S	32	S	30	S	34	S	28	S	22	S
IF3 D4	24	S	8	R	22	S	27	S	16	S	18	S	30	S	32	S	28	S	28	S	23	S	23	S
IF3 D7	24	S	8	R	22	S	28	S	16	R	13	S	30	S	30	S	26	S	28	S	13	S	23	S
IF4 D1	17	R	0	R	0	R	7	R	13	R	12	R	24	S	25	S	27	S	16	R	13	I	18	S
IF4 D4	20	S	0	R	16	S	24	S	18	S	13	S	26	S	28	S	24	S	26	S	24	S	24	S
IF4 D7	17	R	0	R	0	R	7	R	12	R	11	R	22	S	24	S	24	S	14	R	10	R	5	R
IF5 E1	21	S	0	R	16	S	21	S	17	S	13	S	26	S	30	S	24	S	21	S	26	S	22	S
IF6 D1	19	R	10	R	13	R	18	R	0	R	0	R	6	R	23	S	0	R	24	S	23	S	18	S
IF6 D4	15	R	0	R	0	R	10	R	12	R	13	R	19	R	24	S	23	S	21	I	0	R	18	S
IF6 D6	23	S	12	R	14	S	24	S	13	S	13	S	21	R	23	S	24	S	26	S	20	S	23	S
IF7 D1	22	S	0	R	15	S	25	S	20	S	13	S	25	S	30	S	29	S	30	S	24	S	20	S
IF7D2	28	S	20,5	S	20,5	S	29	S	13,5	S	20	S	28,5	S	30,5	S	26	S	26,5	S	28	S	25	S
IF08D1	27,5	S	25	S	12,5	R	26,5	S	21	S	22,5	S	29	S	27,5	S	28	S	28,5	S	32	S	28,5	S
IF08D4	27	S	21	S	22,5	S	28,5	S	22,5	S	24,5	S	33	S	32	S	30	S	27	S	29,5	S	30	S
IF08D8	26	S	23	S	24,5	S	29	S	25	S	26,5	S	31,5	S	31	S	31,5	S	29	S	29	S	30	S
IF9F1	24,5	S	23	S	13	R	23,5	S	20,5	S	21,5	S	27	S	20,5	S	20,5	R	27	S	26,5	S	25,5	S
IF9 F4	15	R	0	R	20	S	20	S	13	R	0	R	18	R	19	I	13	R	20	I	18	S	15	R
IF9 G1	21	S	0	R	13	S	26	S	17	S	13	S	26	S	30	S	24	S	27	S	25	S	23	S
IF10C1	26,5	S	19	S	0	R	13	R	20,5	S	13,5	S	14	R	30,5	S	31,5	S	16	R	30	S	30	S
IF10C4	26	S	25	S	24	R	26,5	S	20	S	21	S	32	S	30,5	S	23	S	27	S	29	S	23	S
IF11D1	25	S	22	S	20		28	S	18	S	19	S	28	S	29,5	S	28,5	S	27	S	27	S	27	S
IF12F1	25,5	S	22,5	S	12,5	R	24,5	S	18,5	S	20,5	S	25,5	S	27	S	27	S	28,5	S	24,5	S	26,5	S
IF12 F4	22	S	8	R	20	S	25	S	22	S	20	S	28	S	30	S	28	S	26	S	24	S	23	S
IF12F7	31	S	23	S	23		27,5	S	20,5	S	17,5	R	33	S	23,5	S	27,5	S	27,5	S	27	S	21	S
IF12 F8	23	S	0	R	12	R	21	S	17	S	17	R	23	S	25	S	25	S	27	S	24	S	19	S
IF13 E1	22,5	S	21,5	S	13	S	23,5	S	19	S	18	S	26,5	S	26,5	S	27,5	S	24	S	23,5	S	22	S
IF13 E2	21	S	0	R	0	R	0	R	8	R	0	R	20	R	14	R	23	S	21	I	20	S	17	R
IF13 E5	22	S	8	R	16	S	29	S	16	S	20	S	28	S	30	S	28	S	26	S	24	S	24	S
IF14 E1	24	S	0	R	11	R	23	S	30	S	19	S	26	S	34	S	28	S	26	S	27	S	24	S
IF15 D1	21	S	8	R	21	S	25	S	3	R	20	S	25	S	27	S	26	S	25	S	25	S	24	S
IF16 D2	22	S	0	R	23	S	21	S	10	R	9	R	20	R	20	I	23	S	21	I	22	S	12	R
IF16 D4	20	S	8	R	13	S	25	S	12	R	14	R	22	S	23	S	25	S	24	S	24	S	12	R
IF16 D5	22	S	12	R	16	S	19	R	12	R	10	R	23	S	22	S	23	S	20	I	22	S	13	S
IF16 D6	44	S	40	S	40	S	42	S	24	S	21	S	34	S	40	S	50	S	22	S	32	S	28	S
IF17F3	25,5	S	23	S	23,5	S	26	S	16,5	R	16,5	R	28	S	26,5	S	26,5	S	28,5	S	25,5	S	23,5	S
IF18C3	26,5	S	23,5	S	23,5	S	27,5	S	17,5	S	17,5	R	28	S	28,5	S	27	S	27	S	26	S	23,5	S
IF18C5	20,5	S	22,5	S	13	R	13,5	R	26,5	S	22,5	S	23,5	S	16,5	I	29	S	8,5	R	23,5	S	23	S
IF18C6	22	S	24,5	S	13,5	S	20,5	S	27,5	S	23	S	26,5	S	28	S	23,5	S	15,5	R	23,5	S	25,5	S
IF18C12	26,5	S	25,5	S	26,5	S	30	S	13,5	S	13,5	S	27,5	S	29	S	28,5	S	24,5	S	13	S	24,5	S
IF19C1	23	S	14	R	21,5	S	25,5	S	16	R	16,5	R	24	S	27,5	S	26,5	S	24,5	S	24,5	S	23,5	S
IF19C4	24,5	S	0,0	R	0,0	R	13,5	R	16,0	R	16,0	R	26,5	S	28,5	S	26,5	S	25,5	S	12,0	I	22,0	S
IF19C7	26,5	S	25,5	S	23	S	28,50	S	24,5	S	24,00	S	27,5	S	23,5	S	27,5	S	23	S	25	S	28,50	S
IF20E1	22,5	S	6,5	R	7	R	21,5	S	17	S	17	R	22,5	S	26	S	24,5	S	21,5	I	22	S	18,5	S
IF20E4	23,5	S	23,5	S	25,5	S	25,0	S	23,5	S	21,5	S	24,5	S	25,0	S	29,5	S	18,5	R	0,0	R	22,0	S
IF20E7	23,5	S	0	R	14	S	25	S	18	S	18	S	24	S	27	S	26,5	S	25	S	28	S	20	S
IF20F3	22,5	S	25,5	S	26,5	S	27,5	S	21,5	S	13,5	S	24,5	S	25,5	S	24,5	S	16,5	R	14,5	S	23,5	S
IF21E2	25	S	15	R	23,5	S	26	S	17	S	16,5	S	28	S	28,5	S	27,5	S	26,5	S	29	S	25	S
IF31E4	23,5	S	12,5	R	21,5	S	25,0	S	17,0	S	17,0	R	25,0	S	27,5	S	27,0	S	27,0	S	0,0	R	22,0	S
IF22D1	23,5	S	10,5	R	20,5	S	26,5	S	17,5	S	16,5	S	23,5	S	23,5	S	23,5	S	26	S	26	S	26,5	S
IF22D2	24,5	S	12,5	R	21,0	S	27,0	S	17,5	S	16,0	S	25,5	S	23,5	S	28,0	S	27,5	S	26,0	S	27,0	S
IF23 F1	28	S	0	R	0	R	16	R	13	S	17	R	28,5	S	31,5	S	30,5	S	26,5	S	0	R	20,5	S
IF23 F3	26,5	S	15,5	R	22,5	S	27,0	S	17,5	S	16,5	R	28,0	S	30,5	S	28,5	S	27,0	S	25,5	S	22,5	S
IF23 F5	25	S	0	R	0	R	17	R	18	S	18	S	28,5	S	30	S	27,5	S	27	S	19,5	S	22,5	S
IF23 F7	26,5	S	0,0	R	0,0	R	16,5	R	13,5	S	13,0	S	28,5	S	30,0	S	28,5	S	27,5	S	20,0	S	23,5	S
IF24 D4	26,5	S	16,5	R	23	S	27	S	13,5	S	18	S	28	S	29,5	S	28	S	27	S	26,5	S	24,5	S
IF24 D7	25,0	S	13,5	R	21,5	S	26,0	S	17,5	S	17,5	R	26,0	S	30,0	S	23,5	S	25,0	S	27,0	S	22,5	S
IF24 D10	26,5	S	0	R	7	R	17,5	R	0	R	22	S	23	S	28	S	25,5	S	28	S	6,5	R	27	S
IF24 D15	27,0	S	13,5	R	21,5	S	27,0	S	17,5	S	17,0	R	27,5	S	28,5	S	25,5	S	26,0	S	25,0	S	24,0	S
IF25 D10	26,5	S	0	R	7	R	24,5	S	13,5	S	13	S	28	S	31,5	S	28	S	25,5	S	27	S	22	S
IF25 D1	25,5	S	13,0	R	22,5	S	21,5	S	16,0	R	17,5	R	25,5	S	30,0	S	26,5	S	26,5	S	26,5	S	23,5	S
IF25 D4	25	S	13,5	R	21,5	S	25	S	17,5	S	16,5	S	26,5	S	28,5	S	26	S	25,5	S	25,5	S	21,5	S
IF25 D7	23,5	S	14,5	R	22,5	S	26,0	S	18,0	S	17,0	R	26,5	S	28,0	S	26,5	S	26,0	S	26,5	S	22,5	S

Table 6. Representation of the susceptibility tests of all the strains. We can see that resistance is observed more in AMC and less in IMP.

The results were found in different samples from different patients used in this study, certain bacterias (IF1D1; IF2A2, A4, B2, C1; IF4D1, D7; IF9D4) have shown strong resistance to the penicillins classes, aminoglycosides, fluoroquinolones, cephalosporine and tetracyclines. In this sense, according to their resistance to more than one classes of antibiotics, they are considered multi-drug-resistant bacteria. The strains IF10D1, D4; IF7D2; IF8D4, D8; IF13E1; IF16D6; IF19C7 do not shown any multi-drug-resistant bacteria.

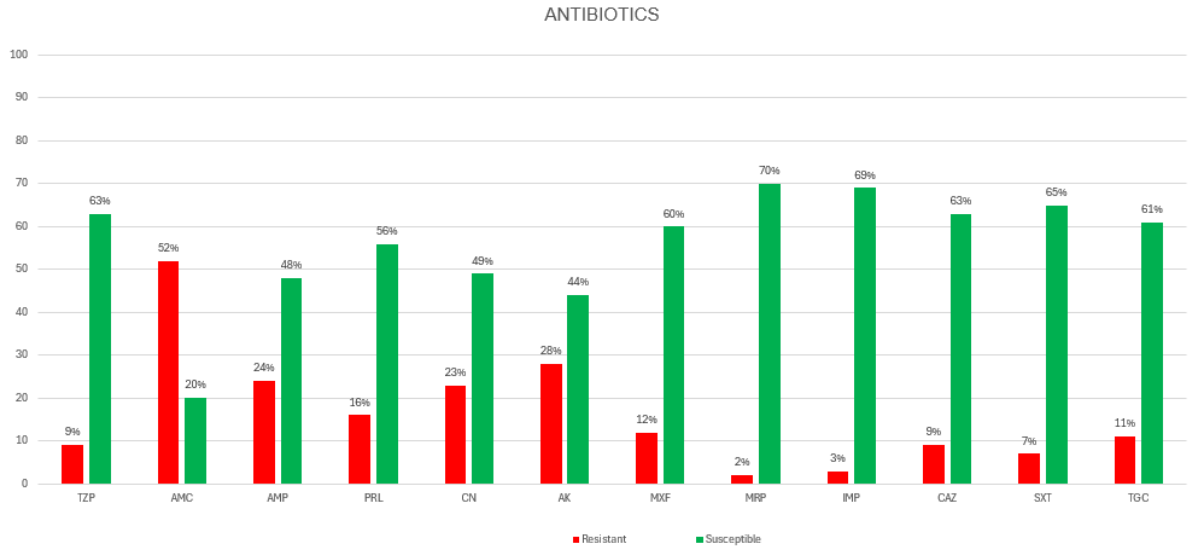

Figure 15. The classes of antibiotics used are penicillins (TZP, AMC, PRL); Aminoglycosides (CN, AK); Fluoroquinolones (MXF); Carbapenems (MRP, IMP) Cephalosporin of third generation (CAZ), Sulfonamides (SXT); Tetracyclines (TGC). These antibiotics was used to determine susceptibility among 72 isolates.

The susceptibility result shows resistance profile (RP) in all the isolates species. In it, we observed the resistance profiles among the different species in each sample of the participants of the study.

- IF1-RP1 resistant to AMC, AMP, PRL, CN, CAZ, SXT, TGC
- IF2-RP2 resistant to TZP, AMC, AMP, PRL, CNE, MXF, MRP, IMP, CAZ, TGC
- IF3- RPR3 resistant to AMC, CN,
- IF4- RP4 resistant to TZP, AMC, AMP, PRL, CN, AK, CAZ, TGC
- IF5- RP5 resistant to AMC
- IF6-RP6 resistant to TZP AMC, AMP, PRL, CN, AK, MXF, SXT
- IF7-RP7 resistant to AMC
- IF8-RP8 resistant to AMP
- IF9-RP9 resistant to TZP, AMC, AMP, CN, AK, MXF, IMP, TGC
- IF10-RP10 resistant to AMP, PRL, MXF, CAZ
- IF11-RP11 does not possess the resistance
- IF12-RP12 AMC, AMP, AK
- IF13-RP13 resistant to AMC, AMP, PRL, CN, AK, MXF, MRP, TGC
- IF14-RP14 resistant to AMC, AMP
- IF15-RP15 resistant to AMC, CN

- IF16-RP16 resistant to AMC, PRL, CN, AK, MXF, TGC
- IF17-RP17 resistant to CN, AK
- IF18-RP18 resistant to AMP, PRL, AK, CAZ
- IF19-RP19 resistant to AMC, AMP, PRL, CN, AK
- IF20-RP20 resistant to AMC, AMP, AK, CAZ, SXT
- IF21-RP21 resistant to AMC, AK, SXT
- IF22-RP22 resistant to AMC
- IF23-RP23 resistant to AMC, AMP, PRL, AK, SXT
- IF24-RP24 resistant to AMC, PRL, CN, AK, SXT
- IF25-RP25 resistant to AMC, AMP, CN, AK

In the order to establish the resistance profile in each sample, among the 24 resistance profiles from 25 samples, the graph was created to illustrate the frequency of profile. The high diversity of the profiles was observed in species of the samples IF1, IF2, IF4, IF6, IF9, IF13, IF16, IF19, IF20. Just in IF11 was not observed resistance profile. IF2-RP2 detained more resistance profile compared to the remaining resistance profile, followed by IF1-RP1, IF4-RP4, IF6-RP6, IF9-RP9, IF13-RP13. IF16-RP16 with six resistance profile. The IF19-RP19, IF20-RP20, IF23-RP23, IF24-RP24 with five resistance profile. The IF18-RP18, and IF10-RP10 with four resistance profile. IF21-RP21, IF12-RP12 with three resistance profile. IF3-RP3, IF14-RP14, IF15-RP15 and IF17-RP17 with two resistance profile. IF5-RP5, IF7-RP7, IF8-RP8, IF22-RP22 all with one resistance profile.

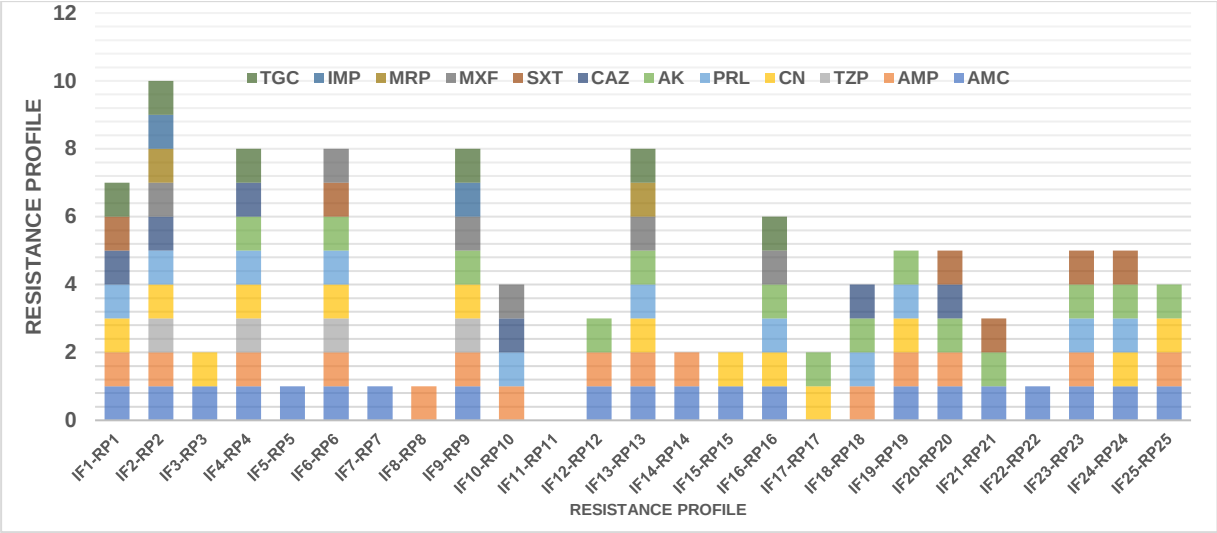

Figure 16. With illustration of resistance profile found among the isolate of the 25 samples.

The resistance detected among isolates, allow to assess multi-drug resistance (MDR) among the antibiotics used in this study. MDR was identified in many isolate samples, as shown in the graph below. The sample IF2 is the one that shows greatest multi-drug resistance compared to the other isolates. At least IF15 present resistance profile in two antibiotics groups.

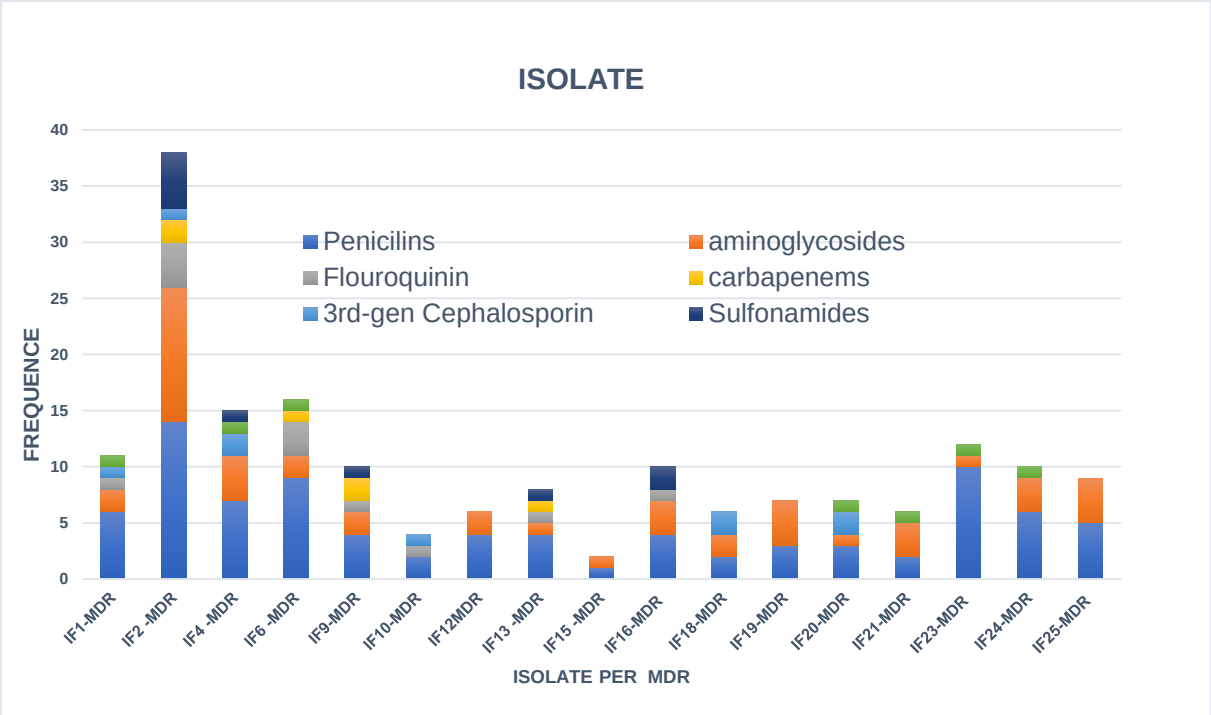

Figure17. representation of resistance frequence within the isolates obtained from 25 samples.

Among the 72 species from the 25 stool samples studies, 17 samples shown 68% (n=17/25) of MDR. Two or more MDR profiles among the antibiotic classes were observed, as represented in figure 17. In IF2 was observed resistance in all its isolates in six classes of antibiotic.

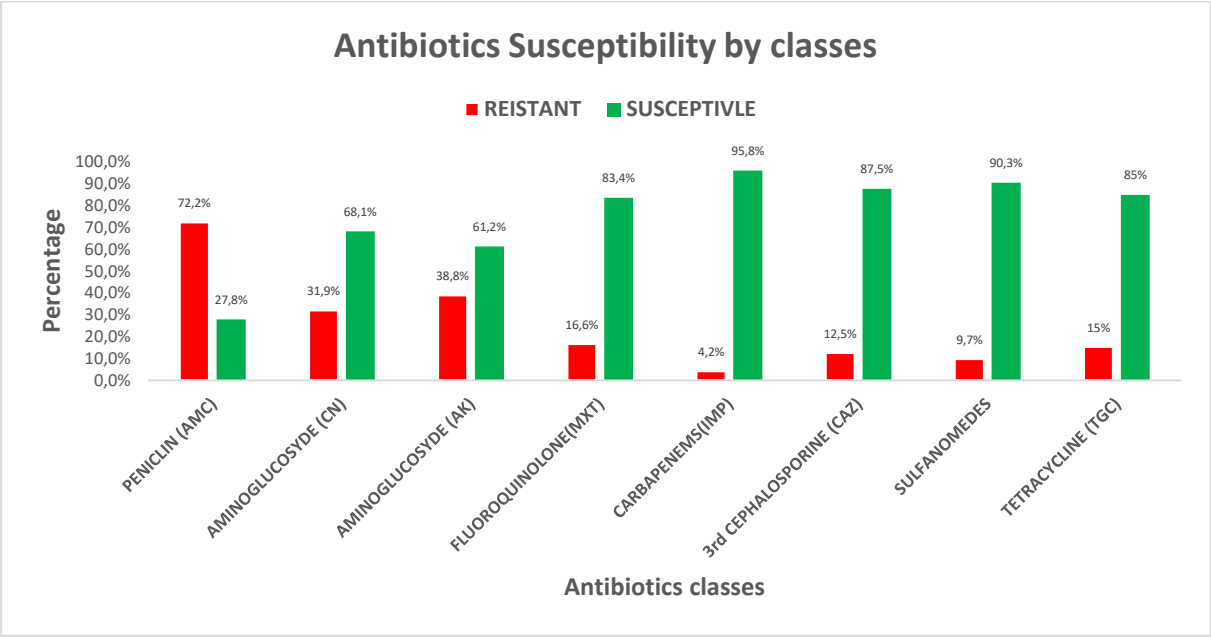

Figure18. The antibiotic resistance classes used in this study The AMC has highest resistance compared to other antibiotics.

4.1.4. BOX-analysis

After extration the DNA, following result was obtained after electrophoresis gel Agar to ensure DNA integrity.

The expected result of the extracted genomic DNA, after electrophoresis gel is shown in the figure19 under UV light. DNA fragment integrity was observed in good quality to BOX-PCR genotyping.

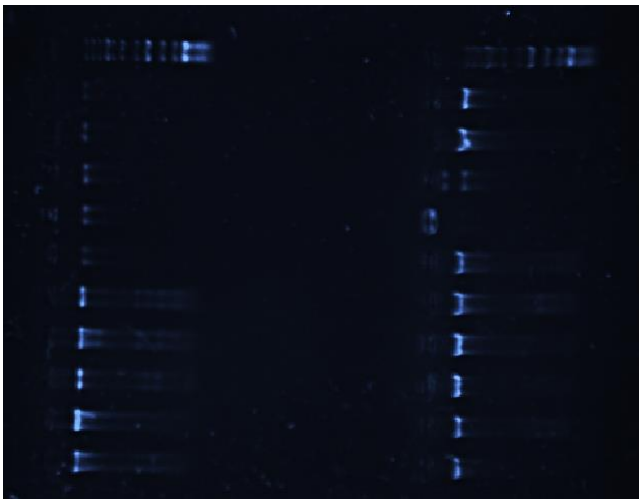

Figure19. demonstration of DNA integrity after running electrophoresis gel Agar. DNA integrity is in good to perform the BOX-PCR

The BOX-PCR, after been running and amplicons obtained were subjected to agarose gel electrophoresis and the gel was stained, visualized under UV illumination (Gene Flash). Their bands in figures 20, 21, 22, 23 below were observed (A, B, C, D). To reach acceptable results, the bands analysis was measured through DNA ladder of 100bp to 1kb.

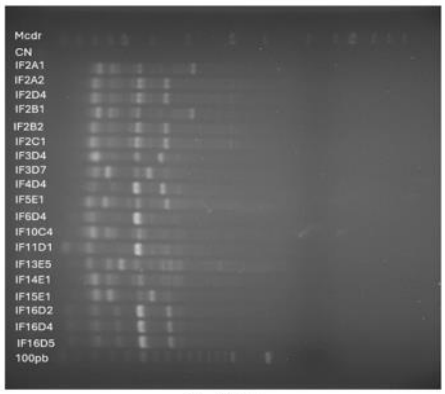

Fig:20

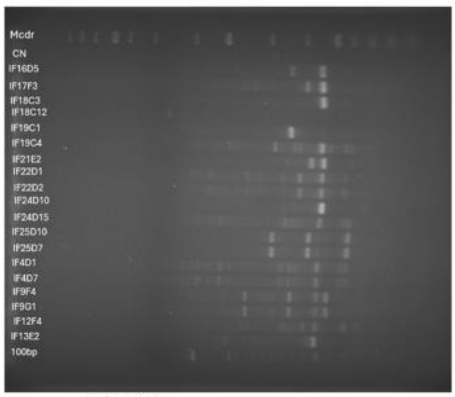

Fig:21

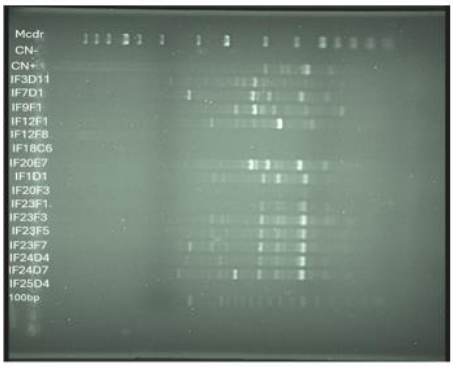

Fig:22

Fig:23

Figure 20, 21, 22, 23 Illustration of the band traces of all 52 samples used in this intergenic consensus study, clearly showing the bands. The negative control showed no band as expected and the ruler also. IF12F8, IF18C6, IF25D4 all was repeated in figure 4, because of not showed clear band in figure 3.

4.2. Phylogenetic analysis

4.2.1. BOX phylogeny

The alignment of the bands was performed using GeneCompar II programme to generate phylogenetic tree for the member of order *Enterobacterales* isolate.

The bands were analyzed using the Dice coefficients, which possesses a range of adjustment of 1.0%. Phylogenetic similarity is considered among the isolate's species, when they present 80% or more genetic similarity patterns. The cluster of patterns in this analysis shows that most of species have high degree of phylogenetic similarity among species.

The range of similarities among the branches varies from 80% to 94.7%. it was observed 4 main cluster which branched to 27 small branches.

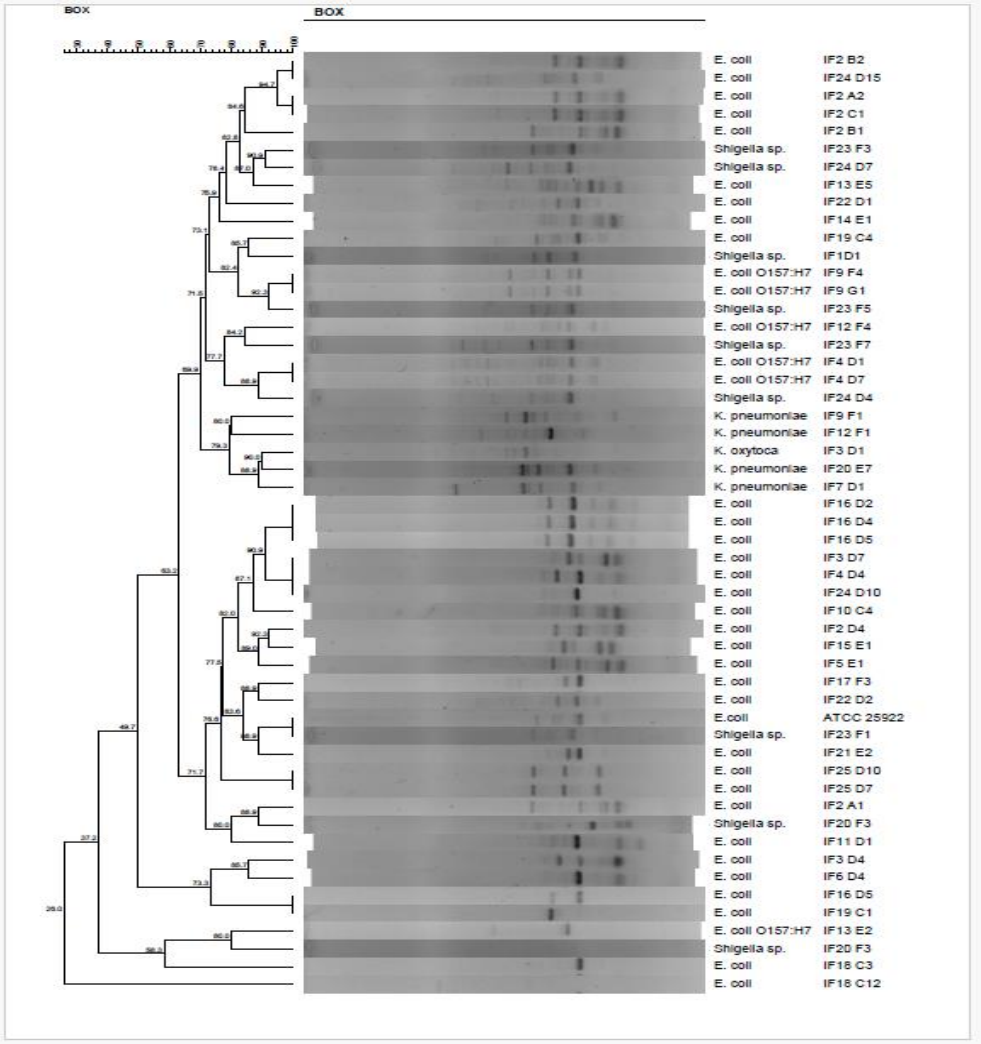

Figure 24. The Box-PCR genotyping analysis of the member of the order *Enterobacterales.* Their 4 main cluster with 27 small branches. The similarity among the *Enterobacteriales* is from 80% to 94.7%.

5. DISCUSION

The member of the order *Enterobacterales* are many bacteria that lives in gastrointestinal tract. The most known is *Escherichia coli* (E. coli), *Klebsiella pneumoniae* and *Shigella* sp. etc. They are facultative anaerobic bacterial species, which is most predominant among the member of the *Enterobacterales* order (Tenaillon et al., 2010b). Many pathogenic variants of *E. coli* are known, such as enterohemorrhagic *E. coli* 0157:H7, which through its toxins, it can cause serious disease (Lim et al., n.d.)

As been said above, the four most common strains that were identified in elderly patients over 60 years which are suffering of faecal incontinence through Remel RapID ONE System are: *E. coli* with 44% of the strains isolated, followed by *Shigella sp* with 11%, *K. pneumoniae* 10% and *E. coli* (0157:H) 9%. Among the four most frequent isolates in these samples, the presence of enterohemorrhagic *E. coli* (0157:H7) is concerning, due to its pathogenic mechanisms, which can unbalance the intestinal bacteriome, resulting in serious illness (Jang et al., 2017). The normal *E. coli* is the most numerous among the isolates detected.

If we look at number of the samples used in this study and bacteria isolated, we can observe different bacterial species by sample, which reveals the existence of diverse members of the *Enterobacterales*. The relationship between members of the *Enterobacterales* and Faecal Incontinence, it is necessary to recognize various aetiological factor that can associate and trigger the faecal Incontinence (Elashri et al., n.d.).

It is understood that *Enterobacterale* can withstand antibiotic action through their adaptive mechanisms (Dandachi et al., 2016). As previously mentioned, antibiotics have various sites of action, including cell wall synthesis, protein synthesis, nucleic acid synthesis, and antimetabolic effects (Calvo & Martínez-Martínez, 2009). In this study, the following antibiotic classes were tested against the isolated bacterial species: penicillins, fluoroquinolones, third-generation cephalosporins, sulfonamides, carbapenems, tetracyclines, and aminoglycosides. Among the antibiotic classes, resistance was observed in these 72 isolates: 72% were resistant to penicillin (AMC), 31.9% to aminoglycosides (CN), 38.8% to fluoroquinolones (AK), 15% to tetracycline (TGC), 16.6% to fluoroquinolones (MXT), 12.5% to sulfonamides, and 8% to carbapenems. These results indicate multiresistance, which can hinder treatment and facilitate the spread of multiresistant strains.

According to various studies, the members of the *Enterobacterales* possesses several adaptive mechanisms to overcome antibiotic efficacy by producing extended-spectrum beta-lactamases (ESBLs) and carbapenemases, which render antimicrobials less effective. The most used antibiotics are beta-lactam antibiotics, such as penicillin (AMC), which are known for their high efficacy. When the bacterium produces beta-lactamase enzymes during treatment, the effectiveness of antibiotics from this group is compromised because these enzymes can neutralize the action of the antibiotics (Bush, 2018; Tooke et al., 2019). Moreover, *Enterobacterale* exhibit resistance to aminoglycosides through enzymes they produce that hinder these antibiotics. Specifically, enzymes like phosphotransferases, acetyltransferases, and adenyltransferases interfere with transport mechanisms and protect the 30S ribosomal subunit, which is the target for aminoglycoside antibiotics such as gentamicin (CN) and amikacin (AK). This modification by these enzymes inhibits aminoglycosides and allows protein synthesis to occur (Smith & Baker, 2002). Such mechanisms facilitate the emergence of resistance among aminoglycosides.

The resistance results constitute 25 resistance profiles for the *Enterobacterale* studied. Among these profiles, RP2 is observed in 21 out of 25 samples, making it the most prominent compared to the others. RP11 shows no resistance in its isolate of *E. coli*. Its genomic plasticity provides many variations that can be observed (Leimbach et al., 2013).

It can be seen from the same isolates in these samples that they do not demonstrate the same resistance profiles (RPs). This variation occurs with isolates from IF2 (E. coli), IF10 (E. coli), IF16 (E. coli), IF19 (E. coli), IF20 (*Enterobacter cloacae*), IF23 (*Shigella* sp.), and IF24 (E. coli). This phenomenon is particularly noted in *E. coli*, *Enterobacter cloacae*, and *Shigella* sp. Some studies indicate that redundant enzymes are essential for *E. coli* growth, and a reduction in these enzymes decreases *E. coli* growth in acidic and alkaline environments (Macheboeuf et al., 2006; Mueller et al., n.d.). Furthermore, the sharing of genetic elements among bacteria, known as mobile gene transfer, can occur through horizontal or vertical transfer among species (Lerminiaux & Cameron, 2019).

On the other hand, the selection criteria and contact with antibiotics are serious conditions that lead to variations in resistance profiles. Therefore, this variability in resistance profile patterns among these samples illustrates the diversity in antibiotic resistance. Given these results, further studies are necessary to ensure appropriate treatment for patients with these bacteria (Lerminiaux & Cameron, 2019).

Regarding multidrug-resistant (MDR) strains, the prevalence in this study's sample revealed that 17 MDR strains were identified in 25 samples, amounting to 68%. The MDR strains detected include those from IF1, IF2, IF4, IF6, IF9, IF10, IF12, IF13, IF15, IF16, IF18, IF19, IF20, IF21, IF23, IF24, and IF25. This data shows that many classes of antimicrobials used in clinical contexts were found to be resistant among several isolates, leading to difficult treatment scenarios. Improper use of antibiotics, self-medication, incorrect medical prescriptions, and negligence in caring for the elderly contribute to rising bacterial resistance. The emergence and increase of MDR in bacteria will complicate treatment and prolong the disease (Jean et al., 2022).

Throughout this study, the increasing antibiotic resistance can be attributed to intestinal dysbiosis due to the aetiological conditions of faecal incontinence, along with the patient's age, which can be a significant factor. Given these results, addressing antibiotic resistance and mitigating its severity through standard antibiotic management is challenging. Necessary measures to contain infections, along with ongoing research, must continue to offer the best solutions to prevent bacterial infections.

The characterization of species identified and determination of antibiotics resistance profile, was done by genetic analyzes to obtain kindship of the isolate through BOX-PCR.

The characterization of identified species and determination of antibiotic resistance profiles were conducted through genetic analyses to establish the kinship of the isolates using BOX-PCR. The phylogenetic tree of the members of the *Enterobacterales* derived from the strains obtained from faecal samples of women aged 60 years suffering from faecal incontinence is composed of four principal groups which branched in 27 small cluster. The programme use is GeneCompar II. The method used is the Dice method, which helps measure band intensity at the site. The first cluster has 73.3% similarity, the second has 80%, the third has 76.6%, and the fourth cluster has 69.9%. Based on these percentages, similarity among the strains is considered significant due to the range of similarity being from 80% to 94.7%. However, upon closer examination of their branches, it becomes evident that all these strains are closely related. The first cluster consists of *E. coli* and *Shigella* sp. The second cluster includes only *E. coli*, while the third cluster branches off from the second, primarily containing *E. coli* and *E. coli ATCC25922*, which is used as a reference, along with two *Shigella* sp. The third cluster exhibits considerable similarity because both species have comparable genomes (Zuo et al., 2013). The fourth cluster contains several branches, with the first branch consisting of

Klebsiella oxytocin and *Klebsiella pneumoniae*. The second branch of the fourth cluster includes *E. coli O157:H7* and *Shigella* sp., which are present in the subsequent branch. Finally, the last branches contain only *E. coli*, which did not show any disagreement regarding similarity.

Additionally, in the fourth cluster, it can be observed that the strains of *E. coli* and *Klebsiella* are distant from each other. However, this distance may be influenced by lifestyle habits, dietary exposure to chemicals, and aging. The BOX-PCR method has been indicated to help select specific elements for the identification of the *Enterobacterale* and relate them according to genetic variation. Other analytical methods, such as whole-genome sequencing (WGS) or 16S rRNA sequencing, can also be utilized in this context.

6. CONCLUSION

The data obtained from this study help us understand the similarities within the members of the order of *Enterobacterales*. During the genotyping of *Enterobacterales*, the species isolated from faecal samples of older women over 60 years old with faecal incontinence revealed notable similarities among all species. The similarity results of this *Enterobacterale* study provide a general view of the resistant characteristics associated with the enterobacteria species that colonize these elderly women.

These member of the *Enterobacterales* isolates exhibit a great diversity of resistance profiles to different antibiotics. Furthermore, the frequency of multidrug-resistant (MDR) profiles was observed in 17 out of 25 faecal samples studied, which is concerning. The detection of these MDR strains poses a significant threat and hindrance to treatment for these patients. Beyond the treatment issues for these women, there is concern about the spread of MDR bacteria among family members, caregivers, and the community, which could lead to an escalation in antibiotic resistance, for which no one is prepared.

Finally, with the use of BOX-PCR, it was possible to visualize the clusters that indicate genetic diversity among the isolates studied. Although this method is useful for determining the genus and species, the variants of the same species remain indistinguishable due to the limitations of the method. In this regard, more studies and analyses using more sophisticated and accurate methods, such as 16S rRNA sequencing and whole-genome sequencing (WGS), are needed to uncover these hidden variants.

7. FUTURE PERSPECTIVE

Faecal incontinence is a condition that affects many women, leading them to seclude themselves due to insecurity within society. The issue of antibiotic resistance poses a significant threat to the world. This study aims to help understand the deeper behavior of our intestinal microbiota and identify the reasons for intestinal dysbiosis related to faecal incontinence. Although the study was able to unveil many antibiotic resistances among the *Enterobacterale*, it also highlighted the importance of identifying antibiotic susceptibility to determine resistance. Multidrug-resistant (MDR) strains were found among these isolates, which raises concerns about patient treatment. Meanwhile, a phylogenetic tree of the isolates was constructed to find similarities among species. In this case, all species demonstrated enterobacterial similarities. Unfortunately, the variants of the same species could not be determined due to methodological issues. Therefore, further studies using advanced tools are needed to uncover evidence of hidden *Enterobacteriale* variants of the same species, and development of new antibiotics with more effectiveness.

Glossary

Bacteriocins: Antimicrobial peptides released by bacteria to inhibit growth of similar or closely related microorganisms.

Commensalism: A relationship between two organisms in which one organism benefits, whereas the other does not

Mutualism: A relationship between two organisms, in which both organisms beneficiate

Dysbiosis: A state of microbial composition that is characterized by an unbalanced proportion of bacteria compared with the proportion in a healthy state.

Horizontal gene transfer: The movement of genetic material from one organism to another, without the need for cell division.

DNA: Deoxyribonucleic acid

PCR- Polymerases chain reaction

IF or FI- faecal incontinence

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