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Universidade Federal do Rio Grande

Programa de Pós-Graduação em Ciências da Saúde

RELAÇÃO ENTRE VARIÁVEIS AMBIENTAIS E PRESENÇA DE ENTEROBACTÉRIAS RESISTENTES A ANTIMICROBIANOS ISOLADAS DE UM ECOSSISTEMA ESTUARINO NO SUL DO BRASIL

Juliana Lemos Dal Pizzol

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal do Rio Grande, como requisito parcial à obtenção do título de Mestre em Ciências da Saúde.

Orientador: Prof. Dr. Pedro Eduardo Almeida da Silva

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“Você não precisa que ninguém te ensine a voar
está no seu espírito
mas é bom ter quem nos lembre
de que temos asas.”
Ryane Leão

Dedicado as mulheres incríveis que me criaram com asas para voar:
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RESUMO

As complexidades das interações biótico-abióticas em ecossistemas dificultam o entendimento da influência das variáveis ambientais que podem selecionar bactérias resistentes aos antimicrobianos. O objetivo deste estudo foi identificar a presença de Enterobactérias fenotipicamente resistentes aos antimicrobianos e investigar a sua relação com variáveis ambientais mensuradas no estuário da Lagoa dos Patos, situada na cidade do Rio Grande, no Rio Grande do Sul, Brasil. Coletou-se amostras de água e aferiu-se os parâmetros físico-químicos em três estações de amostragem. O isolamento bacteriano foi obtido através da técnica de membrana filtrante e o cultivo em Ágar M-ENDO LES. Realizou-se a contagem das Unidades Formadoras de Colônia por mililitro (UFC/mL) de *Escherichia coli* e coliformes totais. A identificação fenotípica foi realizada através de provas bioquímicas e o perfil de sensibilidade das espécies *E. coli*, *Klebsiella* spp. e *Enterobacter* spp. foi definido através do teste de disco-difusão, usando a metodologia Kirby-Bauer. Avaliou-se a relação entre os parâmetros aferidos e a presença de bactérias resistentes por Análise de Componentes Principais (PCA). As estações apresentaram diferentes características físico-químicas, o que refletiu na quantificação, identificação de espécies e no número de cepas resistentes. No Estuário Médio, a temperatura relacionou-se com a presença de bactérias resistentes. Além disso, neste local, observou-se o aumento da temperatura e de UFC/mL de *E. coli* e coliformes totais no mês de novembro. A variável turbidez apresentou relação com bactérias resistentes no Estuário Médio e na Foz, o que pode estar associado a ressuspensão de sedimento e de bactérias devido as chuvas relatadas especialmente no mês de setembro, em que também verificou-se aumento na quantificação de coliformes totais. Na Praia do Cassino, as variáveis pH e oxigênio dissolvido, relacionaram-se com a presença de bactérias resistentes. O aumento da oxigenação é atribuído às algas e ocasiona a morte de bactérias devido à produção de formas tóxicas de oxigênio. A presença de algas também ocasiona o aumento do pH, que atua como bactericida. A avaliação da qualidade ambiental estuarina forneceu dados a serem utilizados em programas de gestão ambiental a fim de reduzir a pressão para seleção de microrganismos resistentes aos antimicrobianos.

Palavras-chave: bactérias resistentes aos antimicrobianos; *Escherichia coli*; *Klebsiella* spp.; *Enterobacter* spp.; estuário; Lagoa dos Patos.

ABSTRACT

The complexities of biotic-abiotic interactions in ecosystems make it difficult to understand the influence of environmental variables that can select bacteria resistant to antibiotics. The objective of this study was to identify the presence of Enterobacteriaceae phenotypically resistant to antibiotics and to investigate their relationship with environmental variables measured in the Patos Lagoon estuary, located in the city of Rio Grande, Rio Grande do Sul, Brazil. Water samples were collected and the physical-chemical parameters were measured at three sampling stations. Bacterial isolation was obtained using the membrane filter technique and cultivation on M-ENDO LES Agar. Colony Forming Units per milliliter (CFU/mL) of *Escherichia coli* and total coliforms were counted. The phenotypic identification was performed through biochemical tests and the sensitivity profile of the species *E. coli*, *Klebsiella* spp. and *Enterobacter* spp. was defined through the disk-diffusion test, using the Kirby-Bauer methodology. The relationship between the measured parameters and the presence of resistant bacteria was evaluated by Principal Component Analysis (PCA). The stations presented different physicochemical characteristics, which reflected in the quantification, identification of species and in the number of resistant strains. In the Middle Estuary, temperature was related to the presence of resistant bacteria. Furthermore, in this location, it was observed an increase in temperature and in CFU/mL of *E. coli* and total coliforms in November. The turbidity variable was related to resistant bacteria in the Middle Estuary and Foz, which may be associated with the resuspension of sediment and bacteria due to the rains reported especially in September, when there was also an increase in the quantification of total coliforms. At Cassino beach, the variables pH and dissolved oxygen were related to the presence of resistant bacteria. The increase in oxygenation is attributed to algae and causes the death of bacteria due to the production of toxic forms of oxygen. The presence of algae also causes an increase in pH, which acts as a bactericide. The estuarine environmental quality assessment provided data to be used in environmental management programs in order to reduce the pressure for the selection of microorganisms resistant to antimicrobials.

Keywords: antibiotic resistant bacteria; *Escherichia coli*; *Klebsiella* spp.; *Enterobacter* spp.; estuary; Patos Lagoon.

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LISTA DE ABREVIATURAS E SIGLAS

BRA	Bactérias resistentes aos antimicrobianos
GRA	Genes de resistência aos antimicrobianos
OMS	Organização Mundial da Saúde
ESKAPEE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* species and *Escherichia coli*
EGM	Elementos genéticos móveis
E.coli	*Escherichia coli*
K. pneumoniae	*Klebsiella pneumoniae*
K. oxytoca	*Klebsiella oxytoca*
RS	Rio Grande do Sul
PELD-EPA	Long-Term Ecological Research group in the Lagoa dos Patos Estuary and Adjacent Marine Coast
EMA	Marine Aquaculture Station
HU-FURG/EBSERH	Dr. Miguel Riet Corrêa Jr. University Hospital of the Federal University of Rio Grande
BHI	Brain-heart infusion broth
ANVISA	Agência Nacional de Vigilância Sanitária
CFU	Colony forming units
MH	Mueller Hinton
CLSI	Clinical and Laboratory Standards Institute
PCA	Principal Component Analysis
Temp	Temperature
Sal	Salinity
TDS	Total dissolved solids
Turb	Turbidity

OD	Dissolved oxygen
pH	Hydrogenion potential
NH_4	Ammonium
ICU	Intensive Care Unit
UTI	Unidade de terapia intensiva
ESBLs	β-lactamases de espectro estendido
ITUs	Infecções do trato urinário

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1 INTRODUÇÃO

Atividades antropogênicas como o lançamento de efluentes hospitalares e industriais, esgotos domésticos e escoamentos urbanos e agrícolas fornecem entrada para microrganismos patogênicos, resíduos de antimicrobianos, bactérias resistentes aos antimicrobianos (BRA) e genes de resistência aos antimicrobianos (GRA) no ambiente, especialmente em compartimentos aquáticos (XU et al., 2019; JIANG et al., 2018; LAFFITE et al., 2016). Estes ecossistemas são considerados potenciais resistomas, por apresentarem um conjunto de genes envolvidos na resistência aos antimicrobianos presentes em bactérias ou mesmo livres no ambiente (ALMAKKI et al., 2019). Consequentemente, os corpos d'água impactados se tornam pontos focais para a proliferação de BRA (BAQUERO et al., 2008).

A promoção, manutenção e disseminação de organismos bacterianos resistentes aos antimicrobianos nos ecossistemas aquáticos depende de diferentes variáveis ambientais. Visto que, fatores abióticos, como a temperatura, pH, níveis de oxigênio, salinidade e nutrientes, representam condições cruciais no processo evolutivo que envolve a resistência aos antimicrobianos (PETIT et al., 2014). No entanto, apesar de estudos anteriores demonstrarem o envolvimento de fatores abióticos na seleção de BRA, ainda permanecem dúvidas de como esta estas interações biótico-abióticas influenciam a presença de BRA nos diferentes ecossistemas (HAN et al., 2021).

A disseminação de patógenos multirresistentes é uma das ameaças emergentes mais relevante para a saúde pública. Atualmente, cerca de 700.000 pessoas morrem a cada ano no mundo devido a infecções causadas por microrganismos resistentes aos antimicrobianos e a Organização Mundial da Saúde (OMS) prevê que esse número pode subir para 10 milhões até 2050 (OMS, 2020), tornando-se a principal causa de morte. Diante deste cenário, a OMS publicou uma lista de patógenos designados pela sigla ESKAPEE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa, Enterobacter* species and *Escherichia coli*) aos quais foi dado o mais alto "status de prioridade", na medida em que infecções com estes microrganismos têm resultado em altas taxas de morbimortalidade e elevados custos econômicos, tornando-se um grave problema de saúde pública (OMS, 2017).

As margens de cursos d'água representam áreas propícias ao desenvolvimento urbano e a crescente pressão demográfica impacta diretamente os ambientes aquáticos. Especialmente os ambientes estuarinos, que se situam na fronteira entre os ecossistemas terrestres/de água doce e marinhos, são um hotspot para impactos antropogênicos (LOTZE et al., 2006). Não há

regulamentações que definam os níveis permitidos de antimicrobianos ou determinantes de resistência aos antimicrobianos considerados limítrofes no meio ambiente. Além disso, a pesquisa na área de resistência a antimicrobianos é tradicionalmente voltada para os microrganismos obtidos de amostras clínicas, humana ou veterinárias. Portanto, o reconhecimento de reservatórios de patógenos resistentes aos antimicrobianos é fundamental para a elaboração de estratégias de controle dentro do conceito de Saúde Única (BAQUERO et al., 2008).

A foz da Lagoa dos Patos, localizada no município do Rio Grande, Rio Grande do Sul (RS), compreende uma região estuarina de múltiplo uso no que diz respeito ao desenvolvimento portuário, industrial e urbano em suas margens. O desenvolvimento da cidade favorece a ocorrência de impactos antrópicos no ambiente aquático, como o lançamento de efluentes de diferentes origens, tratados e não tratados. Sabe-se que o atual sistema de coleta de esgoto do Rio Grande abrange cerca de 32% dos efluentes gerados, sendo inadequado ao seu porte e desenvolvimento (MARRETO et al., 2017).

Nesse sentido, este estudo teve como objetivo avaliar se a região estuarina da Lagoa dos Patos, localizada na cidade do Rio Grande (RS), é um potencial reservatório de Enterobactérias resistentes aos antimicrobianos e investigar a relação da presença destas bactérias com variáveis ambientais.

2 REVISÃO BIBLIOGRÁFICA

2.1 Resistência aos antimicrobianos

Com a descoberta da penicilina ao final dos anos 20, e a sua introdução na prática clínica a partir da metade dos anos 40, doenças que eram fatais começaram a ser tratadas com eficácia terapêutica, reduzindo de forma significativa, as taxas de mortalidade devido a estas enfermidades. Além disso, houve o início de intervenções médicas cuja profilaxia com antimicrobianos é essencial (MOHR, 2016). Dessa forma, os antimicrobianos participam de forma evidente no aumento da qualidade e da expectativa de vida (CROFTS et al., 2017). Entretanto, o uso desses fármacos, especialmente quando feito de forma inadequada, cria uma pressão seletiva de genes e microrganismos resistentes aos antimicrobianos (HU et al., 2013; VRANCIANU et al., 2020).

As infecções por BRA estão associadas ao aumento da morbimortalidade, necessidade de hospitalização, prolongamento do tempo de hospitalização e custos mais elevados de cuidados de saúde (COSGROVE, 2006). A mudança da estratégia terapêutica pressionada pela resistência aos antimicrobianos, conduz a alternativas mais tóxicas e/ou de custo elevado (FRIEDMAN et al., 2016). Atualmente, países desenvolvidos e em desenvolvimento estão avançando para a era pós-antibióticos, em que infecções comuns se tornam intratáveis, estabelecendo uma volta ao passado onde as doenças infecciosas eram fatais devido à falta de alternativa terapêutica (WANG et al., 2020). Por esta razão, a elevação do número de casos de infecções por BRA foram classificados pela OMS como uma das maiores ameaças à saúde global no século XXI (OMS, 2020).

A resistência aos antimicrobianos não está restrita aos espaços de saúde humana ou animal, mas também a saúde ambiental que apresenta um papel fundamental no controle da resistência aos antimicrobianos. O uso excessivo de antimicrobianos na saúde, agricultura e meio ambiente, bem como o consumo inadequado desses fármacos, como escolhas e dosagens inadequadas e baixa adesão às diretrizes de tratamento, contribuem para a disseminação de BRA e determinantes de resistência, dentro e entre esses setores, representando um risco para a saúde humana (HERNANDO-AMADO et al., 2019). Dadas as dimensões humanas, animais e ambientais interdependentes, é lógico adotar uma abordagem de Saúde Única para abordar o problema da resistência antimicrobiana (MCEWEN et al., 2018).

Efluentes provindos de assentamentos domésticos, hospitalares, industriais e instalações agropecuárias, são geralmente caracterizadas por cargas bacterianas altas e concentrações subterapêuticas de antimicrobianos (HERNANDO-AMADO et al., 2019; BERENDONK et al., 2015). Estas águas residuais, após o processo de tratamento, são liberadas em águas superficiais ou solos agrícolas como resultado de irrigação, que posteriormente serão transportadas para corpos d'água adjacentes ou para a profundidade do solo aquífero (CHRISTOU et al., 2017). Assim, ecossistemas aquáticos que servem como destino final para as águas residuais são considerados potenciais resistomas, devido apresentarem um conjunto de genes envolvidos na resistência microbiana presentes em bactérias vivas ou livres no ambiente (ALMAKKI et al., 2019).

2.2 Estuários

Estuários e ambientes costeiros têm sido pontos focais de assentamento humano e uso de recursos aquáticos ao longo da história. Estes ecossistemas desempenham um papel importante no apoio à produtividade biológica, conservação da biodiversidade e atividades econômicas e socioculturais (LOTZE et al., 2006). No entanto, a superexploração, transformação de habitat e poluição promovem a degradação estuarina, com perda de biodiversidade e fragilização da sua resiliência ecológica (MARTÍNEZ-MEGÍAS et al., 2022).

Estas áreas são privilegiadas devido ao seu uso múltiplo, no que diz respeito a ocupação urbana, portuária e industrial. No entanto, o múltiplo uso também é reconhecido pela frequente liberação de elementos antrópicos no ambiente aquático, especialmente o lançamento de águas residuais tratadas e não tratadas e o escoamento urbano (AUDOUIT et al., 2017; WANG et al., 2020). A contaminação química e microbiológica de áreas costeiras e estuários reflete a pressão antrópica exercida sobre suas bacias (PETIT et al., 2014).

Atualmente não há regulamentações que definam os níveis aceitáveis de antimicrobianos ou determinantes de resistência aos antimicrobianos no meio ambiente. Resíduos oriundos de atividades humanas são ricos em compostos recalcitrantes como antimicrobianos, metais e biocidas, que podem atingir corpos d'água e contribuir significativamente para a seleção de fenótipos de BRA (LEITE et al., 2019). No ambiente aquático, o destino destes microrganismos dependerá de sua capacidade de superar o estresse ambiental (salinidade, temperatura e oligotrofia). Quando associados a partículas organominerais, as bactérias de origem fecal e as moléculas mais estáveis de antimicrobianos, seguem a dinâmica das partículas e se depositam em lodaçais, zonas de deposição de partículas finas (PETIT et al., 2014). Dessa forma, a disseminação e o acúmulo de BRA podem ocorrer em lagoas urbanas e representam uma grande ameaça à saúde pública (ZHU et al., 2017; ZHENG et al., 2021).

A importância do ambiente não-clínico para o agravamento do problema da resistência bacteriana aos antimicrobianos ainda não foi totalmente esclarecida. O contato entre bactérias patogênicas resistentes aos antimicrobianos e microrganismos ambientais nos ecossistemas poluídos pode resultar em aumento da resistência à maioria dos antimicrobianos clinicamente significativos nessas populações bacterianas (BAQUERO et al., 2008). Dessa forma, a expansão de cepas resistentes e determinantes de resistência aos antimicrobianos entre microbiomas humanos, animais e ambientais têm o potencial de alterar a genética de populações bacterianas em níveis local e global, modificando a estrutura e a produtividade dos microbiomas onde as BRA podem se expandir (HERNANDO-AMADO et al., 2019).

2.3 Microrganismos indicadores

As comunidades bacterianas estão associadas à qualidade ambiental, devido ao seu papel fundamental para o funcionamento e estabilidade dos ecossistemas, como também por sua sensibilidade a variações ambientais causadas por processos naturais ou antropogênicos (SAXENA et al., 2015). Além disso, as bactérias apresentam características que favorecem sua utilização em estudos *in vitro* como indicadores para a detecção de alterações no ambiente que possam interferir na saúde humana.

Estes microrganismos, ao contrário dos organismos superiores, requerem pouco espaço para o seu cultivo e apresentam uma estrutura celular/molecular menos complexa, além de serem capazes de se adaptar a condições ambientais desfavoráveis e permitirem o estudo da transmissão e modificação do material genético em um curto período de tempo, devido ao seu ciclo de vida rápido (LOGAR et al., 2007). Nesse sentido, estudos metagenômicos em amostras ambientais têm permitido caracterizar com maior acurácia a diversidade de populações microbianas e auxiliar no monitoramento e na implantação de estratégias de biorremediação (TECHTMANN et al., 2016).

As bactérias do grupo dos coliformes, pertencentes à família Enterobacteriaceae, são comumente utilizadas como indicadores microbiológicos. Os coliformes totais representam um grupo de bactérias Gram-negativas, não formadoras de esporos, oxidase/indol negativas, anaeróbicas facultativas em forma de bastonete que fermentam lactose em ácido e gás dentro de 24-48 h a 36 ± 2 °C em um meio contendo bile sais e detergentes (SAXENA et al., 2015). Estes microrganismos não são utilizados como indicadores de contaminação fecal pois não são específicos para o intestino humano. Assim, estes também podem ser encontrados no ambiente natural (ALAV et al., 2018).

Do contrário, coliformes que são capazes de crescer e fermentar a lactose com a produção de ácido e gás a 44,5 °C na presença de sais biliares, são agrupados como coliformes fecais ou termotolerantes, e têm uma correlação positiva com contaminação fecal de animais de sangue quente (TORANZOS et al., 2007). A contagem de coliformes termotolerantes ou *E. coli* é comumente utilizada para monitorar a qualidade das águas recreativas ou potáveis. Estas bactérias são classicamente usadas como indicadoras de contaminação fecal na água, pois são consideradas residentes do trato intestinal de animais homeotérmicos e, portanto, são de importância sanitária (OMS, 2008; PAYMENT et al., 2003). No entanto, outras espécies de bactérias também podem ser utilizadas para avaliar a qualidade das águas marinhas e salobras,

pois a ausência de *E. coli* não significa que não possam existir outros grupos de patógenos (NNADOZIE et al., 2019).

2.4 ESKAPEE

O aumento do número de casos de infecções com microrganismos resistentes aos antimicrobianos é impulsionada pelos patógenos do grupo ESKAPEE (Gram positivos *Enterococcus faecium*, *Staphylococcus aureus*; e Gram negativos *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp. e *Escherichia coli*) (OMS, 2017). Os patógenos ESKAPEE são bactérias ambientais ou comensais, geralmente não patogênicas, mas causadoras de infecções oportunistas em pacientes hospitalizados ou imunocomprometidos (RICE, 2008).

Segundo a OMS, estas espécies são classificadas como patógenos de Prioridade Crítica por serem difíceis de tratar, devido à sua capacidade intrínseca e adquirida de desenvolver rapidamente mecanismos de resistência em resposta ao uso de antimicrobianos ou outros fatores de pressão seletiva (OMS, 2017). Cada uma dessas espécies tem resistência intrínseca ou adquirida a um ou mais antimicrobianos, e cepas individuais podem acumular resistência a diferentes antimicrobianos de famílias químicas dissimilares (DE OLIVEIRA et al., 2020). Cepas multirresistentes destes microrganismos são frequentemente isoladas em ambientes hospitalares e, por esse motivo, são responsáveis pela maioria das infecções hospitalares pacientes de difícil manejo com terapia antibiótica (SANTAJIT et al., 2016).

Os mecanismos de resistência a múltiplos fármacos exibidos por ESKAPEE foram agrupados em três categorias: inativação do fármaco por uma clivagem irreversível catalisada por uma enzima, modificação do sítio alvo de ligação e a redução da tração intracelular, seja devido a redução da permeabilidade do envoltório celular ou pelo mecanismo de efluxo (SANTAJIT et al., 2016). Além disso, estes microrganismos também são capazes de formar biofilmes que inibem a resposta imune, o acesso dos antimicrobianos e protegem as populações bacterianas bactérias dormentes/persistentes (LEWIS, 2007).

A transferência e a obtenção de GRA entre bactérias pode ocorrer por diversos mecanismos. Esses incluem a transferência de gene vertical, que consiste na transmissão de informação genética localizada no cromossomo bacteriano para gerações subsequentes de células-filhas; e a transferência de gene horizontal, mediada por elementos genéticos móveis (EGM) que promovem a mobilidade do DNA por meio de plasmídeos, transposons, integrons e

bacteriófagos (SULTAN et al., 2018). A captura, acumulação e disseminação de GRA são, em grande parte, devido às EGM. Assim, a troca de EGM entre diferentes espécies bacterianas é uma ferramenta fundamental para a sobrevivência e persistência no meio ambiente (PARTRIDGE et al., 2018).

Os patógenos Gram-negativos ESKAPEE são considerados uma grande ameaça, devido ao surgimento de cepas resistentes à maioria dos antimicrobianos disponíveis. O acúmulo de resistência antimicrobiana nestes microrganismos é principalmente devido a transferência horizontal de genes auxiliada por plasmídeos e EGM (REZA et al., 2019).

2.4.1 *Klebsiella pneumoniae*

O gênero *Klebsiella*, pertencente à família Enterobacteriaceae, é representado por bacilos Gram-negativos, fermentadores de carboidratos, imóveis e não esporulados. Estes microrganismos fazem parte da microbiota entérica comensal, como também podem ser encontrados na água, nos vegetais e no solo (PODSCHUN et al., 1998).

K. pneumoniae e *K. oxytoca* são as principais espécies do gênero ocorrendo em espécimes clínicos humanos, sendo a *K. pneumoniae* a espécie clinicamente mais importante, devido à associação com altas taxas de mortalidade e por ser frequentemente envolvida em surtos nosocomiais (NEOG et al., 2021). Estão associados a uma variedade de infecções que afetam as vias urinárias, o trato respiratório, as feridas cutâneas e, em alguns casos, podem causar septicemia e meningite. A fonte de infecção, na maioria dos casos, é a microbiota endógena dos pacientes, principalmente portadores de diabetes mellitus tipo 2, neonatos e imunocomprometidos (PODSCHUN et al., 1998).

K. pneumoniae é um agente causador de infecções em aproximadamente 14-20% do trato respiratório, ducto biliar inferior, feridas cirúrgicas e ITU (De Rosa et al., 2015). Este microrganismo é intrinsecamente resistente à ampicilina devido à presença da penicilinase SHV-1 em seus cromossomo (HOLT et al., 2015). A resistência a fármacos adicionais pode surgir através de mutações cromossômicas, no entanto, a maior parte da resistência antimicrobiana é resultante da aquisição de genes via transferência horizontal de genes, principalmente através de grandes plasmídeos conjugativos (ROZWANDOWICZ et al., 2018). Além disso, é a espécie na qual vários novos genes de resistência antimicrobiana foram descobertos pela primeira vez antes de se espalharem para outros patógenos (por exemplo, genes de resistência a carbapenem KPC, OXA-48 e NDM-1). Por esse motivo, esta espécie pode desempenhar um papel

fundamental na disseminação de genes de resistência antimicrobiana de microrganismos ambientais para patógenos clinicamente importantes (WYRES et al., 2018).

Antimicrobianos da classe das cefalosporinas e carbapenêmicos têm sido a base do tratamento para infecções graves causadas por Enterobacteriaceae, como *K. pneumoniae*, mas a eficácia foi comprometida pela aquisição generalizada de genes que codificam enzimas, como β-lactamases de espectro estendido (ESBLs) e carbapenemases, que mediam a respectiva resistência a esses fármacos (PATERSON et al., 2005; MUNOZ-PRICE et al., 2013). Como os carbapenêmicos são usualmente empregados como alternativa para o tratamento de infecções causadas por bactérias Gram-negativas, *K. pneumoniae* resistente a carbapenêmicos é um importante desafio clínico (OMS, 2018). As cepas de *K. pneumoniae* resistentes a carbapenêmicos são as Enterobactérias resistentes a carbapenêmicos mais clinicamente proeminentes (EARS-Net, 2019).

2.4.2 *Enterobacter* spp.

O gênero *Enterobacter*, pertencentes à família Enterobacteriaceae, inclui bacilos Gram-negativos anaeróbios facultativos com 2 mm de comprimento e móveis por meio de flagelos peritríquios. Esses microrganismos são amplamente encontrados na natureza, sendo saprófitos no ambiente, pois são encontrados no solo e esgoto, e também fazem parte da microbiota entérica comensal do trato gastrointestinal humano (DAVIN-REGLI et al., 2019).

Até o momento, 22 espécies foram encontradas no gênero *Enterobacter*: *E. aerogenes*, *E. amnigenus, E. arachidis, E. asburiae, E. carcinogenus, E. cloacae, E. cowanii, E. dissolvans, E. gergoviae, E. helveticus, E. hormaechei, E. kobei, E. ludwigii, E. mori, E. nimipressuralis, E. oryzae, E. pulveris, E. pyrinus, E. radicincitans, E. soli, E. taylorae* e *E. turicensis*. Dentre essas espécies, sete estão agrupadas dentro do grupo complexo *Enterobacter cloacae*: *E. cloacae, E. asburiae, E. hormaechei, E. kobei, E. ludwigii, E. mori* e *E. nimipressuralis*. Essa nomenclatura é baseada no compartilhamento de características fenotípicas e principalmente genotípicas, determinadas por hibridizações DNA-DNA de todo o genoma (HOFFMANN et al., 2003).

Nas últimas décadas, *Enterobacter* spp. assumiram significado clínico e surgiram como patógenos nosocomiais de pacientes de terapia intensiva (SANDERS et al., 1997). *E. cloacae* e *E. aerogenes* representam as espécies mais frequentemente isoladas descritas em infecções clínicas, principalmente em pacientes imunocomprometidos e internados em UTI, devido à adaptação dessas espécies aos agentes antimicrobianos e seu comportamento como patógenos

oportunistas (DAVIN-REGLI et al., 2019). Seu representante mais importante, *E. cloacae*, surgiu como um patógeno importante para instituições de saúde em todo o mundo. *E. cloacae* é responsável por até 5% da sepse adquirida no hospital, 5% das pneumonias nosocomiais, 4% das infecções do trato urinário nosocomiais e 10% dos casos de peritonite pós-cirúrgica (PAAUW et al., 2008).

Esses patógenos estão frequentemente associados a um fenótipo de resistência a múltiplos fármacos, principalmente devido à sua adaptação ao ambiente hospitalar e sua capacidade adquirir facilmente EGM contendo genes de resistência e virulência. Essas espécies apresentam resistência intrínseca à ampicilina, amoxicilina, cefalosporinas de primeira geração e cefoxitina devido à expressão de um gene *AmpC* constitutivo-lactamase. Além disso, a produção de ESBLs tem sido relatada nestas bactérias, o que dificulta seu tratamento (DAVIN-REGLI et al., 2015; DAVIN-REGLI et al., 2016). A resistência aos antimicrobianos, a regulação dos genes de resistência e as implicações clínicas dessas situações têm sido extensivamente estudadas (PEREZ et al., 2012; DAM et al., 2018; MOLITOR et al., 2018).

2.4.3 *Escherichia coli*

E. coli é uma bactéria Gram-negativa da família Enterobacteriaceae, não esporulada, anaeróbia facultativa, fermentativa, em sua maioria móvel devido aos flagelos peretríqueos e cresce em temperaturas de 18 a 44°C. Metaboliza uma ampla variedade de substâncias como carboidratos, proteínas, aminoácidos, lipídeos e ácidos orgânicos, produzem catalase, e utiliza glicose, amônia e nitrogênio como fontes de carbono (CROXEN et al., 2013).

A maioria de seus sorogrupos fazem parte da microbiota comensal do intestino dos mamíferos, no entanto certos sorotipos são patogênicos para o homem e para outros animais, e não são considerados como integrantes da microbiota intestinal normal. Cepas com potencial patogênico, causam infecções extraintestinais (ExPEC) em humanos, incluindo infecção do trato urinário (UPEC) e meningite neonatal (NMEC) (ALAV et al., 2018). Doenças do tubo digestivo e infecções podem variar desde formas benignas até formas que podem ser mortais, dependendo de fatores como o tipo de estirpes patogênicas, a susceptibilidade do paciente e o grau de exposição (LEE et al., 2018).

A multirresistência em *E. coli* tornou-se uma questão preocupante cada vez mais frequente na clínica. *E. coli* é intrinsecamente suscetível a quase todos os agentes antimicrobianos clinicamente relevantes, mas essa espécie tem grande capacidade de acumular

genes de resistência, principalmente por meio de transferência horizontal de genes de outros membros da família Enterobacteriaceae (DE OLIVEIRA et al., 2020). Os mecanismos em *E. coli* correspondem à aquisição de genes que codificam ESBLs (conferindo resistência a cefalosporinas de amplo espectro), carbapenemases (conferindo resistência a carbapenêmicos), 16S rRNA metilases (conferindo pan-resistência a aminoglicosídeos), genes de resistência a quinolonas mediados por plasmídeo (conferindo resistência a fluoroquinolonas) e genes *mcr* (conferindo resistência a polimixinas) (POIREL et al., 2018).

Considerada a representante do grupo dos coliformes fecais, a contagem de *E. coli* tem sido usualmente empregada no monitoramento de saúde ambiental, especialmente na qualidade das águas. A resolução CONAMA 357/05 afirma que a *E.coli* pode substituir o parâmetro de coliformes termotolerantes, e visto que são restritas ao trato intestinal de animais homeotérmicos, são consideradas por diversos autores como mais significativas que os coliformes totais para expressar poluição ambiental de origem fecal (JANG et al., 2017; DEVANE et al., 2020). Além disso, também tem sido sugerido o uso de *E. coli* resistente a antimicrobianos como indicador em programas de monitoramento ambiental para verificar a ocorrência e os níveis de resistência antimicrobiana no ambiente (ANJUM et al., 2021).

3 OBJETIVOS

3.1 Objetivo geral

Avaliar a relação da presença de *E. coli*, *Klebsiella* spp. e *Enterobacter* spp. resistentes a β-lactâmicos, fluoroquinolonas, aminoglicosídeos e tetraciclina com variáveis ambientais mensuradas em um ecossistema estuarino no sul do Brasil.

3.2 Objetivos específicos

a) Caracterizar a diversidade microbiológica da região estuarina da Lagoa dos Patos e costa marinha adjacente;

b) Quantificar o número de Unidades Formadoras de Colônias por mililitro (UFC/mL) de *E. coli* e coliformes totais na região estuarina da Lagoa dos Patos e costa marinha adjacente;

c) Avaliar o perfil de sensibilidade frente aos antimicrobianos Cefotaxima, Ciprofloxacina, Gentamicina, Imipenem e Tetraciclina das Enterobactérias *E. coli*, *Klebsiella* spp. e *Enterobacter* spp. isoladas da região estuarina da Lagoa dos Patos e costa marinha adjacente.

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Relationship between environmental variables and antibiotic resistant in estuary ecosystem in southern Brazil

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ABSTRACT

Abiotic factors (eg temperature, pH, salinity and nutrients) can drive the evolution, proliferation and spread of antibiotic resistant bacteria (ARB). The complexities of biotic-abiotic interactions result in a lack of understanding of the environmental variables that influence the presence of these microbes. We identified the presence of phenotypically antibiotic-resistant Enterobacteriaceae and related them to environmental parameters measured in the Patos Lagoon estuary, located in the city of Rio Grande, Rio Grande do Sul, Brazil. Water samples were collected and physicochemical parameters were measured. The colony forming units per milliliter (CFU/mL) were counted. The phenotypic identification was performed by biochemical tests and the sensitivity profile by the disk diffusion test, Kirby-Bauer methodology. The relationship between environmental parameters and the presence of ARB was evaluated by Principal Component Analysis (PCA). In the Middle estuary, there was an increase in temperature and CFU/mL of *Escherichia coli* and total coliforms in November. Turbidity was related to ARB in the Middle estuary and mouth. Due to the rains notified mainly in the month of September, there was a resuspension of sediments and adhered bacteria, which also caused an increase in the quantification of total coliforms. At Cassino beach, pH and dissolved oxygen variables influenced the presence of ARB. The presence of algae causes an increase in oxygenation and pH, which causes the production of toxic forms of oxygen and tends to be bactericidal. The estuarine environmental quality assessment provided data to be used in environmental management programs in order to reduce the spread of ARB.

Keywords: antibiotic resistant bacteria; *Escherichia coli*; *Klebsiella* spp.; *Enterobacter* spp.; estuary; Patos Lagoon.

1 INTRODUCTION

Chemical pollutants such as antibiotics and heavy metals were primarily responsible for the spread of antibiotic resistant genes (ARG) in aquatic ecosystems, where they impose selection pressures on microbial communities, increasing horizontal gene transfer and maintaining/increasing the abundance of antibiotic resistant bacteria (ARB) (Zhu et al., 2017; Pal et al., 2017; Dickinson et al.; 2019). In addition, other abiotic factors (e.g. temperature, pH,

salinity and nutrients) may also represent selective stresses driving the evolution, proliferation and spread of ARB (Zhang et al., 2019; Ohore et al., 2020; Wang et al., 2021). However, little information is available on the relative importance of these factors that influence the diversity and abundance of ARB in urban lagoon environments. The complexities of biotic-abiotic interactions in these compartments result in a lack of integrated understanding of the environmental variables that influence the presence of resistant bacteria (Han et al., 2021).

The spread of antibiotic resistance is a global threat to humans, and is estimated to be the cause of 10 million deaths each year by 2050 (WHO, 2019). Overuse of antibiotics in healthcare, agriculture, and the environment, as well as inappropriate consumption of antibiotics such as inappropriate choices, dosage, poor adherence to treatment guidelines, contribute to the increasing selection of antimicrobial resistance (Prestinaci et al., 2015). Faced with increasing antibiotic resistance, the WHO has published a list of pathogens designated by the acronym ESKAPEE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* species and *Escherichia coli*) to which the highest "priority status", as they are the main cause of nosocomial infections worldwide and most of them are multidrug-resistant, which is a huge challenge in clinical practice (WHO, 2017). Thus, understanding of dynamic of antibiotic resistance dissemination in environment is crucial to control these important problem of public health.

ARB and ARG can be enriched in the environment because of anthropogenic impacts, with hospital wastewater potentially acting as an important source this process. This compartment acts as a reservoir for microbes, ARG, plasmids, phages, antimicrobials and other substances, being called a resistome (Wellington et al., 2013; Suzuki et al., 2017). ARG evolution in aquatic environments is mediated by mutations, horizontal gene transfer (HGT) and other genetic mechanisms. Conjugative transfer via mobile genetic elements (e.g. plasmids and transposons i.e. Integrated Conjugative Elements) is thought to be common and has the potential to transfer ARG to bacteria of unrelated phyla (von Wintersdorff et al., 2016).

Estuaries and coastal seas have been focal points of human settlement and economic resource use throughout history and are a hotspot for anthropogenic impacts (Lotze et al., 2006). Patos Lagoon (10,360 km^2), located in southern Brazil, is considered the largest choked lagoon in the world and classified as partially closed (Kjerfve, 1986). In the city of Rio Grande, in Rio Grande do Sul (RS), its mouth and estuarine region are located. The municipality of Rio Grande has port and industrial areas located on one side of the lagoon, while urban occupation develops along the other side. Among the impacting activities, recurring in the region, which can change

the quality of water and fish, are the receipt of untreated domestic sewage and the indirect influences of the disposal of domestic waste in dumps. These impacting activities can put local aquatic life at risk, also threatening the economic exploitation of biological resources, bringing negative effects to the economy and human health, since many extractive activities take place in the region, including artisanal fisheries and other recreative uses (Kalikoski & Vasconcellos, 2012).

The assessment of microbial water quality in multiple-use aquatic ecosystems should be made to propose interventions and protocols that can prevent or delay the recruitment of resistance genes and reduce the environmental spread of resistant pathogens (Larsson et al., 2021). The detection of pathogenic microorganisms in aquatic environments is extremely relevant in terms of public health. These laboratory methodologies are usually difficult, expensive and time-consuming, they are frequently replaced by the assessment of fecal indicator bacteria, such as *Escherichia coli* (Oliveira et al., 2018). However, it is known that effluent discharge may be associated with the release of other pathogenic microorganisms (Berendonk et al., 2015; Hernando-Amado et al., 2019). Thus, it is desirable that the parameter "coliforms" in the indication of the bacteriological quality of the water is accompanied by additional monitoring of the main waterborne pathogens.

The present study aimed to investigate the presence of antibiotic-resistant Enterobacteria (species present in the acronym ESKAPEE) and their relationship with environmental variables measured in the Patos Lagoon estuary in the city of Rio Grande (RS).

2 MATERIALS AND METHODS

Study design and area

A cross-sectional study was made to assess the prevalence of antibiotic-resistant Gram-Negative bacteria isolated from water samples collected in the Patos Lagoon estuary and adjacent marine coast, in the municipality of Rio Grande (RS). Sampling was obtained through monthly collections, from August to November 2021. Water collections were performed along with the Long-Term Ecological Research group in the Patos Lagoon Estuary and Adjacent Marine Coast (PELD-EPA) and occurred in three fixed stations: 1) middle estuary: pier of the Rio Grande Yacht Club; 2) mouth of estuary: pier of Prainha in the "4º Secção da Barra" section; 3) Cassino beach: in front of the Marine Aquaculture Station (EMA) (Figure 1). At each

sampling station, the physicochemical parameters: temperature, salinity, total dissolved solids, turbidity, dissolved optical oxygen, hydrogenion potential (pH) and ammonium (NH_4), were measured with a YSI PRODSS multiparameter probe (YSI Incorporated, Yellow Springs, OH, USA).

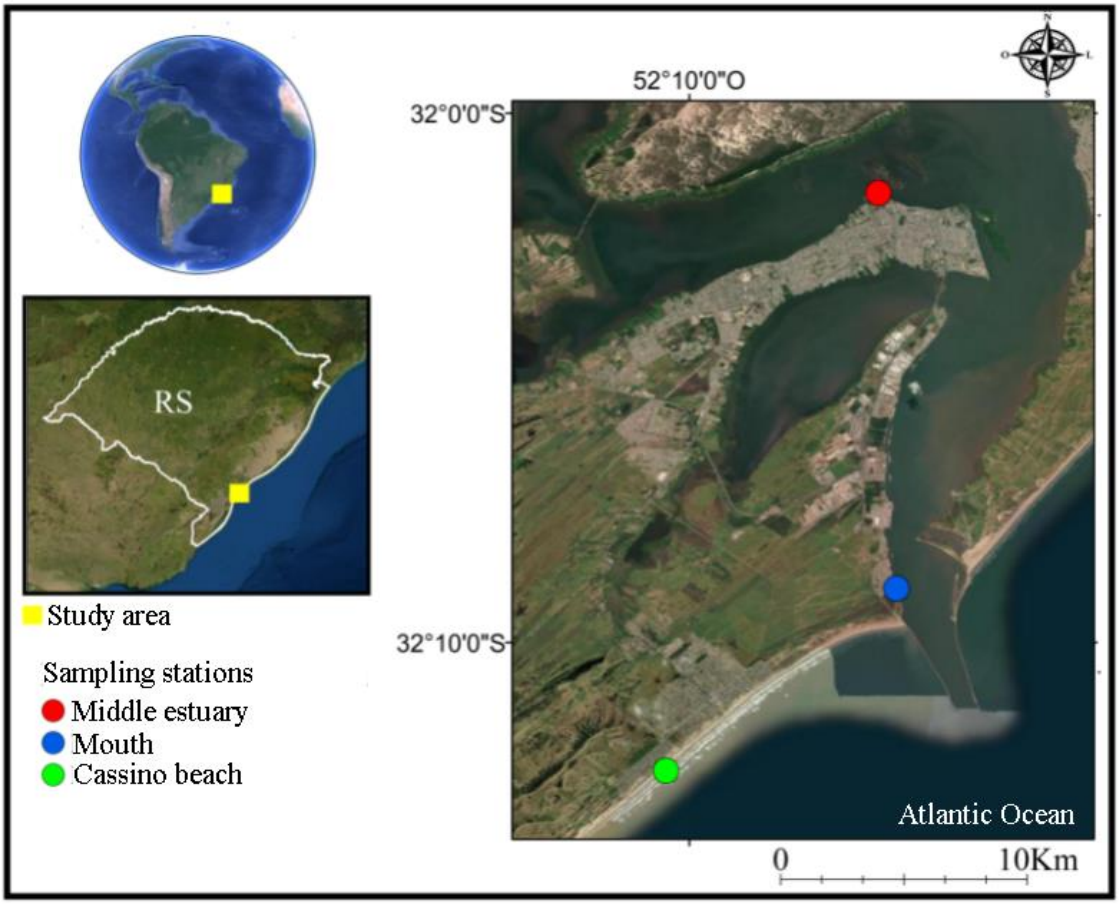

Figure 1. Study areas located in the Patos Lagoon estuary and adjacent marine coast, in the city of Rio Grande (RS).

A total volume of 1.000 mL of water was collected at each sampling station using buckets and subsequently stored in sterile amber glass bottles. All water samples were stored in a refrigerated isothermal box until the moment of analysis at the Laboratory of Molecular Microbiology and Cell Culture, located at the Dr. Miguel Riet Corrêa Jr. University Hospital of the Federal University of Rio Grande (HU-FURG/EBSERH).

Culture

The isolation of bacteria present in the water samples was obtained through the filtering membrane technique. Vacuum filtration of the samples was performed using a filtering membrane of mixed cellulose esters, with a diameter of 47 mm and pores of 0.45 µm (Millipore), in a polysulfone filtration system (Millipore). 100 mL of each water sample collected at the three sampling stations were filtered for the isolation of Enterobacteriaceae. For each filtration, a separate filter membrane was used.

The filter membranes containing the microorganisms retained after the filtration process were added with the aid of sterile forceps to Petri dishes with the selective culture medium Agar M-ENDO LES and cultivated at 36 ± 1°C for 22 ± 2 hours. The culture medium was prepared according to the manufacturers' instructions. After the incubation time, the number of colony forming units per milliliter (CFU/mL) of water sample was calculated. Membranes that presented between 30 and 300 CFU were considered.

Identification

After bacterial growth in M-ENDO LES Agar culture medium containing the filter membranes, a new bacterial culture was performed to obtain isolated colonies. For this, each colony was transferred, with the aid of a sterile disposable bacterial loop, to an eppendorf tube containing brain-heart infusion broth (BHI), in order to obtain bacterial growth. After incubation at 36 ± 1°C for 22 ± 2 hours, an aliquot of the eppendorf bacterial growth was transferred and seeded in a Petri dish containing MacConkey Agar, with the aid of a sterile disposable bacterial loop. The same incubation conditions mentioned above were used.

Each colony of bacteria isolated in MacConkey Agar was submitted to phenotypic identification through biochemical tests, such as the use of citrate, production of phenylalanine deaminase, decarboxylation of lysine, fermentation of lactose, production of gas (CO_2), production of hydrogen sulfide (H_2S), motility, indole production and urea hydrolysis. All the culture media for the biochemical tests were prepared according to the manufacturers' instructions and the results were interpreted according to the National Health Surveillance Agency (ANVISA, 2004).

Drug susceptibility test

The antibiotic resistance profile was evaluated using the disk diffusion test, using the Kirby-Bauer methodology proposed by CLSI (Clinical and Laboratory Standards Institute, 2019). For this, a bacterial suspension was prepared with the addition of previously isolated bacterial colonies to tubes containing sterile saline solution (NaCl 0.85%), until obtaining a turbidity compatible with the 0.5 MacFarland Scale ($1x10^8$ CFU/mL). Then, a new seeding on plates containing Mueller Hinton Agar was performed using a swab soaked in the bacterial suspension. After the surfaces of the plates had dried, disks impregnated with antibiotics were added to the surfaces of the inoculated media. The plates with the disks were incubated in a bacteriological oven at 35 ± 2°C for 16 to 18 hours (24 hours for cefoxitin and vancomycin). The results were interpreted with the aid of a ruler or caliper, used to measure the diameter of the inhibitory halos generated by each antibiotic disk. The samples were classified as susceptible, intermediate or resistant to the antibiotics tested. Cutoff points established by the CLSI were considered (CLSI, 2019; Table 1).

Table 1. Inhibitory halo values established by CLSI for Enterobacteriaceae.

		Inhibition halo diameter (mm)		
Antibiotic	**Disk concentration**	**Resistant**	**Intermediary**	**Sensitive**
Cefotaxime	30 µg	≤22	23-25	≥26
Ciprofloxacin	5 µg	≤21	22-25	≥26
Gentamicin	10 µg	≤12	13-14	≥15
Imipenem	10 µg	≤19	20-22	≥23
Tetracycline	30 µg	≤11	12-14	≥15

Source: CLSI, 2019.

Statistical analysis

To evaluate the relationship between the physical chemical parameters measured and the prevalence of antibiotic-resistant bacteria, Principal Component Analysis (PCA) was performed using the number of resistant bacteria quantified each month per sampling station. All statistical analysis was performed in the R programming language (RStudio, 2018).

3 RESULTS

Physicochemical parameters

The physical-chemical parameters Temperature (Temp), Salinity (Sal), Total Dissolved Solids (TDS), Turbidity (Turb), Dissolved Optical Oxygen (OD), Hydrogenion potential (pH) and Ammonium (NH_4), measured at each sampling per month, are expressed in Table 2.

Table 2. Values of Temperature (Temp), Salinity (Sal), Total Dissolved Solids (TDS), Turbidity (Turb), Dissolved Optical Oxygen (OD), Hydrogenion potential (pH) and Ammonium (NH_4) measured in three sampling stations of the Patos Lagoon estuary and adjacent marine coast evaluated from August to November.

Months	Temp (°C)	Sal (psu)	TDS (mg/L)	Turb (FNU)	pH	OD (mg/L)	NH_4 (µM)
Middle estuary							
Aug	12	6,3	7196	2,3	6,9	10,3	6,1
Sep	18	4,4	5178	26,1	7,7	8,6	2,5
Oct	16	3,4	3989	26,6	7,5	8,8	2,8
Nov	22	1,7	2183	68,1	7,5	7,7	2,9
Mean (±SD)	**17(±4.2)**	**3.9(±1.9)**	**4583.5(±2104.2)**	**26.4(±27.3)**	**7.5(±0.3)**	**8.7(±1.1)**	**2.9(±1.7)**
Mouth							
Aug	12	5,3	6187	7,4	7,1	10,6	4,9
Sep	17	2,9	3526	14,5	7,7	9	2,6
Oct	16	12,1	13145	56,2	7,1	8,6	3,1
Nov	23	1,5	1890	65	7,6	8	3,1
Mean (±SD)	**16.5(±4.5)**	**4.1(±4.7)**	**4856.5(±4965.2)**	**35.35(±29)**	**7.3(±0.3)**	**8.8(±1.1)**	**3.1(±1)**
Cassino beach							
Aug	12	26,3	26759	12,9	8,1	9,3	3,8
Sep	17	29,1	29256	49,7	7,8	8,3	2,4
Oct	18	28,5	28658	65,8	7,8	8,3	2,4
Nov	20	30,7	30649	45,4	7,9	7,5	2,7
Mean (±SD)	**17.5(±3.4)**	**28.8(±1.8)**	**28957(±1613.4)**	**47.5(±22.2)**	**7.8(±0.1)**	**8.3(±0.7)**	**2.5(±0.7)**

Quantification of bacterial colonies

The number of Colony Forming Units per milliliter (CFU/mL) of *E. coli* found at each station during each sampling month are shown in Figure 2. It was observed that in November

there was an increase in CFU/mL at the stations Middle estuary and Mouth, while at Cassino beach station the presence of *E. coli* was not verified in any of the months (Figure 2).

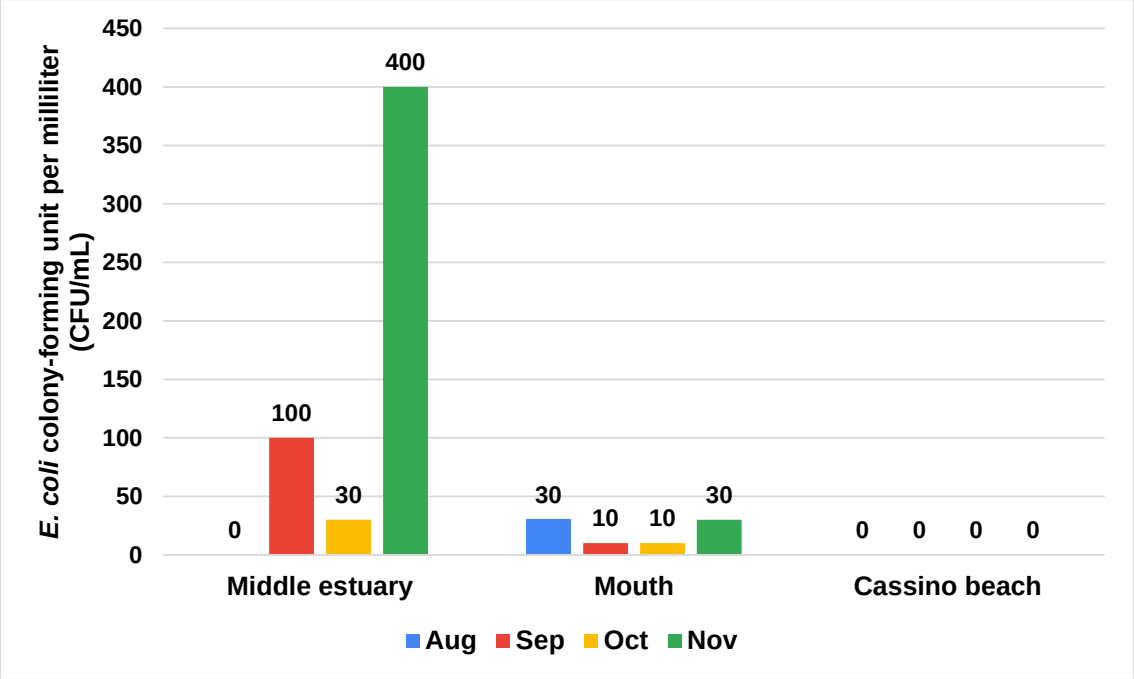

Figure 2. Colony Forming Units per milliliter (CFU/mL) of *E. coli* isolated from three different sampling stations (Middle estuary, Mouth and Cassino beach) during the months of August, September, October and November.

The number of Colony Forming Units per milliliter (CFU/mL) of total coliforms found at each station during each sampling month are shown in Figure 3. It was found that in September and November there was an increase in CFU/mL in the stations Medium estuary and mouth, while at Cassino beach station there was a progressive increase in the number of CFU/mL of total coliforms over the months (Figure 3). In October, the quantification of total coliform colonies was not possible at the Middle estuary and Mouth sampling stations, due to the excessive growth of microcolonies (Figure 4).

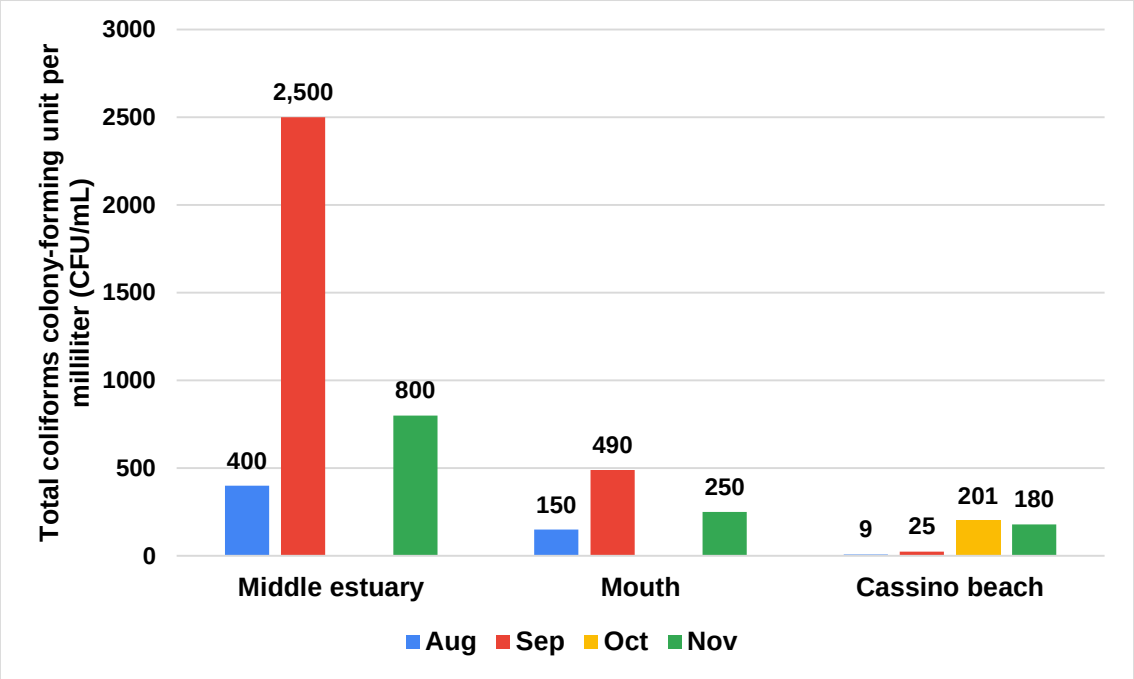

Figure 3. Colony Forming Units per milliliter (CFU/mL) of total coliforms isolated from three different sampling stations (Middle estuary, Mouth and Cassino beach) during the months of August, September, October and November.

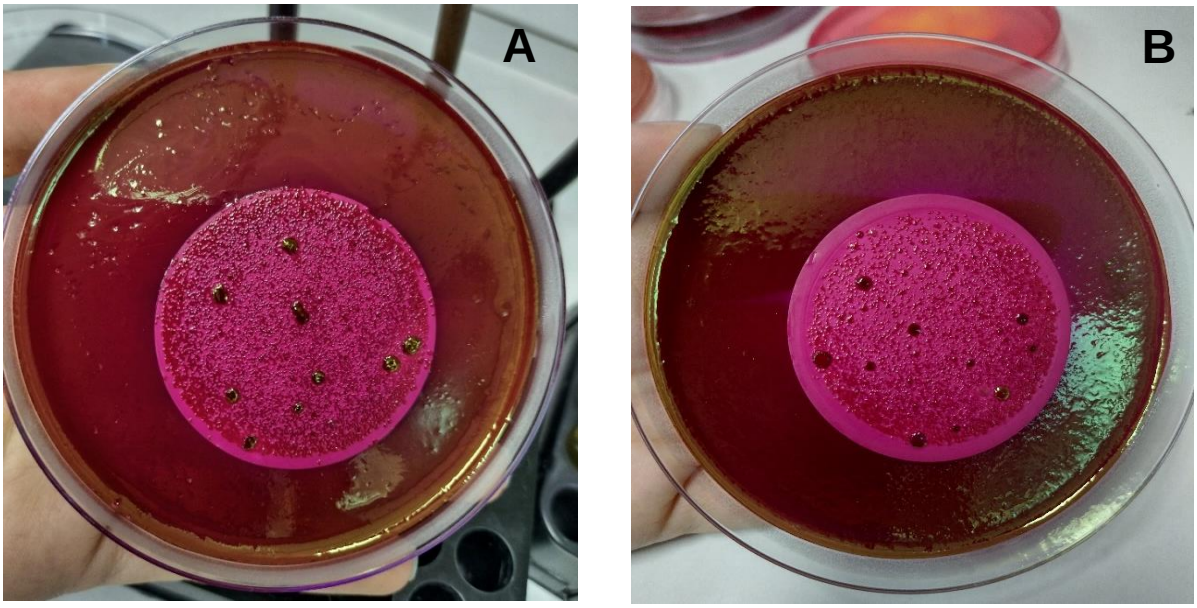

Figure 4. Petri dish containing microcolony overgrowth on representative filter membranes from the Middle estuary (A) and Mouth (B) sampling stations.

Identification of the bacterial community

Species of Gram-negative bacteria isolated at the three sampling stations (Middle estuary, Mouth and Cassino beach) in each month are described in Table 3. The presence of the same homogeneous group was observed regardless of the sampling station.

Table 3. Species of Gram-negative bacteria isolated from three sampling stations of the Patos Lagoon estuary and adjacent marine coast evaluated from August to November.

Bacterial Organism	**Middle estuary (*n*)**	**Mouth (*n*)**	**Cassino beach (*n*)**	**Total number of isolates per species (*n*)**
Non-fermenting bacteria	6	3	4	**13**
Citrobacter koseri	4	0	2	**6**
Pantoae agglomerans	17	12	14	**43**
Morganella morganii	1	1	0	**2**
Yersinia enterocolitica	7	1	16	**24**
Klebsiella spp.	8	3	1	**12**
Hafnia alvei	2	2	1	**5**
Enterobacter cloacae	4	8	1	**13**
Enterobacter aerogenes	1	0	0	**1**
Escherichia coli	8	8	0	**16**
Proteus vulgaris	2	0	0	**2**
Citrobacter freundii	1	1	0	**2**
Edwardsiella tarda	0	6	1	**7**
Klebsiella oxytoca	0	0	3	**3**
Total number of isolates per site (*n*)	**61**	**45**	**43**	**Total *n* = 149**

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Antibiotic resistance profile

The antibiotic resistance profile of Gram-negative bacteria *E. coli*, *Klebsiella* spp. and *Enterobacter* spp. isolated at the three sampling stations for each month are described in Tables 4, 5 and 6. It was verified that in the Middle estuary and Cassino beach, there was a greater number of strains of *Klebsiella* spp. resistant compared to the other species (Table 4 and 5), while in Mouth there was a greater number of resistant strains of *E. coli* (Table 6).

Table 4. Value of the diameter of the inhibitory halos of Gram-negative bacteria identified as *E. coli*, *Klebsiella* spp. and *Enterobacter* spp. isolated from the Middle estuary sampling station

and their classification according to the cutoff points for the antimicrobials Cefotaxime (CTX), Ciprofloxacin (CIP), Gentamicin (GEN), Imipenem (IPM) and Tetracycline (TET), established by the Clinical Laboratory Standard Institute (CLSI, 2019).

Middle estuary						
Months	**Species**	**CIP**	**IPM**	**GEN**	**TET**	**CTX**
Sep	*Klebsiella* spp.	Sensitive	Sensitive	**Intermediary**	Sensitive	Sensitive
Oct	*Klebsiella* spp.	Sensitive	**Resistant**	**Intermediary**	**Resistant**	Sensitive
	Klebsiella spp.	**Intermediary**	Sensitive	Sensitive	Sensitive	Sensitive
	Klebsiella spp.	**Resistant**	Sensitive	Sensitive	Sensitive	Sensitive
	Klebsiella spp.	**Resistant**	Sensitive	**Resistant**	Sensitive	**Resistant**
	E. aerogenes	**Intermediary**	Sensitive	**Sensitive**	Sensitive	**Sensitive**
	E. cloacae	**Sensitive**	Sensitive	**Sensitive**	Intermediary	**Resistant**
Nov	*E. coli*	**Resistant**	Sensitive	**Intermediary**	**Resistant**	Sensitive
	E. cloacae	Sensitive	Sensitive	Sensitive	**Intermediary**	**Resistant**

Table 5. Value of the diameter of the inhibitory halos of Gram-negative bacteria identified as *E. coli*, *Klebsiella spp*. and *Enterobacter spp*. isolated from the Mouth sampling station and their classification according to the cutoff points for the antimicrobials Cefotaxime (CTX), Ciprofloxacin (CIP), Gentamicin (GEN), Imipenem (IPM) and Tetracycline (TET), established by the Clinical Laboratory Standard Institute (CLSI, 2019).

Mouth						
Months	**Species**	**CIP**	**IPM**	**GEN**	**TET**	**CTX**
Oct	*Klebsiella* spp.	**Intermediary**	Sensitive	Sensitive	Sensitive	Sensitive
	E. coli	**Intermediary**	Sensitive	**Intermediary**	**Resistant**	Sensitive
	E. cloacae	**Resistant**	Sensitive	**Resistant**	**Intermediary**	**Resistant**
Nov	*E. coli*	**Intermediary**	Sensitive	**Intermediary**	**Resistant**	Sensitive
	E. coli	**Intermediary**	Sensitive	Sensitive	Sensitive	Sensitive

Table 6. Value of the diameter of the inhibitory halos of Gram-negative bacteria identified as *Klebsiella* spp. isolated from the Cassino beach sampling station and their classification according to the cutoff points for the antimicrobials Cefotaxime (CTX), Ciprofloxacin (CIP), Gentamicin (GEN), Imipenem (IPM) and Tetracycline (TET), established by the Clinical Laboratory Standard Institute (CLSI, 2019).

Cassino Beach

Month	Species	CIP	IPM	GEN	TET	CTX
Aug	*E. coli*	**Intermediary**	Sensitive	**Intermediary**	Sensitive	Sensitive
	K. oxytoca	Sensitive	Sensitive	**Intermediary**	Sensitive	Sensitive
	K. oxytoca	Sensitive	Sensitive	Sensitive	Sensitive	**Intermediary**
	Klebsiella spp.	**Resistant**	Sensitive	Sensitive	Sensitive	Sensitive

Principal Component Analysis and correlation panel

There was a consistent linear relationship between the pH variable and the presence of bacteria resistant to CIP, IPM, GEN, TET and CTX at the Middle estuary sampling station (vector positions in the PCA and correlation coefficients). The temperature (temp) and turbidity (turb) variables were also related to the presence of bacteria resistant to CIP, TET and CTX in this same season (Figure 5).

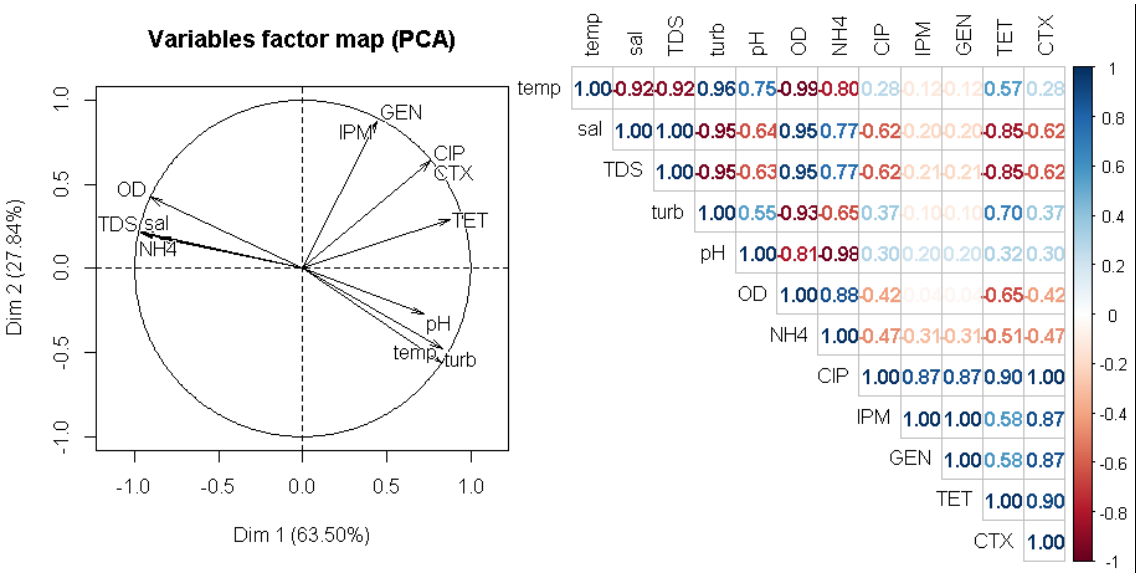

Figure 5. Middle Estuary: Linear correlations between the number of antibiotic resistant bacteria (CIP, IPM, GEN, TET and CTX) and the following physicochemical parameters: temperature (temp), salinity (sal), total dissolved solids (TDS), turbidity (turb), dissolved optical oxygen (OD), hydrogenion potential (pH) and ammonium (NH_4). Red values represent negative relationships and blue values represent positive relationships.

In the mouth, it was observed that the salinity (salt), total dissolved solids (TDS) and turbidity (turb) variables were related to the presence of bacteria resistant to CIP, IPM, GEN, TET and CTX (Figure 6).

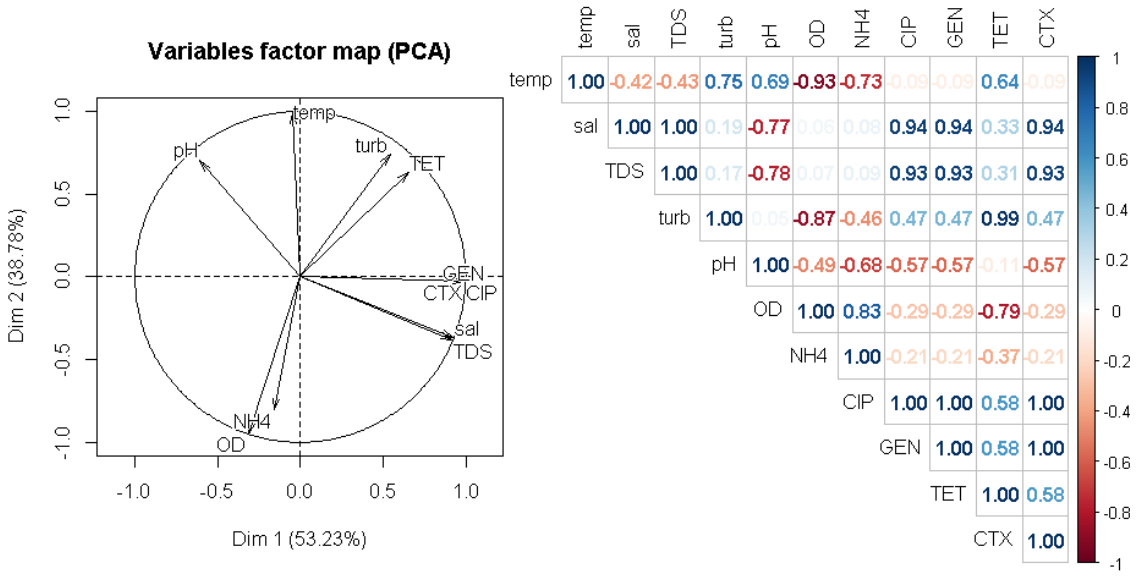

Figure 6. Mouth of Estuary: Linear correlations between the number of antibiotic resistant bacteria (CIP, GEN, TET and CTX) and the following physicochemical parameters: temperature (temp), salinity (sal), total dissolved solids (TDS), turbidity (turb), dissolved optical oxygen (OD), hydrogenion potential (pH) and ammonium (NH_4). Red values represent negative relationships and blue values represent positive relationships.

At the Cassino beach sampling station, the variables that were related to the presence of CIP-resistant bacteria were hydrogenion potential (pH), ammonium (NH4) and dissolved optical oxygen (OD) (Figure 7).

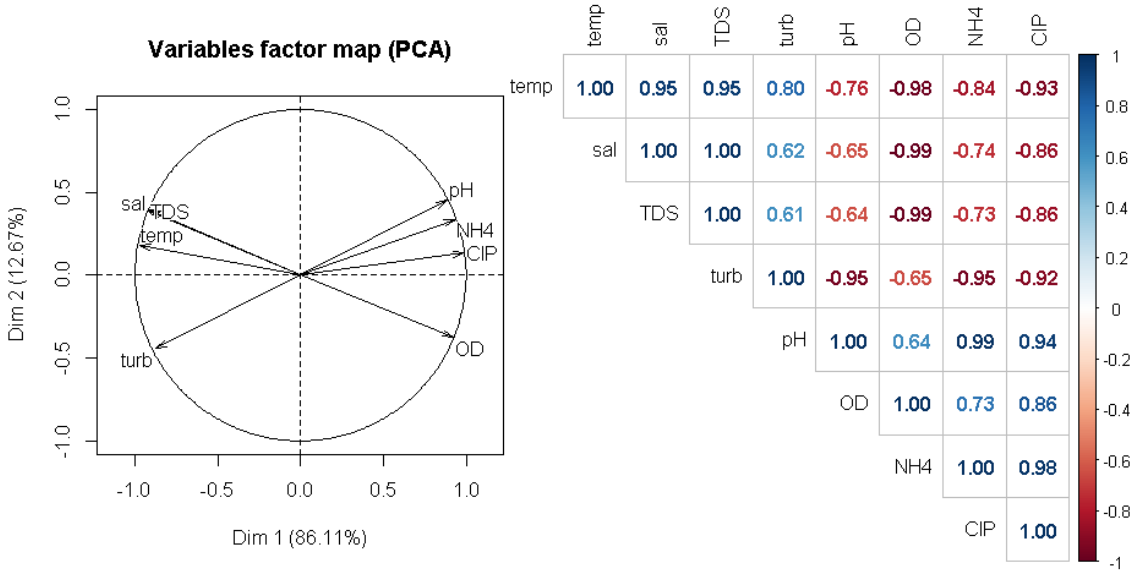

Figure 7. Cassino Beach: Linear correlations between the number of antibiotic resistant bacteria (CIP) and the following physicochemical parameters: temperature (temp), salinity (sal), total dissolved solids (TDS), turbidity (turb), dissolved optical oxygen (OD), hydrogenion potential

(pH) and ammonium (NH_4). Red values represent negative relationships and blue values represent positive relationships.

4 DISCUSSION

Quantification of E. coli and total coliforms

E. coli quantification differed especially at Cassino beach sampling station compared to Middle estuary and Mouth. The absence of *E. coli* at this site may be associated with higher salinity values. Baliarsingh et al. (2021) found that the mean *E. coli* count was highest during low tide and lowest during high tide attributed to salinity fluctuations of the Mahanadi estuary. In naturally saline estuarine and marine environments, salt concentrations reduce survival rates of *E. coli* and its efficacy as a pathogen indicator (Anderson et al., 1979).

Decreased *E. coli* survival in high salinity environments is caused by hyperosmotic stress and inactivation of crucial cellular processes. When released into the sea, the bacteria are subjected to hyperosmotic shock resulting in cell “closure”, that is, a faster degradation of the energy load and a decrease in the nutrient transport capacity (Troussellier et al., 1998; Rozen et al., 2001). The results show that the use of *E. coli* as an indicator of coliforms is inappropriate in places in areas with marked oscillations of salinity (estuarine areas). On the other hand, the quantification of total coliforms in this environmental compartment was not influenced by salinity, which allows the presence of other bacterial species resistant to higher salinity levels.

A relevant fact was that the number of CFU/mL for *E. coli* and total coliforms was higher in the Middle estuary compared to other sampling stations. It is known that this compartment is located on the edges of the urbanization and suffers direct anthropic influence, such as the release of treated and untreated wastewater from urban runoff. *E. coli* strains have been described as capable of growing efficiently under oligotrophic conditions. An association with biotic (algae, plants) or abiotic characteristics promotes the survival of *E. coli* in water, mainly due to the more efficient absorption of nutrients from the water column (Moreira et al., 2012).

In the Middle estuary and Mouth sampling stations, there was a higher number of CFU/mL of *E. coli* representative of November compared to the number of CFU/mL of the other months. *E. coli* is a fecal indicator microbe that circulates between two main habitats, endothermic intestines (primary habitat) and environmental water, sediments, and soils (secondary habitats). These habitats differ markedly in terms of physical conditions (eg

temperature). The temperature remains relatively constant (approximately 37°C) in the primary habitat, but can vary greatly in the secondary habitat (Freter, 1976). This data may be associated with the increase in temperature stimulating the growth of these microorganisms, since the optimal growth temperature is 37ºC.

The quantification of total coliforms showed that the month of September presented higher numbers of CFU/mL in relation to the other months in the sampling stations of the Middle estuary and Mouth. It was also observed that the quantification of total coliforms and the quantitative values of TDS were higher in these regions compared to Cassino beach, possibly as a consequence of the resuspension of microorganisms in the sediment. The action of rain, for example, can cause the resuspension of sediments and adhered bacteria.

Bacteria are strongly associated with particulate matter present in aquatic environments, which provides their persistence in the environment, offering physical and chemical protection against biotic and abiotic stresses. Thus, the release of bacteria during sediment resuspension also poses a risk to the microbiological quality of the water (Hassard et al., 2016). In estuaries, frequent resuspension and sediment deposition cause the transport of bacteria adhered to particles and possibly induce bacterial responses such as growth, degradation or changes in attachment (Fries et al., 2008). In this sense, a finite supply of sediment-associated bacteria is available for resuspension during heavy rainfall events.

Identification of bacteria

The most frequently species isolated were *P. agglomerans* (*n* = 43) and *Y. enterocolitica* (*n* = 24), respectively. *P. agglomerans* is an environmental bacterium frequently isolated from plants, soil, water, and food. This organism is an opportunistic pathogen that can be associated with trauma caused by the penetration of vegetative material during agricultural activities, gardening or children's play, as well as secondary bacteremia, or nosocomial infections related to medical equipment such as intravenous catheters or contaminated intravenous fluids (Cruz et al., 2007; Dutkiewicz et al., 2016). Bicudo et al (2007) described an outbreak of sepsis caused by this bacterium in the pediatric emergency department of a tertiary hospital in Brasília, Federal District. The transfer tube used in the intravenous hydration therapy was contaminated with *P. agglomerans*, characterizing the outbreak of common origin (Bicudo et al., 2007).

The second most frequently isolated species, *Y. enterocolitica*, is widely distributed in nature in reservoirs ranging from the intestinal tract of mammals to terrestrial and aquatic niches

(Fàbrega et al., 2012). Studies have suggested that dogs, sheep, wild rodents may be reservoirs of pathogenic strains, however, the usual route of infection of this pathogen is through contaminated food or water (Black et al., 1978; Keet et al., 1974). Human pathogenic strains are usually confined to the intestinal tract and cause enteritis/diarrhea (Younis et al., 2019).

Another relevant factor was the prevalence of *Klebsiella* spp. in all stations sampled, especially in the middle estuary (n = 8). *K. pneumoniae* and *K. oxytoca*, are well-known opportunistic hospital pathogens with the potential to exit hospitals via effluent systems (King et al., 2020). Barati et al. (2016) isolated strains of *K. pneumoniae* from sites with anthropogenic influence and control sites (less human activity), indicating that *K. pneumoniae* is ubiquitous in the environment. However, the detection of potentially virulent strains downstream from urbanization suggested to be associated with a source of anthropogenic contamination.

Lepuschitz et al. (2019) found genetic agreement between isolates of *K. pneumoniae* from river water samples and clinical isolates from patients, indicating that such a pathogen finds its way from hospitals to rivers. Due to the frequent cause of hospital illnesses, including pneumonia, urinary tract infections and bacteremia, hospital patients could be a source of Gram negative organisms spilling into rivers when hospital effluents are not properly treated. (Prado et al., 2008; Brink et al., 2012).

Furthermore, at the middle estuary sampling station, strains of non-fermenting bacteria were also isolated (n = 6), which allow to infer possible contamination by hospital effluent at that location. Non-fermenting bacteria are widespread in the environment and are a growing cause of serious infections in hospital practice, mainly affecting the growing population of patients immunocompromised by disease or medical and surgical treatments. The species of non-fermenters that often cause significant problems in hospital practice are: *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia* and members of the *Burkholderia cepacia* complex (Enoch, 2007).

Antibiotic resistance profile of E. coli, Klebsiella spp. and Enterobacter spp.

In addition to presenting a wide distribution between sampling stations, the species belonging to the genus *Klebsiella* also showed a higher rate of antibiotic resistance compared to the other microorganisms tested. Interestingly, the sampling station that presented the highest number of *Klebsiella* spp. together with *Enterobacter* spp. resistant was Middle estuary (n = 5 and n = 3, respectively).

This result may be related to the geographic location of the sampling station, which is close to the hospital complex of the city of Rio Grande, comprised of the Santa Casa and University Hospital. Although our results do not allow us to confirm a relationship between a high level of antibiotic resistance and the proximity to urbanization and the hospital, further studies could bring this information to light, and relate anthropogenic pressure caused by antibiotic use and environment pollution with antibiotic resistance.

Furthermore, in the Middle estuary, we found two strains of *Klebsiella* spp. and one strain of *E. coli*, resistant to antibiotics of different classes. This finding indicates the possibility that multidrug resistance is generated by one of two mechanisms: 1) these bacteria can accumulate several genes, each encoding resistance to a single drug, within a single cell. This accumulation typically occurs in resistance plasmids or 2) multidrug resistance can also occur by overexpression genes related to multidrug efflux, expelling a wide range of drugs (Nikaido, 2009).

Another relevant factor is the strain of *Klebsiella* spp. multi-resistant to TET, IPM and intermediate to GEN. Carbapenems are considered antibiotics of last resort for serious infections caused by members of the Enterobacteriaceae. Resistance to these antibiotics may be caused by reduced permeability of porins, efflux systems and carbapenemase production, which have considerably reduced the effectiveness of these antibiotics (Nordmann et al., 2009).

At the Mouth sampling station, there was a predominance of resistant *E. coli* strains (n = 3). On the shores of this place, there is urbanization, in addition to being a point of arrival and departure for small fishing boats. Thus, it is possible that this point has greater influence of cloacal sewage. Indeed, once released into the water, human-derived *E. coli* strains can be selected based on their survivability, and the resulting bacterial population differs from the original in terms of phenotypic characteristics, including virulence factors and antibiotic resistance (Bergholz et al., 2011; Van Elsas et al., 2010; Berthe et al., 2013).

Enterobacter spp. was the genus with the lowest number of resistant isolates present in Middle estuary (n = 3) and Mouth (n = 1). Especially the two species isolated in this study, *E. aerogenes* and *E. cloacae*, represent the most frequently Enterobacter species isolated in clinical infections, especially in immunocompromised patients admitted to the intensive care unit (ICU) (Davin-Regli et al., 2019).

In Mouth, we obtained an *E. cloacae* multiresistant strain (resistant to CIP, GEN and CTX, and intermediate to TET). It is known that these pathogens are often associated with a multiresistant phenotype, mainly due to their adaptation to the hospital environment and their

ability to acquire mobile genetic elements containing resistance and virulence genes. These species show intrinsic resistance to ampicillin, amoxicillin, first-generation cephalosporins and cefoxitin due to the expression of a constitutive AmpC-lactamase. Furthermore, the production of extended-spectrum β-lactamases (ESBLs) has been reported in these bacteria (Davin-Regli et al., 2015).

Another relevant result in our study was the number of ciprofloxacin-resistant bacteria. Ciprofloxacin, a second-generation fluoroquinolone, which has excellent antimicrobial activity and pharmacokinetic properties, as well as few side effects, has been introduced into clinical practice for the treatment of various bacterial infections for about 3 decades. However, ciprofloxacin resistance in Intensive Care Unit (ICU) is increasing, and the use of this antimicrobial agent as empirical therapy for ICU should be reconsidered. Policy restrictions on the use of ciprofloxacin should be reinforced especially in developing countries (Fasugba et al., 2015).

Relationship between environmental variables and the presence of antibiotic resistant bacteria

In the Middle estuary, temperature was related to the presence of ARB. Temperature affects bacterial growth as well as modulates the transfer of genomic material, which includes genes encoding antibiotic resistance (Lorenz et al., 1994; Philipsborn et al., 2016). Horizontal gene transfer, an important mechanism for acquiring antibiotic resistance, is directly related to temperature rise (Pietikäinen et al., 2005). MacFadden et al. (2018) demonstrated that increasing local temperature, as well as population density, are associated with increased antibiotic resistance in common pathogens (*E. coli, K. pneumoniae*, and *S. aureus*) (MacFadden et al., 2018). Thus, increases in temperature and population density, as seen in this study (section Quantification of *E. coli* and total coliforms), may have caused an increase in antibiotic resistance rates through the mechanism of vertical gene transfer.

Likewise, turbidity was a variable that correlated with ARB at the Middle estuary and Mouth stations, which can be explained by the resuspension of sediments and associated resistant bacteria as a function of rainfall. In estuarine environments, these bacteria survive longer associated, mainly due to the decrease in the effect of U.V light that is partially adsorbed by the turbid waters (Flint, 1987). Still, in the sampling station of Mouth, it was verified that the TDS was also a significant variable. The survival of microbes in water can be influenced by the

concentrations of suspended solids in terms of how easily they can bind to these particles (Petersen et al., 2020).

Sediment association can increase the availability of nutrients and organic matter, particularly when suspended solids include organic material, while providing optimal light exposure (Drummond et al., 2015). Furthermore, the proximity of microbes associated with suspended particles may facilitate the horizontal transfer and proliferation of resistance genes. Horizontal transfer of genetic material can be accelerated when two microbes come into close contact with each other and remain so until the transfer of genetic material is complete (Allen et al., 2010).

At the Cassino beach sampling station, the variables that influenced the presence of ARB were pH, NH_4 and OD. The reduction in oxygenation is attributed to the presence of algae causing the death of fecal bacteria due to the production of toxic forms of oxygen (Curtis et al., 1992).The presence of algae also leads to high pH levels that tend to be bactericidal even in the absence of high concentrations of oxygen (Maynard et al., 1999). Furthermore, fluctuations in pH are known to negatively affect *E. coli* survival (Awuah, 2006), and therefore can result in significant removal of faecal bacteria.

The month of August, at Cassino beach, was the only month in which resistant strains were isolated. In this month, we also observed a higher value of NH_4. Bernier et al. (2011) demonstrated that exposure to ammonia released from stationary phase bacterial cultures modifies the spectrum of antibiotic resistance of Gram-negative and Gram-positive bacteria. Exposure to ammonia increases the level of intracellular polyamines, leading to changes in membrane permeability to different antibiotics, as well as increased resistance to oxidative stress (Bernier et al., 2011).

Limited information is available on ARB population dynamics after release in estuarine environments. This is concerning if the subsequent wastewater treatment process does not remove such bacteria. Possible horizontal transfer of resistance genes from clinical isolates to previously susceptible environmental strains may therefore occur, as was previously reported (Allen et al. 2010). In particular, the influence of environmental change on ARB retention in estuarine environments is unclear.

While it is necessary to strengthen the proper use of antibiotics and accelerate the new strategies to mitigate the spread of antibiotic resistance, it is also critical to understand the movement of ARB in environments. Thus, a more complete understanding of the dynamics of

antibiotic resistance in natural aquatic environments is important to reduce the impact of antibiotic resistance and limit its spread.

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6 CONCLUSÕES

Com o exposto ao longo do presente estudo, pode-se concluir que:

1) As espécies da Família Enterobacteriacea mais frequentemente isoladas foram *Pantoae agglomerans* (*n* = 43) e *Yersinia enterocolitica* (*n* = 24).

2) O uso de *E. coli* como indicador de coliformes é inadequado em locais em áreas com fortes oscilações de salinidade (áreas estuarinas).

3) A região com maior isolamento de microrganismos resistentes foi o Estuário Médio (*n* = 9), o que pode estar relacionado à sua proximidade a urbanização e ao complexo hospitalar.

4) Isolados pertencentes ao gênero *Klebsiella* spp. apresentaram maior índice de resistência a antimicrobianos em relação aos demais microrganismos testados (*n* = 9).

5) O aumento da temperatura influenciou na densidade populacional e, consequentemente, nas taxas de resistência a antimicrobianos, no Estuário Médio.

6) A turbidez apresentou relação com a presença de BRA no Estuário Médio e Foz, possivelmente devido a ação das chuvas que ressuspende o sedimento contendo bactérias resistentes associadas.

7) A exposição à maiores valores de amônia pode ter colaborado para a presença de BRA no mês de agosto na Praia do Cassino.

7 CONSIDERAÇÕES FINAIS

O uso inadequado de antimicrobianos e a remoção ineficiente de BRA e resíduos de antibióticos durante o tratamento de esgoto podem contribuir para o surgimento e disseminação de resistência no ambiente, tornando-o um reservatório natural. Gerenciar as concentrações de microrganismos e, por extensão, a qualidade de corpos d'água receptores requer uma compreensão dos fatores que influenciam os seus ciclos de vida nesse habitat secundário. Entender as influências de parâmetros ambientais é fundamental para a implementação adequada de estratégias de manejo eficazes. As condições do habitat secundário poderão inibir ou estimular o crescimento e a sobrevivência de micróbios, dependendo de seus limites de tolerância.

A ocorrência de espécies bacterianas em hospitais e no ambiente - especialmente aquelas com fenótipos resistentes aos antimicrobianos- é uma importante questão ambiental e de saúde pública. A avaliação da qualidade ambiental estuarina permite que seja realizado o acompanhamento do impacto das atividades antrópicas na contaminação local, além de fornecer dados a serem utilizados em programas de gestão ambiental. Assim, os resultados gerados nesse estudo poderão ser utilizados para propor intervenções e protocolos que possam impedir ou reduzir a disseminação ambiental de patógenos resistentes.