UNIVERSITY OF LATVIA

Faculty of Biology

Dita Gudrā

DOCTORAL THESIS

The long-term impact of *Helicobacter pylori* eradication therapy on the human gastrointestinal microbiome and extended-spectrum β-lactamase (ESBL) coding gene levels

Promotion to the degree of Doctor of Biology

Molecular Biology

Supervisor: Dr. Biol. Dāvids Fridmanis

Rīga, 2023

The doctoral thesis was carried out at the Latvian Biomedical Research and Study Centre, Department of Human Genetics and Disease Mechanisms, "Exotic" Site Microbiome and G-protein Coupled Receptor Functional Research Group from 2016 to 2023.

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The research was supported by the European Regional Development Fund (ERDF) grant No. 1.1.1.1/16/A/272 "Long-term effects of *H. pylori* eradication on gastro intestinal tract microbiome and development of screening system for detection of extended-spectrum beta-lactamase coding genes within faeces samples" and by the European Social Fund (ESF) grant No. 8.2.2.0/20/I/006 "Strengthening of the capacity of doctoral studies at the University of Latvia within the framework of the new doctoral model".

The form of this thesis is a collection of research papers in biology, subfield – molecular biology.

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The thesis will be defended at the public session of the Doctoral Committee of Biology, University of Latvia, on February 23rd, 2024, at 13:00 at Latvian Biomedical Research and Study Centre, Ratsupites Str.1, k-1.

The thesis is available at the Library of the University of Latvia, Kalpaka blv. 4.

This thesis is accepted for the commencement of the degree of Doctor of Biology on November 13th, 2023, by the Doctoral Committee of Biology, University of Latvia.

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ABSTRACT

The current international guidelines advocate for a "test-and-treat" strategy for *Helicobacter pylori* infection to reduce the incidence of gastric cancer, primarily in countries with a high prevalence of *H. pylori* infection and gastric cancer. This would necessitate antibiotic treatment for approximately 79% of the population in Latvia. Nevertheless, the potential adverse effects of such therapies on the gastrointestinal microbiome and its antimicrobial resistance genes remain insufficiently studied.

The aim of this study was to investigate the long-term impact of first-line *H. pylori* eradication therapy on the gastrointestinal microbiome and on the levels of genes encoding extended-spectrum β-lactamases (ESBLs). In our first study we demonstrate the suitability of the Faecal Immunochemical Test sample containers, that are commonly used in colorectal cancer screening programmes, for faecal sample collection in gastrointestinal microbiome studies. Through the employment of targeted next-generation sequencing technologies, we further present that the taxonomic composition of the gastrointestinal microbiome remains stable following the *H. pylori* eradication treatment during long-term observation. Although there were significant differences in some low-abundant genera between the treatment states, the overall taxonomic structure of the microbiome appears to be more influenced by subject-specific parameters rather than by the eradication therapy itself. In the third part of this work, we introduce an ESBL coding gene panel that was used to assess the abundance and prevalence of ESBL coding genes in patients undergoing first-line *H. pylori* eradication therapy. Employing both targeted and shotgun metagenomic sequencing approaches, we showed that the distribution and abundance for most of the ESBL coding genes remained stable throughout the long-term observation. However, some of these genes exhibited differences in abundance between the treatment states, with a tendency to decrease during the post-eradication period. In addition, we observed diversification of the resistome profile of the gastrointestinal microbiome during the post-eradication period. Thus, these results indicate that patients underwent minor yet specific alterations in their microbial community profiles during antibiotic therapy. Moreover, these results suggest β-lactamase recolonization during gastrointestinal microbiome restoration, implying the need for greater microbiome control following the antibiotic treatment.

KOPSAVILKUMS

Pašreizējās starptautiskās vadlīnijas iesaka *Helicobacter pylori* infekcijas gadījumā pielietot "testē-un-ārstē" stratēģiju, lai samazinātu kuņģa vēža sastopamību galvenokārt tajās valstīs, kurās ir augsta *H. pylori* infekcijas un kuņģa vēža izplatība. Latvijā tas radītu nepieciešamību piedāvāt antibiotiku kursu aptuveni 79% iedzīvotāju. Taču šādas stratēģijas terapijas iespējamā nelabvēlīgā ietekme uz kuņģa-zarnu trakta mikrobiomu un tajā esošajiem antimikrobiālās rezistences gēniem joprojām ir maz pētīta.

Šī pētījuma mērķis bija izvērtēt pirmās līnijas *H. pylori* eradikācijas terapijas ilgtermiņa ietekmi uz kuņģa-zarnu trakta mikrobiomu un tās ietekmi uz paplašināta spektra β-laktamāžu (ESBL) kodējošo gēnu līmeņiem. Uzsākot pētījumu tika validēta fēču paraugu izmantošana kuņģa-zarnu trakta mikrobioma pētījumiem, kas ievākti Fēču Imūnķīmiskajos Testa paraugu konteineros, kas pamatā tiek izmantoti kolorektālā vēža monitoringa programmās. Izmantojot uz mērķsekvencēšanu balstītu nākamās paaudzes sekvencēšanas tehnoloģiju, tika novērots, ka pēc *H. pylori* eradikācijas terapijas kuņģa-zarnu trakta mikrobioma taksonomiskais sastāvs ilgtermiņā saglabājas stabils. Salīdzinot pirms un pēc eradikācijas terapijas indivīdu mikrobioma paraugus, tika novērotas būtiskas atšķirības dažās, galvenokārt, zemi pārstāvētās ģintīs, tomēr, mikrobioma kopējo taksonomisko struktūru būtiskāk ietekmēja indivīdu raksturojošie parametri, nevis eradikācijas terapija. Šī darba trešajā daļā tika ieviests ESBL kodējošo gēnu panelis, lai izvērtētu ESBL kodējošo gēnu dažādību un izplatību indivīdiem, kuriem tika veikta pirmās līnijas *H. pylori* eradikācijas terapija. Izmantojot gan mērķsekvencēšanu, gan visa metagenoma pilno sekvencēšanu, tika novērots, ka ilgtermiņā, vairumam ESBL kodējošo gēnu, to izplatība un daudzveidība saglabājas stabila. Tomēr dažiem no šiem gēniem bija vērojamas atšķirības to relatīvās sastopamības biežumā starp pirms un pēc eradikācijas stāvokļiem, ar tendenci samazināties. Papildus tam, novērota kuņģa-zarnu trakta mikrobioma kopējā antimikrobiālās rezistences profila bagātināšanās pēc-eradikācijas periodā. Līdz ar to, šie rezultāti norāda uz to, ka pacientiem pēc antibiotiku terapijas ilgtermiņa periodā ir vērojamas nelielas, tomēr specifiskas izmaiņas mikroorganismu kopienas profilos. Turklāt iegūtie rezultāti liek secināt, ka kuņģa-zarnu trakta mikrobioma atjaunošanās laikā notiek β-laktamāžu re-kolonizācija, kas norāda uz nepieciešamību pēc lielākas mikrobioma atjaunošanās kontroles pēc tā ārstēšanas ar antibiotikām.

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ABBREVIATIONS

aac – aminoglycoside resistance gene
AIC – Akaike Information Criterion
All-post-erad – all subjects in the post-eradication group
All-pre-erad – all subjects in the pre-eradication group
AME – aminoglycoside-modifying enzyme
AMR – antimicrobial resistance
ANOVA – Analysis of Variance
ARG – antimicrobial resistance gene
AU-ROC – Area Under the Receiver Operating Characteristics
BabA – blood-group antigen-binding adhesin
BL – β-lactamase
bla – β-lactam resistance gene
BLDB – β-lactamase Database
CARD – Comprehensive Antibiotic Resistance Database
CDS – protein-coding features
CFU – colony forming units
CKD – diabetes and chronic kidney disease
CRC – colorectal cancer
erm – macrolide resistance gene
ESBL – extended-spectrum β-lactamase
F-post-erad – subjects in the post-eradication group with ineffective *H. pylori* eradication
F-pre-erad – subjects in the pre-eradication group with ineffective *H. pylori* eradication
fca – fluoroquinolone resistance gene
FDR – False Discovery Rate
FIT – Faecal Immunochemical Test
FXR – farnesoid X receptor
GALT – gut-associated lymphoid tissue
gFOBT – guaiac-based Faecal Occult Blood Test
GIT – gastrointestinal tract
GO – Gene Ontology
HMP – Human Microbiome Project
HPE – *H. pylori* eradication
IARC – International Agency for Research on Cancer
IBD – inflammatory bowel disease
LPS – lipopolysaccharide
mcr – colistin resistance gene
mdr – multidrug resistance gene
MLS – macrolide- lincosamide-streptogramin resistance
NGS – next-generation sequencing
NLR – nod-like receptors
OTU – Operational Taxonomic Unit
PCoA – Principal Coordinate Analysis
PPI – proton pump inhibitor
PPR – pattern recognition receptors
RDA – Redundancy Analysis
RECUH – Riga East Clinical University Hospital
RGI – Resistance Gene Identifier
S-post-erad – subjects in the post-eradication group with successful *H. pylori* eradication
S-pre-erad – subjects in the pre-eradication group with successful *H. pylori* eradication
SabA – sialic acid-binding adhesin
SCFA – short-chain fatty acid
sIgA – secretory IgA
SILT – small intestinal lymphoid tissue
sul – sulfonamide resistance gene
tet – tetracycline resistance gene
TLR – toll-like receptors
UBT – ^{13}C-urea breath test
VacA – vacuolating cytotoxin A
van – vancomycin resistance gene
wFIT – faecal samples stored in Faecal Immunochemical Test container
woFIT – faecal samples stored without media

INTRODUCTION

The microbiome is characterized by a complex relationship with the human host and has been linked to various health and disease states. In cases of bacterial infection, antimicrobial compounds are often used to eradicate the pathogen; however, they can also affect the viability of commensal bacteria. One of the most common infections is *Helicobacter pylori*, which primarily resides in the human gut mucosal layers. While *H. pylori* infection is often asymptomatic, in some cases, it can progress to gastric inflammation, which may lead to such disorders as gastric cancer. Notably, the International Agency for Research on Cancer (IARC) has classified *H. pylori* as a Class I carcinogen, thus highlighting its potentially hazardous effect on the host's gastrointestinal health. Therefore, a "test-and-treat" strategy for this pathogen infection has recently been proposed for both symptomatic and asymptomatic individuals. However, given the high global prevalence of *H. pylori*, this recommendation is accompanied by concerns regarding the increased antibiotic consumption worldwide. Furthermore, adverse effects of combined broad-spectrum antimicrobial therapies on the gastrointestinal microbiome remain insufficiently studied, especially over a prolonged period of time, as these effects may vary depending on factors such as the specific antibiotic substance used, geographic location, resistance profiles, bacterial strains, and other variables.

Importance of this work: Until now, a limited number of studies have evaluated the long-term impact of the first-line *H. pylori* eradication therapy on the human gastrointestinal microbiome and antimicrobial resistance gene profile. New knowledge on treatment of biological material that has been collected for other purposes is essential to fulfil its scientific potential. Furthermore, knowledge on the microbiome stability following antibiotic treatment enhances our understanding of the long-term effects of antimicrobial agents on the gastrointestinal microbiome and the development of antimicrobial resistance. Therefore, presented results might contribute to the development of the national *H. pylori* eradication strategy.

Aim of the study: To evaluate the long-term effects of *H. pylori* eradication on the gastrointestinal microbiome and its effects on the abundance and prevalence of extended-spectrum β-lactamase coding genes.

Specific tasks to reach the aim:

1) Assess the applicability of biological material stored within the Faecal Immunochemical Test sample container for next-generation sequencing-based gastrointestinal microbiome studies.

2) Investigate the impact of the first-line *H. pylori* eradication therapy on the gastrointestinal microbial community taxonomic structure before starting the eradication therapy and two years after the treatment.
3) To identify the repertoire of extended-spectrum β-lactamase (ESBL) coding genes in the gastrointestinal microbiome samples.
4) To assess the differences in the relative abundance of various ESBL coding genes within individual samples before the eradication therapy and two years after.

1 LITERATURE REVIEW

1.1 The microbiome

The human body is home to a complex and abundant microbial ecosystem of eukaryotes, protozoa, and archaea. Different definitions are used to characterise this ecosystem; however, the most used are microbiota, microbiome, and metagenome to specify various aspects of the system. A microbiota is a community of microorganisms (bacteria, archaea, viruses, and some unicellular eukaryotes) that inhabit a given habitat. Conversely, the microbiome is a broader collection of microbial communities, their products (nucleic acids, proteins, lipids, polysaccharides, toxins, and others), and the environmental interactions between the microbiota and the human host. The metagenome, however, is limited to the genes and genomes of the microbiota (Berg *et al.* 2020). Recent studies have determined that the number of bacterial cells in and on the human host is comparable to that of human cells, with an estimated ratio of 1:1 (Sender *et al.* 2016a, 2016b). Although the exact number of genes encoded by the microbial metagenome is unknown, the most accepted estimate is two million, 100 times more than the number of human genes (Qin *et al.* 2010; Gilbert *et al.* 2018). From a taxonomic perspective, humans are estimated to host approximately 10^5 bacterial generations per human generation (Cho and Blaser 2012). Consequently, humans have two distinct genomes: one inherited from their ancestors and one acquired during life. Notably, the inherited genome is relatively stable, while the microbiome is highly dynamic and can be affected by various external influences (Cénit *et al.* 2014; Berg *et al.* 2020; Shoaie *et al.* 2021).

Numerous studies have investigated the microbial composition of health and disease states, demonstrating considerable intra-individual variability over time and the presence of distinct microbial signatures in various body sites. The goal of the Human Microbiome Project (HMP) Consortium was to analyse and characterise the healthy human microbiome, which has been achieved through the development of metagenomic protocols that have provided the scientific community with reference genomes of bacterial, viral, and eukaryotic microorganisms. The HMP has generated a substantial amount of data and has established the boundaries of the normal compositional range of the microbiome. The first phase of HMP focused on five primary body sites: the oral cavity, skin, nasal cavity, gastrointestinal tract, and urogenital tract. Studies revealed that microbial composition significantly varied based on anatomical location, with the gut possessing the highest number of bacterial species. These primarily belonged to a few phyla, mainly *Firmicutes*, *Bacteroidetes, Actinobacteria* and *Proteobacteria,* with the latest in lesser abundance than the other three (Dethlefsen *et al.* 2007;

Methé *et al.* 2012; D'Argenio and Salvatore 2015). The second phase of the HMP project was dedicated to the elucidation of the relationship between the human host, microbiome and the microbiome-associated conditions utilising multi-omic approaches (iHMP Research Network Consortium 2014; Proctor *et al.* 2019). This endeavour, along with other projects such as MetaHit project, which established a human gut microbial gene catalogue (Qin *et al.* 2010), and the emergence of next-generation sequencing technologies, enabled the formation of various regional population microbiome study cohorts, such as the Estonian Microbiome Project (Aasmets *et al.* 2022), Swedish population cohorts (Bäckhed *et al.* 2015; Hugerth *et al.* 2020), Finnish population cohorts (Korpela *et al.* 2019; Ruuskanen *et al.* 2022), British Gut Project (http://www.britishgut.co.uk), American Gut Project (Daniel *et al.* 2018), Latvian Microbiome Project (https://latvijasmikrobioms.lv) and other.

From a particular standpoint, the human microbiome can be segregated into two components – a variable portion that is not uniform across individuals and a fixed collection of common microbial populations. This persistent portion is commonly referred to as the core microbiota, and specific fluctuations in its composition are correlating and can be indicative of various pathologies. Though the microbiota composition is relatively stable at higher taxonomic levels, greater diversity can be observed at lower levels (Cho and Blaser 2012).

1.2 The gastrointestinal microbiome

The gastrointestinal tract (GIT) is a long muscular tube extending from the mouth to the anus, through which ingested material is physically broken down into a suspension of tiny particles and nutrients. Digestion, the process of macromolecule breakdown, occurs in the stomach and involves gastrointestinal motility to mix the ingested bolus, pH regulation, and the secretion of biological detergents and enzymes, such as pepsin, that promote protein hydrolysis. Following digestion, a bolus is delivered to the small intestine, where most lipid digestion and absorption occur. During absorption, digested macronutrients such as carbohydrates, proteins, vitamin B_{12} and bile salts are transferred from the intestines into the blood supply or lymphatic system (Guida and Venema 2015; Liu *et al.* 2019). Undigested food components are further transported to the large intestine, where they undergo bacterial fermentation. During this stage of digestion, water is absorbed, and various vitamins, including vitamin K, B_1, B_2 and B_{12}, are released (Liu *et al.* 2019).

The intestinal barrier is composed of a monolayer of intestinal epithelial cells, among which are goblet cells, Paneth cells, intestinal epithelial stem cells and others. Goblet cells secrete mucus, a highly hydrated gel that contains gel-forming glycosylated proteins, primarily

mucin 2. This mucus layer consists of a single layer in the small intestine and two layers in the stomach and colon, which plays a critical role in GIT transport, serving both as a lubricant and as a defilade layer that hampers bacterial invasion. The shielding and lubricating properties of mucus are essential for sustaining intestinal homeostasis. Bacterial cells adhere to the mucus layer via lectins and glycosidases expressed by specific bacteria, thereby highlighting the existence of a selective microbial habitat. Despite the active attachment of bacteria to the mucous layer, its structure, composed of interconnected sheets with pores too small for bacterial cells to pass through, limits their presence to the outer layer, leaving the inner layer nearly sterile (Bäckhed *et al.* 2015; Lee *et al.* 2021). Mucin glycans also serve as a nutrition source for mucus-associated bacteria (Li *et al.* 2015), resulting in the colonisation of both – commensal and, in some instances, pathogenic bacteria (Etienne-Mesmin *et al.* 2019; Lee *et al.* 2021). In addition, certain microbial species, such as *Lactobacillus* spp. and *Bifidobacterium* spp., have been demonstrated to stimulate mucin production, thus increasing the thickness of the mucus layer, providing an additional barrier to pathogenic bacteria (Lee *et al.* 2021).

The surface area of the human intestines reaches approximately 200 m^2, thus forming one of the main surfaces for microbial colonisation. The GIT microbiome is predominantly populated by strict anaerobes, which are present in more significant numbers than facultative anaerobes and aerobes. Variation in bacterial load and diversity is observed throughout the GIT tract (Figure 1) – bacterial load is lower in proximal GIT (stomach and duodenum – 10^1-10^3 CFU/mL), and diversity increases towards the distal tract (jejunum and ileum – 10^4-10^7 CFU/mL and colon 10^{10}-10^{11} CFU/mL) (Sekirov *et al.* 2010; Clarke *et al.* 2019). Although the composition of the microbiome varies from site to site in the GIT, in healthy adults, the common core is mainly composed of *Bifidobacterium*, *Clostridium*, *Peptococcus, Enterobacter*, *Eubacterium*, *Lactobacillus*, *Prevotella*, *Ruminococcus*, *Streptococcus* and others (de Vos and de Vos 2012; Dekaboruah *et al.* 2020). Generally, the microbial composition of the GIT is relatively stable; however, perturbations, such as those caused by

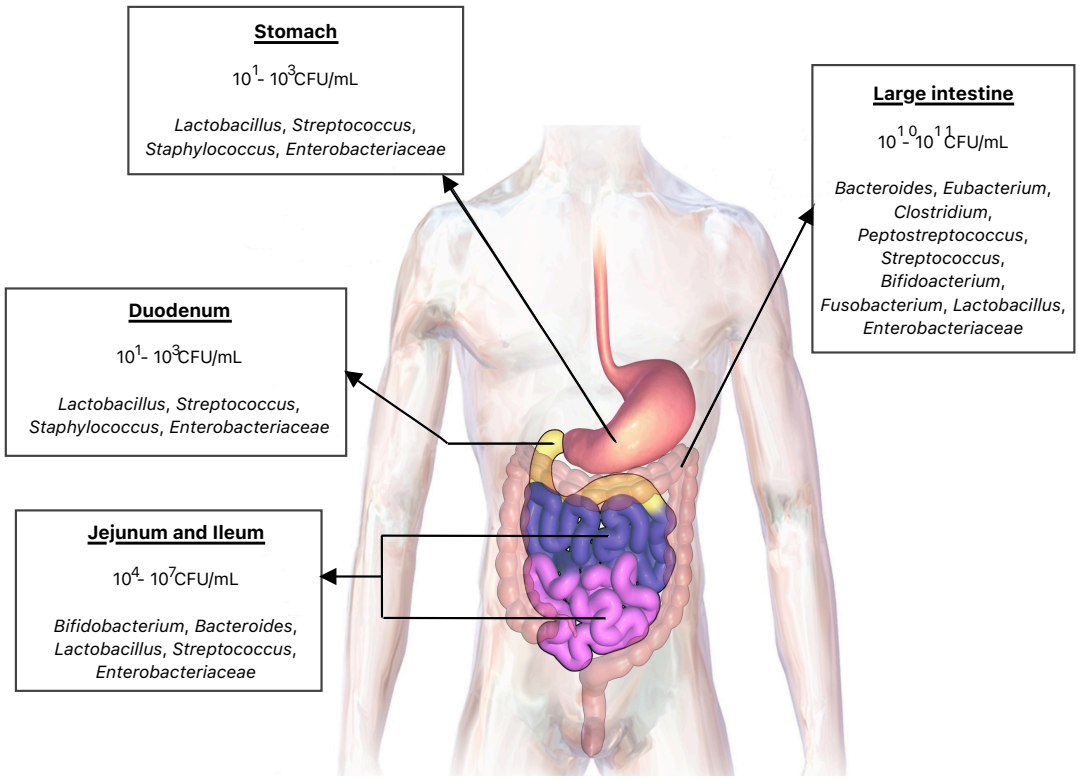

Figure 1. Spatial organization of gastrointestinal microbiome constituents (Blausen.com 2014; Clarke *et al.* 2019). Figure adapted from (Blausen.com, 2014). Abbreviations: CFU – colony forming units.

disease or drugs may cause a change in the microbiome, although it typically returns to its original state (Scott *et al.* 2013).

1.2.1 Gastrointestinal microbiome functions

1.2.1.1 Metabolic and trophic function

The GIT microbiome has been demonstrated to produce many metabolites, which can exhibit considerable diversity among individuals. This variability primarily stems from differences in the GIT microbiome composition and an individual's dietary choices. Notably, there is an exchange of metabolites between the host and the GIT microbiome, suggesting a co-metabolism that can significantly affect the physiology of the host (Sharon *et al.* 2014; Donia and Fischbach 2015; Kho and Lal 2018).

Explorations into the GIT microbiome have unveiled its wealth of metabolic pathways associated with polysaccharides, amino acids, xenobiotics, and micronutrients, hinting at these components' potential metabolic advantages on the host. Previous research has uncovered the presence of bacterial communities adept at producing enzymes like glycoside hydrolase and synthesizing vitamins not encoded within the human genome. These microorganisms may play a critical role in the digestion of dietary fibres and complex carbohydrates, including cellulose, xylans, resistant starch, and inulin (Kho and Lal 2018; Dekaboruah *et al.* 2020). Moreover, the

microbial fermentation of dietary components yields short-chain fatty acids (SCFAs), encompassing butyrate, propionate, acetate, and pentanoate, providing the host with an additional 10% of their daily energy requirements for diverse metabolic processes (Payne *et al.* 2012; den Besten *et al.* 2013). In general terms, SCFAs serve as residual by-products utilized by the microbial population to maintain equilibrium in the anaerobic environment of the GIT. Nevertheless, from the host's perspective, SCFAs play a critical role, contributing to 70% of ATP production in the colon, particularly emphasising butyrate as the preferred energy source for colonocytes (Donohoe *et al.* 2011). Over recent decades, the notion that SCFAs might hold significance in preventing and treating conditions such as metabolic syndrome (Hu *et al.* 2010), bowel disorders, and specific types of cancer (Scharlau *et al.* 2009; Tang *et al.* 2011) has gained prominence. Additionally, several clinical investigations have unveiled the positive impact of SCFA administration in the management of ulcerative colitis, Crohn's disease, and antibiotic-associated diarrhoea (Parada Venegas *et al.* 2019).

Previous research has demonstrated that the GIT microbiota can synthesize many B groups vitamins and vitamin K, with the latter being essential in decreasing vascular calcification, elevating high-density lipoproteins, and lowering cholesterol levels in the human host (Magnúsdóttir *et al.* 2015; Kho and Lal 2018). Eight B-group vitamin biosynthetic pathways have been identified in the GIT metagenomic profile. These are responsible for the synthesis of biotin (B_7), cobalamin (B_{12}), folate (B_9), niacin (B_3), pantothenate (B_5), pyridoxine (B_6), riboflavin (B_2), and thiamine (B_1). Results from studies indicated that the distribution of these pathways within bacterial genomes was diverse, with B_2 and B_3 being the most commonly synthesized vitamins. Specifically, it was observed that the phyla *Bacteroidetes*, *Fusobacteria*, and *Proteobacteria* were the most likely to possess the requisite pathways for producing B vitamins, particularly B_2 and B_7. In the case of vitamin B_{12}, all the *Fusobacteria* were predicted to be producers, compared with only 10-50% of the other four phyla. Additionally, it was predicted that members of *Bacteroidetes* form the majority of B vitamin producers, with genomes of over 90% of species containing the necessary gene sets, those that are required for vitamin B_{12} production excluded (Magnúsdóttir *et al.* 2015). The synthesis of acetylcholine and cortisol, essential for the proper functioning of the nervous system, is just one of many biochemical processes in the host that rely on vitamins B_5 and B_{12} as coenzymes. Deficiencies in these vitamins have been linked to GIT discomfort, insomnia, neuropsychological and haematological disorders (Shipton and Thachil 2015).

Furthermore, the gut microbiota plays a fundamental role in the co-metabolism of bile acids. Cholesterol derivatives synthesized in the liver are conjugated with taurine or glycine before their storage in the gall bladder and secretion into the duodenum, where they aid in digestion, cholesterol synthesis, and lipid metabolism. Approximately 95% of bile acids are reabsorbed at the distal ileum (Staels and Fonseca 2009), while the remaining 5% are deconjugated by bile salt hydrolases, which are secreted by such members of colonic microbiota as *Clostridium perfringens* and *Clostridium scindens* and are then partially reabsorbed and transported back to the liver for conjugation. The host then excretes the unabsorbed secondary bile acids (Gopal-Srivastava and Hylemon 1988; Ajouz *et al.* 2014; Kho and Lal 2018). The initiation of signalling through the host nuclear farnesoid X receptor (FXR), triggered by both primary and secondary bile acids, has been demonstrated to regulate glucose metabolism and bile acid synthesis and potentially impact hepatic autophagy (Lee *et al.* 2014; Nie *et al.* 2015). Furthermore, the antimicrobial properties of secondary bile acids can modify the integrity of microbial cell membranes, leading to the release of intracellular contents and, thus, the inhibition of the growth of microorganisms' intolerant to bile acids (Nie *et al.* 2015; Kho and Lal 2018).

1.2.1.2 Protective function

Although the precise mechanism by which the microbiome protects humans against pathogen colonization and expansion is not fully understood, two mechanisms have been postulated: (1) direct interactions between the human microbiome and pathogens, where they compete for shared niches and nutrients, and (2) use or enhancement of host defence mechanisms by the microbiome leading to suppression of pathogen invasion (Kho and Lal 2018).

Direct interaction of the indigenous microbiome with pathogenic invader includes, but is not limited to, competitive niche exclusion, which involves nutrient consumption to ultimately outcompete the invader (Kamada *et al.* 2013; Maltby *et al.* 2013); production of such fermentation products and toxins as SCFAs and bacteriocins, which can alter the pH of the environment to inhibit pathogen growth (Kamada *et al.* 2013; Kho and Lal 2018); production of such antibiotic detoxification agents as β-lactamases and efflux pumps, which act as a microbial self-defence mechanism and removes toxic molecules (McNally and Brown 2015; Kho and Lal 2018). On the other hand, the GIT microbiome can stimulate or enhance host defence mechanisms by inducing the production of such host antimicrobial peptides as defensins, cathelicidins, and C-type lectins, which disrupt the surface structures of both

commensal and pathogenic bacteria, thus regulating the composition and abundance of bacterial cells that inhabit the GIT (Sekirov *et al.* 2010; Kho and Lal 2018). Additionally, the GIT microbiome induces the secretion of IgA and proinflammatory cytokines that facilitate the recruitment of immune cells to eradicate pathogens. The GIT microbiome also interacts with such local pattern recognition receptors (PRRs) as toll-like receptors (TLRs) and nod-like receptors (NLRs) to facilitate the maintenance of intestinal immunity homeostasis (Chiu *et al.* 2017; Kho and Lal 2018). Peptidoglycans present in microbial cell walls signal through TLR2, which helps to foster the integrity of the intestinal epithelium by maintaining tight junctions and reducing apoptosis. Therefore, microbiome signalling through mucosal TLRs appears essential for maintaining GIT epithelial homeostasis and tissue repair following injury (Sekirov *et al.* 2010; Chiu *et al.* 2017).

1.2.1.3 Immune homeostasis

Over the course of evolution, a complex symbiotic relationship has developed between the human host and its microbial cells, which is essential in the development and function of the mucosal immune system. The ability of the host's immune system to coexist harmoniously with microbial entities in the GIT is of great importance to both parties. In the human body, the intestinal mucosa is the most extensive surface that is constantly exposed to external antigens. As a result, the majority of antigens encountered by immune cells come from the GIT microbiome. Activation of pattern recognition receptors, such as TLRs and NLRs, by these antigens promotes the production of cytokines, chemokines, and various other soluble immune mediators. Consequently, the GIT microbiome plays a key role in modulating the production of these immune mediators, thereby contributing to the regulation of the immune response within the GIT (Sommer and Bäckhed 2013; Chiu *et al.* 2017; Kho and Lal 2018). In general, the mucosal immune system comprises several elements, including gut-associated lymphoid tissue (GALT), small intestinal lymphoid tissue (SILT), lymphoid aggregates in the colon, and diffuse immune cells within the lamina propria of the intestines. These immune cells are closely connected to the rest of the immune system through local mesenteric lymph nodes (Sekirov *et al.* 2010; Jandhyala *et al.* 2015). A diverse range of immune cell types, including both effector and regulatory T cells, IgA-producing B cells, Group 3 innate lymphoid cells, as well as resident macrophages and dendritic cells, are essential in immunomodulation (Jandhyala *et al.* 2015).

Disruption of immune homeostasis can be caused by lipopolysaccharides (LPS) present on the outer membrane of Gram-negative bacteria. The host's immune response to LPS can

lead to septic shock, resulting in death. However, there are several ways to preclude septic shock. Intestinal alkaline phosphatase mediates the dephosphorylation of the endotoxin component of LPS, thereby reducing LPS toxicity (Sekirov *et al.* 2010; Jandhyala *et al.* 2015). Physical separation of microbial cells by the host's mucosal immune system is another strategy for preventing septic shock. In this regard, plasma cells located in the lamina propria produce secretory IgA (sIgA), which is transcytosed through the intestinal epithelium into the lumen (Sommer and Bäckhed 2013; Jandhyala *et al.* 2015). Once in the lumen, sIgA recognizes microbial antigens and coats microbial cells, thereby controlling their numbers locally. Dendritic cells activate plasma cells that produce sIgA and are confined to the local mesenteric lymph node. This local confinement prevents a systemic response to the GIT inhabiting microbial cells. In addition, AMPs act similarly to sIgA by preventing microbial cells from escaping the luminal compartment (Sekirov *et al.* 2010; Jandhyala *et al.* 2015).

1.2.2 *Helicobacter pylori* infection

Helicobacter pylori, a bacterium with a Gram-negative, spiral-shaped morphology that thrives in low-oxygen environments, is recognized for its colonization of the gastric mucosa (Hooi *et al.* 2017). This bacterium stands as the leading instigator of persistent gastritis and is associated with the emergence of severe gastroduodenal disorders in certain individuals. These conditions encompass peptic ulcers in the stomach and duodenum, gastric cancer, and lymphoma in the gastric mucosa-associated lymphoid tissue (Malfertheiner *et al.* 2023).

It is generally estimated that *H. pylori* infection affects more than half of the world's population; however, this prevalence has decreased to 43% within the last decade (Li *et al.* 2023). This decline can be attributed mainly to improvements in socioeconomic status, living standards, and hygiene conditions (Malfertheiner *et al.* 2023). Approximately 80% of individuals carrying *H. pylori* show no symptoms; however, gastritis, i.e., inflammation of the stomach lining, can develop in all infected individuals, which, if left untreated, leads to such outcomes as gastric cancer (Plummer *et al.* 2015; Malfertheiner *et al.* 2023). Although no current data is available on the spread of *H. pylori* in Latvia over the last decade, a study conducted in 2011 estimated that almost 80% of the population carried the infection (Leja *et al.* 2012). As a result, the scientific community continues to regard Latvia as a country with high *H. pylori* prevalence.

As the stomach is a harsh environment with pH 1.5-2.0 and regular clearance of the contents (Fujimori 2020), fundamental mechanisms facilitating *H. pylori* colonization include motility, urease production, adhesion, and the secretion of pathogenic proteins that interact

with host tissues (Malfertheiner *et al.* 2023). *H. pylori* employs flagella-driven motility as an essential means to cross the mucus layer and establish a reservoir within the gastric mucosa. Motility is accomplished through chemotaxis, enabling the bacterium to detect and respond to a range of environmental and bacterial signals in the stomach. Through chemotaxis, *H. pylori* can navigate towards its favoured niche within the gastric mucosa and glands (Johnson and Ottemann 2018; Malfertheiner *et al.* 2023). Furthermore, *H. pylori* produces a nickel-containing urease enzyme, which plays a fundamental role in colonization by neutralizing gastric acid and generating ammonia and carbonic acid by hydrolysis of urea. The ammonia produced by urease activity assists in bacterial protein synthesis. Subsequently, urease production can also cause direct damage to host tissues due to ammonia generation and indirectly trigger inflammatory responses (Mobley 1996). Moreover, to establish effective colonization despite challenges such as epithelial cell shedding and gastric emptying forces, *H. pylori* utilizes surface molecules adhesins to adhere to gastric epithelial cells. Adhesins interact with host cell glycan receptors, enabling *H. pylori* to maintain close contact and high colonization levels. The most studied adhesins in respect are blood-group antigen-binding adhesin (BabA) and sialic acid-binding adhesin (SabA) (Matos *et al.* 2021; Malfertheiner *et al.* 2023). Furthermore, *H. pylori* produces various proteins contributing to its pathogenicity in the gastric environment. One such notable protein is vacuolating cytotoxin A (VacA), which has been implicated in multiple effects on host cells, including vacuolization and the induction of apoptotic cell death or necrosis (Ansari and Yamaoka 2019; Malfertheiner *et al.* 2023).

As a result, colonization of *H. pylori* in the gastric mucosa initiates a pro-inflammatory response in gastric epithelial cells, leading to the recruitment of anti-inflammatory T-regulatory cells. This process results in the development of chronic-active gastritis, which in a majority of affected individuals remains asymptomatic for several decades (Robinson *et al.* 2008; Cook *et al.* 2014). The severity of inflammation varies significantly among patients and can be attributed to various factors, including previously described bacterial characteristics, as well as host genetics, and environmental factors, such as smoking, dietary patterns (e.g., low iron and high salt consumption) (Amieva and Peek 2016; Li *et al.* 2019), and single nucleotide polymorphisms in cytokine and growth factor genes and their receptors, such as TLR1, NOD1, IL-2, IL-6 among others (Rudnicka *et al.* 2019; El-Omar 2022).

Invasive and non-invasive procedures have been developed for an accurate diagnosis of *H. pylori* infection. Non-invasive tests, such as ^{13}C-urea breath test (UBT), serological antibody detection, stool antigen test, and *H. pylori* direct detection in stool via PCR, offer

valuable alternatives to the more invasive procedures like gastroscopic biopsies (Malfertheiner *et al.* 2023). Among these methods, the UBT is one of the most widely employed techniques due to its high – over 95% specificity and sensitivity (Best *et al.* 2018; Talebi Bezmin Abadi 2018). This diagnostic approach is particularly suitable for asymptomatic, elderly, and paediatric individuals (Talebi Bezmin Abadi 2018). The basis of the UBT lies in *H. pylori*'s ability to metabolize the administered ^{13}C-urea in a patient's stomach into ammonia and labelled bicarbonate. The labelled bicarbonate is then transported to the lungs, where labelled carbon dioxide is produced, which can be detected by specialized equipment, confirming the presence of *H. pylori* infection (Talebi Bezmin Abadi 2018; Cardos *et al.* 2022).

Given the high prevalence and pathogenicity of *H. pylori*, a "test-and-treat" strategy has been proposed for healthy asymptomatic adults in areas with an increased incidence of gastric cancer (Leja *et al.* 2017; Malfertheiner *et al.* 2022). However, while this approach holds potential benefits, there are apprehensions about its unintended consequences, primarily a rise in general antibiotic consumption and subsequent antibiotic resistance among non-*H. pylori* bacterium (Leja and Dumpis 2020; Malfertheiner *et al.* 2022). The Maastricht VI/Florence consensus report offers guidance on *H. pylori* eradication therapy, considering geographical variations in antibiotic resistance patterns. In regions with low rates of resistance to clarithromycin (i.e., <15%), like Latvia, a standard 10-14 day eradication regimen is recommended, which typically involves the administration of a proton-pump inhibitor (PPI), such as esomeprazole, in combination with clarithromycin, amoxicillin, or metronidazole (Malfertheiner *et al.* 2022). Further aspects regarding the impact of eradication therapy on GIT are described in Chapter 1.4.

1.3 Gastrointestinal microbiome and antimicrobial resistance

Although the microbiome provides many beneficial functions, the high density of microorganisms in the GIT environment also increases the potential of antimicrobial resistance gene (ARG) horizontal transfer to potentially pathogenic bacteria. This phenomenon can lead to the spread of ARGs in the population and limit the effectiveness of antimicrobial therapy, thus posing a significant challenge to public health care (Lamberte and van Schaik 2022; Murray *et al.* 2022).

Bacteria employ a variety of resistance mechanisms that can be classified as 'intrinsic' or 'acquired'. Intrinsic mechanisms are those in which bacteria use genes that they already have to survive exposure to antibiotics, while acquired mechanisms involve the acquisition of new genetic material that provides the bacteria with novel capabilities to mediate their survival

(Blair *et al.* 2015; Darby *et al.* 2022). Intrinsic resistance is postulated to primarily arise from the impermeability of cellular envelopes, the action of multidrug efflux pumps, or the absence of suitable targets for a specific class of drugs. The manifestation of intrinsic resistance is unrelated to prior antibiotic exposure and does not result from horizontal gene transfer (Olivares Pacheco *et al.* 2013). In contrast, the development of acquired resistance is a complex process influenced by the intricate interplay among plasmids, hosts and ARGs, which ultimately determine the spread of genes, vectors, and strains (Darby *et al.* 2022). Acquired resistance can also arise by accepting mutations in bacteria's chromosomal DNA (Reygaert 2018).

1.3.1 Horizontal gene transfer of antimicrobial resistance genes

The horizontal transfer of ARGs is one of the primary mechanisms underlying the spread of antibiotic resistance in bacterial populations and is recognized as an essential factor driving bacterial evolution and contributing to the widespread distribution of ARGs (Tao *et al.* 2022). Fundamental mechanisms that facilitate horizontal gene transfer of ARGs include transformation, conjugation, transduction, and membrane vesicles (Figure 2) (McInnes *et al.* 2020; Tao *et al.* 2022) which are described in more detail below.

Conjugation stands as a primary mechanism for horizontal gene transfer, facilitating the transfer of mobile genetic elements, including plasmids, integrative conjugative elements, and conjugative transposons, from a donor bacterium to a recipient through direct contact (McInnes *et al.* 2020; Virolle *et al.* 2020). Specifically, bacterial conjugation involves the transfer of DNA from a donor to a recipient bacterium through a membrane-associated macromolecular machinery known as the Type IV secretion system. This process occurs when the two bacteria are in close proximity. Despite considerable research on bacterial conjugation, its mechanisms within the GIT microbiome remain poorly characterized (Neil *et al.* 2021). Among these genetic elements, plasmids play a pivotal role in disseminating ARGs due to their capacity to carry multiple resistance genes, facilitated by their relatively large size (typically around 90 kilobases). Plasmids often feature one or more toxin-antitoxin modules that help stabilize them in their microbial hosts (Pal *et al.* 2015). Furthermore, the presence of a mating-pair stabilization complex is essential to ensure that the donor and recipient bacteria remain in close proximity for a sufficient period, facilitating the successful transfer of DNA during conjugation. In this context, the mating-pair stabilization complex could serve as a potential target for interventions aimed at reducing the spread of ARGs (Neil *et al.* 2021). Conjugation is present in both Gram-positive and Gram-negative bacteria, and it is recognized as a

significant driver in the rapid evolution of bacterial genomes (McInnes *et al.* 2020; Virolle *et al.* 2020).

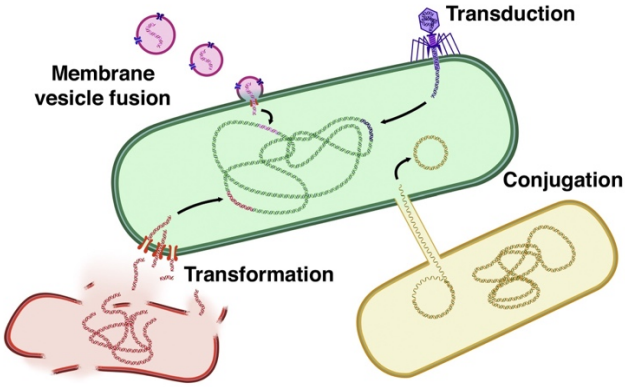

Figure 2. Mechanisms of horizontal antimicrobial resistance gene transfer. Figure adapted from (McInnes *et al.* 2020).

Transduction is a chromosomal and extrachromosomal DNA transfer between bacteria via bacterial virus–bacteriophage. During bacteriophage propagation, host bacterial DNA can occasionally become encapsulated in viral particles, forming transducing particles, which exhibit similarities to mature bacteriophage particles, except that upon infecting new cells, they release bacterial DNA instead of their viral genome. This bacterial DNA can subsequently integrate into the recipient chromosome via recombination or replicate as a plasmid within the new bacterial host cell (Chiang *et al.* 2019). There are three main transduction mechanisms, which, in accordance with underlying processes, are named generalised, specialised, and lateral transduction. In generalized transduction, bacteriophages can package any segment of bacterial DNA during capsid synthesis and transfer it to a recipient bacterium, whereas in specialized transduction, bacteriophages can package and transfer only a specific set of genes (Chiang *et al.* 2019; McInnes *et al.* 2020). On the other hand, lateral transduction occurs when prophages initiate DNA replication while still integrated within the host genome. This results in the generation of multiple DNA copies prior to their excision. Following excision, the DNA, which can encompass the phage genome and up to several hundred kilobases of the adjacent bacterial genome, is packaged into new phage particles and transferred to other bacterial strains (McInnes *et al.* 2020). The GIT microbiome harbours a diverse and extensive community of bacteriophages, which raises concerns about the dissemination of ARGs via transduction. This mechanism of ARG distribution may occur on a larger scale than other horizontal gene transfer

mechanisms (Debroas and Siguret 2019), thus highlighting the potential impact of bacteriophages on the human GIT microbiome dynamics.

Transformation is a genetic process in which bacteria uptake "naked" DNA molecules from the extracellular environment and incorporate them into their genomes. Most of the extracellular DNA can be attributed to plasmid DNA and fragmented DNA, which are discharged through active secretion or lysis of bacterial cells (Tao *et al.* 2022). The bacteria must be naturally transformable or competent for this phenomenon to occur. Although the stimuli responsible for inducing competence in naturally transformable bacteria are only partially understood, nutrient starvation, the presence of competence-inducing peptides, and mechanical stimuli have been identified as factors that trigger this process (McInnes *et al.* 2020; Kasagaki *et al.* 2022). Several clinically significant bacterial pathogens, such as *Vibrio cholerae*, *Neisseria gonorrhoeae* and *Streptococcus pneumoniae*, have acquired antibiotic resistance through this mechanism (Tao *et al.* 2022).

Eukaryotic, archaeal, and bacterial cell secretion can produce membrane vesicles. These are lumen-containing spheres of lipid bilayers derived from the cell surface. Their diameter varies between 10 to 500 nm (Brown *et al.* 2015), with membrane vesicles of Gram-negative bacteria ranging from 10-300 nm (Furuyama and Sircili 2021) and those of Gram-positive bacteria ranging from 10-400 nm (Liu *et al.* 2018). From a functional perspective, membrane vesicles are well-known cargo delivery tools; however, their amount and content can vary within and between species and populations. Proteins, polysaccharides, and nucleic acids are only a few examples of the contents found in membrane vesicles, which can function as toxins, virulence factors and elements of antibiotic breakdown (Domingues and Nielsen 2017). Cargo delivery by membrane vesicles is accomplished by fusing with the target cell membrane and then releasing its cargo (Domingues and Nielsen 2017; McInnes *et al.* 2020). Within the context of ARGs, it has been shown that several GIT commensals from genus *Bacteroides* were able to produce membrane vesicles whose cargo constituted of β-lactamases, thus disseminating the defence mechanism against β-lactam antibiotic substances not only among commensal bacteria but also to pathogenic ones (Stentz *et al.* 2015). Similarly, a recent study by Lee *et al.* (2022) demonstrated that membrane vesicles produced by a methicillin-resistant *Staphylococcus aureus* strain contained β-lactamases in their cargo. The study also showed that these vesicles fused with the membrane of the antibiotic-susceptible *Escherichia coli* strain, leading to the transfer of resistance against β-lactam compounds to the latter (Lee *et al.* 2022).

1.3.2 Largest antimicrobial resistance gene classes

In the literature, ARGs are most often associated with the class of antibiotics to which they are resistant. These classes include aminoglycosides (*aac*), β-lactams (*bla*), colistin (*mcr*), fluoroquinolones (*fca*), macrolides (*erm*), tetracyclines (*tet*), sulfonamides (*sul*), vancomycin's (*van*), multidrug (*mdr*) and other (Jian *et al.* 2021). However, one of the most commonly used ARG database in next-generation sequencing-based research – Comprehensive Antibiotic Resistance Database (CARD) – presents a more extensive classification system with major branches consisting of antibiotic molecules, resistance-modifying agents (such as antibiotic adjuvants or other inhibitors of antibiotic resistance), mechanism of antibiotic resistance, determinant of antibiotic resistance (such as mutation, SNP, gene), antibiotic target, and antibiotic biosynthesis (McArthur *et al.* 2013; Alcock *et al.* 2023). The CARD database has recognised 458 gene families, 64 drug classes, and eight resistance mechanisms (Alcock *et al.* 2023).

1.3.2.1 Beta-lactamases

One of the largest ARG classes encodes β-lactamases (BLs). These enzymes confer resistance to β-lactam antibiotics, and they are mostly produced by Gram-negative bacteria (Tooke *et al.* 2019). Over time, advancements in molecular biology technologies have led to the identification of an increasing number of BLs. At the time of thesis preparation, the β-lactamase database (BLDB) comprised nearly 8'000 BL enzymes characterized at various levels (Naas *et al.* 2017). The primary resistance mechanism of these enzymes involves hydrolyzation of the amide bond within the four-membered β-lactam ring. There are four classes of BLs: the active-site serine BL (classes A, C, and D) and the zinc-dependent or metallo-BL (class B) (Figure 3) (Tooke *et al.* 2019; Sawa *et al.* 2020). Although all four classes are widely distributed across numerous species of clinically significant and environmental bacteria, specific enzyme families within each class have exhibited remarkable success and have spread extensively among the most critical bacterial pathogens. These pathogens are primarily associated with opportunistic healthcare-associated infections in immunocompromised patients. The bacteria include *Enterobacteriaceae*, such as *E. coli*, *Klebsiella pneumoniae*, as well as *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* (Tooke *et al.* 2019).

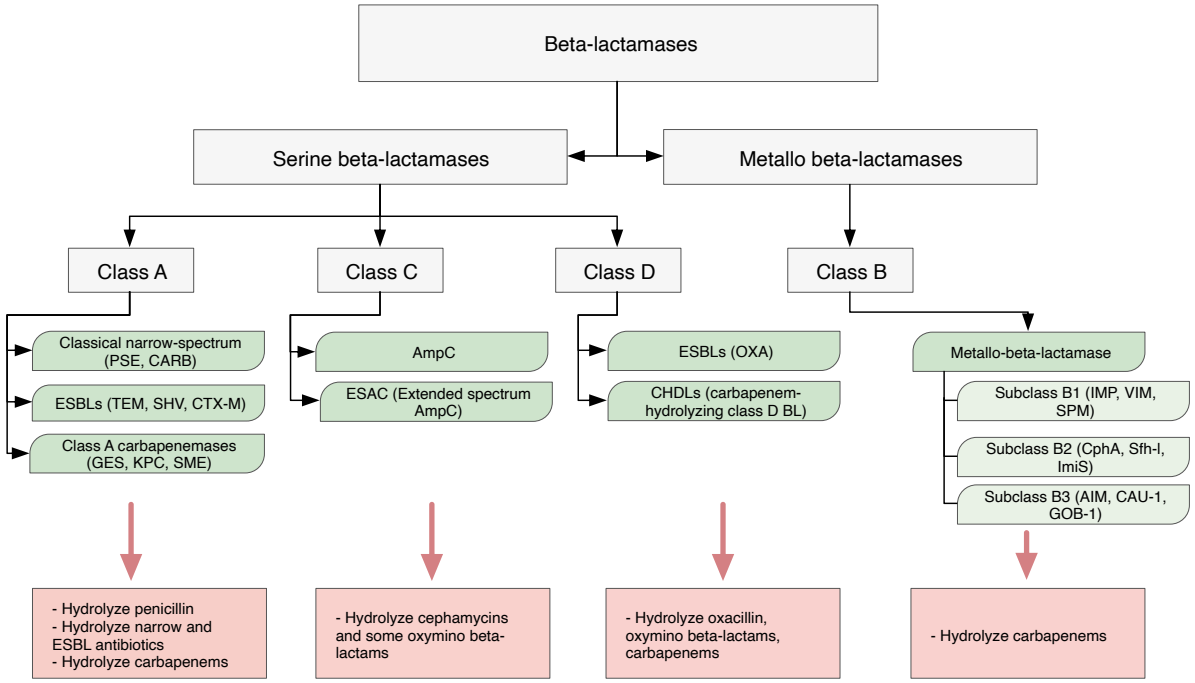

Figure 3. According to the Ambler classification method, β-lactamase classification is based on specific amino acid sequence motifs. Figure adapted from (Toussaint and Gallagher 2015; Sawa *et al.* 2020).

Class A enzymes represent the most widely distributed and extensively studied group of BLs. According to BLDB, 1'896 class A BL enzymes have been identified so far (Naas *et al.* 2017). These include a multitude of clinically relevant enzymes, with some having historical significance in the development of antimicrobial resistance. For instance, PC1 is a plasmid-encoded enzyme implicated in the failure of penicillin and ampicillin treatment (Yuan *et al.* 2011), whereas TEM was identified as the first plasmid-borne BL in Gram-negative bacteria and exhibits high activity against penicillins and early generation cephalosporins (Palzkill 2018; Tooke *et al.* 2019). Another significant enzyme, SHV, was initially identified on the chromosome of *K. pneumoniae* and has subsequently been mobilized onto plasmids, enabling its dissemination among various bacterial strains and environmental niches (Chaves *et al.* 2001; Liakopoulos *et al.* 2016; Tooke *et al.* 2019). Enzyme CTX-M demonstrates inherent activity against cefotaxime and ceftriaxone (Zeynudin *et al.* 2018). This enzyme has rapidly disseminated worldwide, raising substantial concerns about its impact on antimicrobial resistance (Zeynudin *et al.* 2018; Tooke *et al.* 2019). Lastly, *K. pneumoniae* carbapenemase enzyme KPC is another significant class A enzyme associated with carbapenem resistance in *K. pneumoniae* (Tooke *et al.* 2019). KPC enzymes confer resistance to penicillins, cephalosporins, carbapenems and most BL inhibitors. Notably, carbapenems are usually used as a last resort when other treatment options have failed; thus, the spread of KPC enzymes is of high concern (Hobson *et al.* 2020; Mehta *et al.* 2021). The success of enzymes from the

TEM, SHV, and CTX-M families can be attributed to their dissemination on plasmids and other mobile genetic elements, which has facilitated the spread among various Gram-negative pathogens, particularly within the *Enterobacteriaceae* family. Moreover, these enzymes can expand their spectrum of activity as new substrates are introduced in clinical settings. The acquisition of point mutations by TEM and SHV enzymes has enabled to hydrolyse oxyimino-cephalosporins, leading to the development of the "extended-spectrum" phenotype, known as extended-spectrum BLs (ESBLs). Similarly, CTX-M enzymes have accumulated mutations to extend their activity, resulting in resistance against an enlarged range of β-lactam antibiotics. Consequently, ESBLs pose a significant threat to the efficacy of cephalosporins in many clinical settings (Palzkill 2018; Tooke *et al.* 2019).

Class B metallo-BLs constitute a diverse and widely distributed group within the metallohydrolase superfamily, characterized by zinc at the enzyme's active site (Tooke *et al.* 2019; Sawa *et al.* 2020). According to the BLDB, 849 class B BL enzymes have been identified (Naas *et al.* 2017). These enzymes can be categorized into three classes based on their molecular architecture: B1, B2, and B3 (Sawa *et al.* 2020). Notably, they can hydrolyse nearly all β-lactam antibiotics except monobactams (Palzkill 2013). Among the class B metallo-BLs, IMP and VIM are the predominant enzymes that exhibit a continuous process of evolution, leading to the constant emergence of new variants (Sawa *et al.* 2020). Specific mutations in these variants significantly impact their spectrum of carbapenem activities, including their effectiveness against imipenem, meropenem, and doripenem. IMP and VIM enzymes are frequently found within integron structures, coexisting with other resistance genes. These integron structures can be integrated into chromosomal DNA or reside within plasmids, facilitating the dissemination of multidrug-resistant bacteria (Palzkill 2013; Sawa *et al.* 2020).

Class C BLs are prominently distributed on the chromosomes of Gram-negative species, specifically among *Enterobacteriaceae*, *P. aeruginosa*, and *Acinetobacter* spp. (Page 2020). The BLDB has identified nearly 4'000 class C BL enzymes, making them the second most numerous group after class A BLs (Naas *et al.* 2017). Many of the most significant opportunistic Gram-negative pathogens harbour chromosomal genes encoding class C enzymes, while in *Enterobacteriaceae*, genes encoding these enzymes have also been observed on plasmids (Philippon *et al.* 2002). A notable representative of class C enzymes is AmpC, carried on the genomes of bacteria belonging to the *Enterobacteria* genus. AmpC exhibits resistance to clavulanic acid but remains susceptible to cephamycin's. Typically, AmpC expression levels are low; however, administering penicillin or clavulanic acid may elevate

them, leading to carbapenem resistance when present in significant quantities (Codjoe and Donkor 2017; Sawa *et al.* 2020). Other BLs within this group, such as CMY, ACT, FOX, DHA, and MIR, are encoded on plasmids (Tooke *et al.* 2019; Sawa *et al.* 2020).

Class D BLs encompass a diverse group comprising 14 families, with a significant proportion of member enzymes belonging to the OXA family, the most prominent family across all BLs (Yoon and Jeong 2021). The BLDB has identified 1'250 class D BL enzymes (Naas *et al.* 2017). These enzymes are named for their ability to hydrolyse oxacillin, and over time, they have evolved to confer resistance not only to cephalosporins but also to carbapenems and other antibiotic compounds (Toth *et al.* 2016). The genes encoding class D BLs are commonly located on the bacterial chromosome, serving as intrinsic resistance determinants in environmental bacteria. Some of these genes are also present in mobile genetic elements carried by clinically significant pathogens. Notably, OXA BLs are often innate in Gram-negative bacteria. For instance, the OXA-22-like enzyme is intrinsic to *Ralstonia* spp., OXA-42-like enzymes to *Burkholderia pseudomallei*, and OXA-61-like enzymes innate to *Campylobacter* spp. (Yoon and Jeong 2021). On the other hand, there are also class D BLs that are found in plasmids and integrons, including enzymes like OXA-1, OXA-2 and its derivatives, OXA-10, and others (Poirel *et al.* 2010). OXA-48 is a clinically significant and particularly interesting enzyme in this class. It is able to hydrolyse carbapenem in clinical settings. This enzyme, along with its variants, has become widespread in *K. pneumoniae* as well as other *Enterobacteriaceae* and, recently, has also been reported in *A. baumannii;* thus, it is one of the most concerning developments in carbapenem resistance over the last decade (Evans and Amyes 2014). Additionally, other non-OXA BLs have been identified in the chromosomes of Gram-positive bacteria. Typical representatives of this group are BSD and BSU from *Bacillus subtilis*, BAC from *Bacillus clausii*, and CDD from *Clostridioides difficile* (Toth *et al.* 2016; Yoon and Jeong 2021). The rise of OXA enzymes capable of conferring resistance to carbapenems, particularly in *A. baumannii*, has raised awareness in medical society and facilitated the elevation of class D BLs from a minor into a major concern (Evans and Amyes 2014).

1.3.2.2 Other antimicrobial resistance genes

As outlined in the previous sub-chapter, ARGs represent a diverse and extensive genetic component within microorganisms. Due to their continual evolution, the identification and characterization of these genes are often challenging and lag behind their actual prevalence in nature. In addition to the bacterial resistance enzymes described earlier, most other ARGs are

predominantly associated with the specific class of antibiotics to which they confer resistance. Consequently, the resistance against various classes of antibiotics, such as aminoglycosides, tetracyclines, macrolides, vancomycin, and others, holds significant clinical importance, as these types of ARGs are prevalent in the human GIT (Qiu *et al.* 2020).

Aminoglycosides represent a potent class of broad-spectrum antibiotics derived from actinomycetes. They are renowned for their ability to inhibit protein synthesis and induce microbial cell death. They are highly effective against a wide range of infections caused by Gram-negative and some Gram-positive bacteria, including members of the *Enterobacteriaceae* family, *Yersinia pestis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and others (Krause *et al.* 2016; Zhang *et al.* 2023). In clinical practice, aminoglycosides are frequently employed either as single agents or in combination with other antibiotics, particularly when confronted with multi-drug resistant pathogens (Zhang *et al.* 2023). However, the clinical usefulness of aminoglycosides has been challenged due to the increasing prevalence of aminoglycoside-modifying enzyme (AME) genes within bacterial strains, such as *K. pneumoniae* (Wang *et al.* 2020). These AMEs efficiently modify and deactivate aminoglycoside drugs, rendering them ineffective and promoting resistance to aminoglycosides. The dissemination of AME genes occurs through various mechanisms, including conjugative plasmids, transformation, or transduction, all of which facilitate their transmission among diverse bacterial types (Tolmasky 2014; Lalitha Aishwarya *et al.* 2020). Moreover, bacterial resistance to aminoglycosides can be enhanced by modifications in 16S rRNA ribosomal proteins or by the actions of methylation enzymes. These modifications result in reduced binding affinity between 16S rRNA and aminoglycoside antibiotics, thereby increasing bacterial resistance to multiple antibiotics within the aminoglycoside class (Xia *et al.* 2011; Zhang *et al.* 2023).

Tetracyclines represent a class of broad-spectrum antibiotics extensively employed against both Gram-negative and Gram-positive bacteria, acting by disrupting protein synthesis and thus inhibiting the growth of susceptible bacteria (Schnappinger and Hillen 1996; Jahantigh *et al.* 2020). Due to their numerous advantages, such as wide availability, affordability, and minimal side effects, the use of tetracyclines to treat various infections has increased in recent years. However, increased use has led to the emergence of tetracycline-resistant bacteria, limiting the effectiveness of these antibiotics (Garcia *et al.* 2011). Tetracycline resistance genes are widespread in the human GIT (Hu *et al.* 2013) and are commonly encoded in plasmids and transposons, which facilitates their transmission through

conjugation. The main mechanisms of tetracycline resistance through the acquisition of *tet* genes involve the employment of efflux pumps, ribosomal protection, and enzymatic deactivation (Koo and Woo 2011; Jahantigh *et al.* 2020).

Macrolides represent a significant class of broad-spectrum antibiotics extensively employed in the treatment of diverse infections, exerting their therapeutic effect by inhibiting bacterial protein synthesis in both Gram-positive and Gram-negative bacteria (Tenson *et al.* 2003; Miklasińska-Majdanik 2021). These agents continue to play a crucial role in the therapeutic management of various infections, including community-acquired pneumonia, sexually transmitted diseases, *Campylobacter* spp. infections, among others (Fyfe *et al.* 2016). Nevertheless, the emergence of bacterial resistance against macrolide antibiotics has become a pressing concern, with resistance mechanisms involving three primary strategies: (1) target-site modification by methylation or mutation, impairing the antibiotic's binding to its ribosomal target; (2) active efflux of the antibiotic from the bacterial cell; and (3) drug inactivation (Leclercq 2002; Miklasińska-Majdanik 2021). The genetic basis of macrolide resistance in bacterial genomes involves the presence of specific resistance genes, including (1) *erm* genes encoding 23S rRNA methylases; (2) *msr* genes encoding ABC-F proteins, critical for ribosome protection; (3) *mef* genes encoding efflux proteins; (4) *mph* genes encoding macrolide phosphotransferases; and (5) *ere* genes encoding macrolide esterases. Many of these genes are found in mobile genetic elements like plasmids, transposons, and prophages, facilitating horizontal gene transfer across strains, species and sometimes genus boundaries (Feßler *et al.* 2018). This transferability further exacerbates the dissemination of resistance and poses challenges to effective antibiotic therapy in various clinical settings.

1.3.3 Antimicrobial resistance gene distribution and influencing factors

Numerous studies have increasingly demonstrated that the abundance and diversity of ARGs in individuals exhibit significant variations depending on geographic location, dietary habits, medical history, and other individual-specific factors. Notably, a study conducted by Feng *et al.* (2018) revealed the presence of shared genes within the human GIT microbiome across diverse populations, spanning countries such as Austria, France, Germany, Iceland, Sweden, China, Japan, Canada, USA, Peru, and Salvador. These shared ARGs encompassed vancomycin resistance genes *vanR* and *vanS*; tetracycline resistance genes *tetW* and *tetM*; multidrug resistance genes ABC transporter and *acrB*; bacitracin resistance gene *bacA*; and aminoglycoside resistance gene *aadE*. Additionally, this study highlighted the high prevalence of other ARGs across studied populations, with examples of tetracycline resistance genes *tet32*

and *tetQ*; β-lactam resistance genes *CfxA2* and class A BLs; macrolide-lincosamide-streptogramin (MLS) resistance genes *ermB* and *macB*. However, regional discrepancies were also observed in the distribution of ARGs. For instance, Chinese populations exhibited high MLS resistance gene *ermF* and *ermC* levels, while Peru and Salvador demonstrated comparatively low levels (Feng *et al.* 2018). In a similar study conducted by Ghosh *et al.* (2013), regional variations in the abundance of ARGs were observed in the human GIT across seven distinct populations. Remarkably, the Chinese population exhibited the highest levels of ARGs compared to other studied populations. In addition, the authors identified and characterized four different clusters of gut microbiomes based on their antibiotic resistance profiles, which they termed 'resistotypes' (Ghosh *et al.* 2013).

In smaller-scale studies, investigations into the abundance of ARGs have revealed differences among individuals with varying dietary habits. For instance, Losasso *et al.* (2018) conducted a study in which they quantified representative ARGs associated with sulphonamides (gene *sul2*), tetracyclines (gene *tetA*), β-lactams (gene *blaTEM*) and aminoglycosides (gene *strB*) in vegan, vegetarian, and omnivore faecal samples. While their results were not conclusive in the determination of the impact of diet on ARG repertoire in the human GIT, they did identify variations in gene loads across different diets. For example, *sul2* and *strB* were more frequently detected in omnivores and vegetarians than in vegans, indicating a potentially higher transfer of these ARGs from meat or animal-derived foods than from vegetables (Losasso *et al.* 2018). A similar conclusion was obtained by other researchers, revealing that individuals who consumed more diverse diets, characterized by higher fibre content and limited animal protein intake, exhibited lower abundances of ARGs (Oliver *et al.* 2022).

The presence of certain diseases is associated with the diversity of ARGs. For instance, variations in ARG abundances within the GIT microbiome have been observed among individuals with cirrhosis, inflammatory bowel disease (IBD), diabetes and chronic kidney disease (CKD). In a study by Bajaj *et al.* (2021), cirrhosis patients were found to exhibit higher abundances of β-lactam, vancomycin, quinolone, and macrolide resistance genes compared with CKD, IBD, and diabetes (Bajaj *et al.* 2021). Moreover, investigations into gastrointestinal inflammation have revealed inflammatory responses and, consequently, the emergence of disease-specific ARG profiles (Stecher *et al.* 2012; Vich Vila *et al.* 2018). A separate comprehensive study by Qiu *et al.* (2020) compared ARG profiles of the GIT microbiome in various diseases, such as colorectal cancer, type 2 diabetes, liver cirrhosis, rheumatoid arthritis,

hypertension, psoriasis, and ankylosing spondylitis. Although changes in overall resistance abundance were observed in most diseases, no consistent pattern of ARGs was identified (Qiu *et al.* 2020). These inconclusive findings suggest that further in-depth characterization in this area is required to better understand the relationship between disease and ARG dynamics.

Other significant factors contributing to the spread of ARGs include hospital settings, where patients may share ARGs, and the use of antibiotics (Crits-Christoph *et al.* 2022), the latter of which will be described in more detail in the next chapter.

1.4 Impact of antimicrobials on the microbiome

The conventional wisdom is that antibiotic use results in the elimination of the intended bacteria, opening up ecological niches for the proliferation of other bacterial species. Furthermore, subsequent generations of bacterial cells have the potential to acquire resistance genes via horizontal gene transfer (McInnes *et al.* 2020; Lamberte and van Schaik 2022). To mitigate the colonization by drug-resistant bacteria, strategies such as faecal microbiota transplant and the utilization of live biotherapeutics have been proposed. However, their efficacy in achieving this goal remains uncertain (Figure 4) (Lamberte and van Schaik 2022).

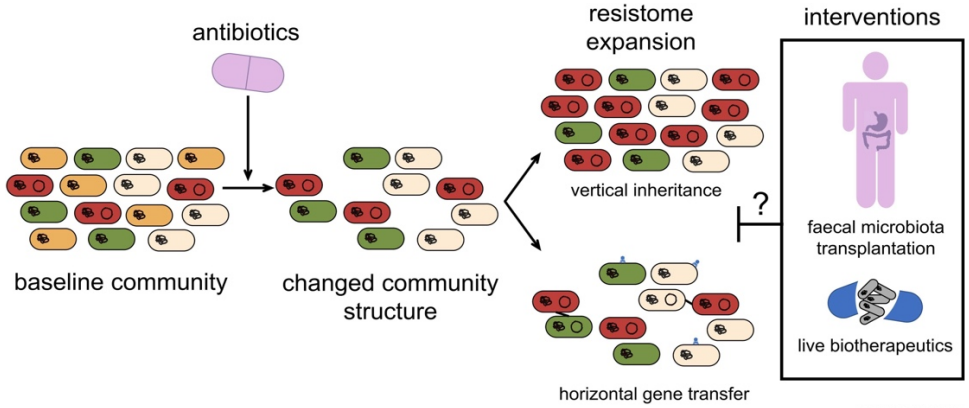

Figure 4. Schematic representation of the spread of antimicrobial resistance genes after microbiome perturbation with antibiotic compounds. Figure adapted from (Lamberte and van Schaik 2022).

Antibiotic treatment has been extensively studied to determine its effect on the diversity and composition of the GIT microbiome. Many studies have demonstrated that such treatment reduces the overall diversity of the microbiome, resulting in the loss of some crucial taxa. This reduction can have various consequences, including metabolic shifts, increased susceptibility to colonization, and bacterial resistance to antibiotics (Lange *et al.* 2016; Palleja *et al.* 2018; Ramirez *et al.* 2020). Even short-term use of antibiotics, particularly during the first two years of childhood, can have long-term impacts on the GIT microbiome (Luchen *et al.* 2023). Recent research has shown that infants who required broad-spectrum antibiotic treatment during their

first week of life exhibited significant shifts in their GIT microbiome and ARG profile directly following treatment. Although the microbial community normalized over 12 months, the effects of the therapy remained measurable at that age compared to a healthy birth cohort (Reyman *et al.* 2022). In healthy adults, studies have shown acute declines in species richness, and the microbiome's recovery is incomplete for up to six months after antibiotic treatment (Dethlefsen *et al.* 2008; Palleja *et al.* 2018; Anthony *et al.* 2022). Remarkably, in some cases, the altered composition persists for up to four years post-treatment (Jakobsson *et al.* 2010; Yap *et al.* 2016).

The changes observed at the community level are characterized by a significant decrease in the relative abundance of key members of the phyla *Firmicutes*, *Bacteroidetes* and *Actinobacteria*, notably *Faecalibacterium prausnitzii*, *Eubacterium* spp., *Roseburia* spp., *Bifidobacterium* spp., and *Ruminococcus* spp., among others (Raymond *et al.* 2016; Palleja *et al.* 2018; Dubinsky *et al.* 2020). These lost species are essential for performing critical metabolic functions in the GIT microbiome. Notably, antibiotic use is associated with reduced SCFA levels, leading to decreased bile acid transformation capacity and a shift in colonocyte energy utilization from SCFAs to glucose (Zarrinpar *et al.* 2018; Kelly *et al.* 2019; Fishbein *et al.* 2023), ultimately affecting the whole body. Furthermore, antibiotic treatment is frequently linked with an increased relative abundance of facultative anaerobes, which were initially less prevalent members of the community, such as *Enterobacteriaceae*, *Enterococcus* spp., *Clostridium* spp., and *Streptococcus* spp. (Palleja *et al.* 2018; Dubinsky *et al.* 2020; Fishbein *et al.* 2023). Considering the variation in the spectrum of activity against the different members of the commensal GIT microbiome, selecting antibiotics with a narrower spectrum may lead to lesser disruption of the microbiome and accelerate community recovery (Ajami *et al.* 2018).

The effect of *H. pylori* eradication therapy on the GIT microbiome has also attracted increasing attention because of its potential impact on overall human health. Studies investigating the short-term effects of *H. pylori* eradication therapy on the GIT microbiome have revealed that the results depend on the specific eradication regimen. Although different regimens had a different impact on short- and long-term GIT microbiome abundance, a common trend of decreased microbiota diversity was observed in all regimens during short-term follow-up periods (Chen *et al.* 2022). A meta-analysis by Chen et al. encompassing 21 articles exploring the effects of triple eradication therapy (different types of PPIs, clarithromycin, and amoxicillin) identified significant reduction at the phylum level in *Actinobacteria*, persisting for over six months. At the genus level, *Lactobacillus* also exhibited

a marked decrease within seven days post-eradication. In contrast, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* showed no substantial variation throughout the follow-up period. Nevertheless, the authors acknowledged the limitations of their meta-analysis due to the relatively small sample size and the influence of individual studies on the overall analysis (Chen *et al.* 2022). Examining smaller-scale studies, a Malaysian investigation utilizing triple eradication therapy (pantoprazole, clarithromycin, and amoxicillin) reported minimal shifts in microbial diversity before and after the eradication therapy, although a decrease in *Bacteroidetes* abundance and an increase in *Firmicutes* were observed (Yap *et al.* 2016). Another study focusing on Japanese high-school students who received a different triple therapy (vonoprazan, amoxicillin, and clarithromycin) revealed transient reductions in species richness, particularly for *Actinobacteria* at post-eradication, with subsequent recovery to baseline levels within 8-12 weeks (Kakiuchi *et al.* 2021). A comprehensive study evaluated the short-term and long-term effects of *H. pylori* triple eradication therapy (lansoprazole, amoxicillin, and clarithromycin) on the GIT microbiome, ARGs, and metabolic parameters. Results indicated a reduction in species richness and alterations in beta diversity two weeks post-eradication, with subsequent restoration at eight weeks and one year. Moreover, transient increases in *E. coli* resistance rates to such antibiotics as ampicillin-sulbactam, cefazolin, levofloxacin and others returned to baseline levels at eight weeks and one year. Notably, no significant differences in ARG prevalence were observed at these follow-up points. Additionally, despite increasing body mass index and body weight, a decrease in insulin resistance and triglyceride concentrations suggested potential beneficial metabolic effects following *H. pylori* eradication (Liou *et al.* 2019).

Although existing research has focused primarily on the short-term effects of *H. pylori* eradication therapy on the GIT microbiome, few studies address potential long-term consequences. Investigating long-term changes in the GIT microbiota resulting from antimicrobial therapy is critical to comprehensively understanding the dynamic relationship between *H. pylori* eradication and the GIT microbiome, thus guiding clinical decisions and contributing to the ongoing debate about microbial therapy.

1.5 Microbiome sample collection and treatment

In the era of microbiome research, many studies have been devoted to uncovering the potential of the microbiome for its use in both diagnostics and treatment of various diseases. Therefore, the development of reliable sample collection systems and careful sample handling remains essential to guarantee the accuracy of clinical diagnostics and scientific research.

Sample stability is especially important in epidemiological studies where samples must be transported from the collection site to a processing laboratory. Several studies have investigated the stability of faecal microbial communities using varying temperature conditions, storage durations, and diverse collection containers. In essence, the taxonomic composition of faecal samples – with no added preservation additives – remains relatively stable for up to 24 hours when stored at 4°C, with slight variations in abundance of phyla *Firmicutes* and *Bacteroidetes* (Holzhausen *et al.* 2021; Zreloff *et al.* 2023). While the most frequently recommended approach for fresh samples is immediate freezing at least at -20°C to prevent bacterial growth (Song *et al.* 2016; Wang *et al.* 2018; Wu *et al.* 2019), this isn't always feasible due to factors such as individual sanitary concerns related to using household freezer for faecal sample storage, as well as the challenge of transporting frozen samples to the laboratory. Thus, various sample collection systems have been introduced, which aim to preserve the microbial community structure, ensuring the safe transportation of samples even at ambient temperature. Among the most widely used systems are OMNIgene-GUT (DNA Genotek Inc, Canada) and DNA/RNA shield-fecal collection tubes (Zymo Research, USA), both of which are prefilled with preservation solution.

OMNIgene-GUT is one of the most extensively validated sample collection systems designed for the GIT microbiome studies. According to the manufacturer, this collection system stabilises the microbial community by inhibiting microbial growth and preventing DNA degradation (DNA Genotek 2020). Recent studies indicate that the community structures of faecal samples stored within this container remain comparable to those rapidly frozen, even when stored at ambient temperature for up to seven days. Moreover, samples preserved in such a way remain stable for over two years of storage at -80°C (Chen *et al.* 2020; Neuberger-Castillo *et al.* 2020). While OMNIgene-GUT displays high performance in microbial DNA preservation, the performance in RNA preservation is suboptimal (Maghini *et al.* 2022). However, some reports highlight that this sample collection container demonstrates the ability to preserve metabolites, such as bile acids and SCFAs (Lim *et al.* 2020; Neuberger-Castillo *et al.* 2021), but for that purpose a more recent container, OMNImet-GUT, has been introduced. This newer product is better suited for long-term storage of faecal material that is intended for targeted and untargeted metabolomics analyses (DNA Genotek 2021).

Another commonly used product in the GIT microbiome studies is the DNA/RNA shield-fecal collection tube. This collection system achieves microbial community stabilisation by inactivating microbial growth and preserving both DNA and RNA (Zymo Research 2023).

The performance of this tube in preserving the microbial community structure is comparable to that of OMNIgene-GUT, yet it offers an additional capability to extract RNA from the same sample (Maghini *et al.* 2022). This system has also demonstrated the ability to preserve the microbial community structure even after repeated freeze-thaw cycles and is able to maintain community stability under various temperature conditions for up to three weeks. Furthermore, faecal samples collected using the DNA/RNA shield-fecal collection tube remain stable during the long-term storage, for up to 18 months (Kim *et al.* 2023). However, since this product is relatively recent, additional applications for samples stored in this container have not been tested yet.

1.5.1 Faecal immunochemical test sample containers

With the launch of various population screening initiatives aimed at detecting such diseases like colorectal (CRC) or cervical cancer, healthcare providers have collected substantial quantities of patient samples. Once the primary analysis is completed, these samples potentially become accessible for additional research endeavours.

The faecal immunochemical test (FIT or iFOBT) is the most widely employed screening method worldwide for the detection of hidden blood in faecal samples and its application facilitates the early-stage diagnosis of CRC (Navarro *et al.* 2017; Chiu *et al.* 2021; Shaukat and Levin 2022). Successful testing requires that immediately after collection faecal sample is placed in a sample storage container, which is filled with a haemoglobin stabilizing buffer and transported to the analysis site. Public mail delivery is the standard approach for transporting samples to a centralized laboratory. One of the advantages of FIT is that there are no requirements for dietary restrictions, as the antibody specifically detects human globulin. This is in sharp contrast with guaiac-based faecal occult blood test (gFOBT), which detects both human and non-human haem. The latter might have been ingested as food and its detection leads to false positive results (Faivre 2010; Navarro *et al.* 2017). The sensitivity of FIT in detecting CRC ranges between 75 and 79%, while in the case of advanced adenomas, it is around 40%. The specificity of this test in detection of CRC is approximately 95% (Shaukat and Levin 2022). However, FIT tests exhibit low sensitivity towards precancerous lesions (12-18%) (Chang *et al.* 2017) and first-stage CRC (T1, 40%) (Niedermaier *et al.* 2020). Furthermore, factors such as smoking, advancing age and use of nonsteroidal anti-inflammatory medication might exert a negative effect on the specificity of the FIT test (Stegeman *et al.* 2013). Given the established contribution of the GIT microbiome to the development of CRC (Tjalsma *et al.* 2012; Rebersek 2021), over the past decade, there has

been a growing interest in combining GIT microbial community signatures with the FIT test to reveal bacterial markers that could facilitate the detection of early-stage GIT malignancies (Krigul *et al.* 2021; Khannous-Lleiffe *et al.* 2022).

However, incorporating FIT test tubes into microbiome research requires a validation process to ensure that the haemoglobin-stabilizing buffer does not interfere with the composition of microbial communities within the faecal samples. Thus, in recent years, various FIT collection containers have been tested across a range of temperature and storage regimens. The validation of FIT sample containers for microbiome studies usually involves collecting faecal samples from generally healthy individuals, which are then aliquoted into smaller portions. Typically, one aliquot is immediately frozen at -80°C, while others are distributed among FIT sample containers subjected to diverse temperature and storage conditions. For instance, a study by Krigul *et al.* (2021) evaluated the compatibility of FIT sample containers for microbiome studies by assessing microbial community structures of faecal samples under four different regimens: immediate storage of fresh samples at -20°C; immediate storage of samples collected in FIT containers at -20°C; storage of samples collected in FIT container at room temperature for four days; and storage of samples collected in FIT container at room temperature for seven days (Krigul *et al.* 2021). As a result, several recent studies have demonstrated that faecal microbiome diversity and taxonomic profiles remain consistent across various FIT sample storage conditions, such as storing containers for 3-10 days in room temperature (depending on the research design) followed by storage at -80°C (Masi *et al.* 2020; Krigul *et al.* 2021; Brezina *et al.* 2023).

1.6 Current methods to study the gastrointestinal microbiome

In the last decade, the scientific community has made significant progress in understanding the complexity of microbial communities and their functionality in diverse environments. This progress has been achieved mainly due to the development of next-generation sequencing (NGS) technologies, which nowadays is an essential tool for analysing microbial communities. Regarding the microbiome analysis, there are three primary sequencing strategies, each characterized by distinct sample preparation, sequencing methods, and subsequent bioinformatical analyses. These strategies are marker gene analysis, whole metagenome analysis, and metatranscriptome analysis (Knight *et al.* 2018).

Marker gene sequencing is a well-established technique that is utilized to uncover microbial communities within a given sample by targeting taxa-specific or otherwise relevant regions of genes. Prominent examples of such genes include bacterial *16S rRNA* or eukaryote

18S rRNA genes. Their amplification and subsequent sequencing have become a robust, efficient, and cost-effective approach that offers a low-resolution insight into microbial communities (Knight *et al.* 2018; Gotschlich *et al.* 2019; Bokulich *et al.* 2020). Marker gene sequencing is especially valuable for samples with high levels of host DNA contamination due to its selective targeting of microbial DNA. However, the technique's limitations often include primer-induced biases and restricted taxonomic resolution (Knight *et al.* 2018).

Metagenomics involves sequencing the whole DNA repertoire within a sample, which, depending on the sample source and type, may contain a wide variety of genomes, including viral, prokaryotic, and eukaryotic genomes. Thus, this technique surpasses the limitations of marker gene sequencing by providing enhanced genomic insight and taxonomic resolution. However, its use is associated with increased sample preparation, sequencing, and bioinformatic analysis costs. Metagenomic sequencing allows for detailed taxonomic classifications at species or even strain levels and the opportunity to assemble complete microbial genomes, thus delving into the functional attributes of microbial communities (Knight *et al.* 2018; Gotschlich *et al.* 2019). However, there are also challenges. For example, there are biases that are introduced during library preparation and the assembly process. In addition, reference databases that are used for annotation can introduce inaccuracies in taxonomic and functional assignment, requiring strict quality control and methodological improvements to mitigate biases (Knight *et al.* 2018).

Metatranscriptomics represents an approach that employs RNA sequencing to uncover the intricacies of transcription within microbiomes. Unlike metagenomics, which provides a static view of microbial genetic potential, metatranscriptomics captures the dynamic interplay of actively transcribed genes. This approach allows us to identify and annotate even lowly expressed genes to grasp microbial functions comprehensively. These transcripts can be further mapped to intricate metabolic pathways, revealing the molecular basis of microbial activities. However, organisms characterized by higher transcription rates may dominate the transcriptomic landscape, thus influencing the representation of less transcriptionally active members (Knight *et al.* 2018; Shakya *et al.* 2019). Therefore, the combination of metatranscriptomic and metagenomic approaches might facilitate the detection of differential expression patterns based on the microbial community genes present in the environment (Franzosa *et al.* 2014).

In addition to NGS-based research, other microbiome research methodologies focus on microbial protein and metabolite profiles, namely metaproteomics and metabolomics. These

approaches most commonly use high-throughput mass spectrometry technologies (Xu and Yang 2021). Furthermore, an increasing number of studies are integrating multiple -*omics* techniques to investigate various pathophysiological conditions in more detail, thereby offering a broader understanding of microbial functional interactions and relationships with the human host.

2 MATERIALS AND METHODS

2.1 Study design and sample collection

Publication I: A widely used sampling device in colorectal cancer screening programmes allows for large-scale microbiome studies

Stool samples were acquired from five healthy male adults at the Riga East Clinical University Hospital (RECUH) to assess the stability of the samples in FIT tubes. Participants had no dietary restrictions or gastrointestinal disorders and had not received antibiotics for at least six months before the sampling. Figure 5 shows the various conditions to which 80 samples were immediately exposed.

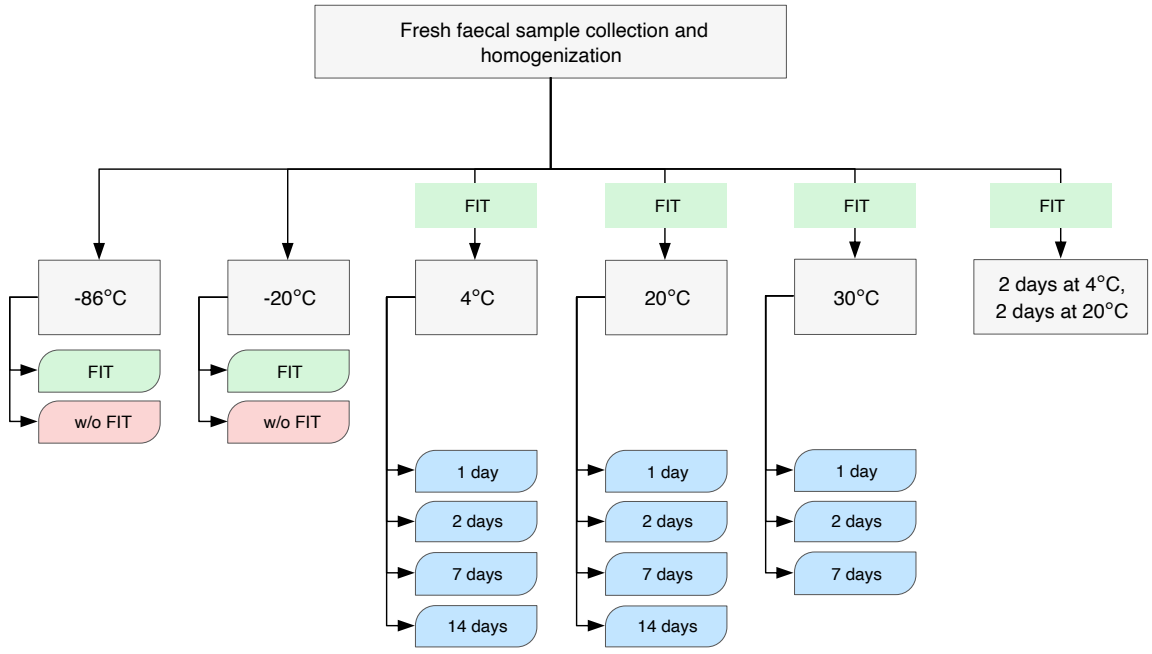

Figure 5. Stool sample stability was assessed by exposing them to different storage conditions. For the first two conditions, baseline sample aliquot shortly after the defecation was transferred either to OC-Sensor tube (FIT) or standard stool collection tube and immediately frozen. Conditions 3-5 – exposure of sample aliquots in the FIT containers to a specific temperature for 1, 2, 7, and 14 days, after which samples were immediately frozen at -86°C. Last condition – exposure of sample aliquots in the FIT containers at 4°C for 2 days following storage at 20°C for two days, after which samples were immediately frozen at -86°C.

Publication II: Lack of significant differences between gastrointestinal tract microbial population structure of *Helicobacter pylori*-infected subjects before and two years after single eradication event

Sixty individuals with positive *H. pylori* infection who met the criteria (men and women aged 40-64; self-reported alcohol consumption 2-3 times per month or less; no history of colon

or rectum polyps since age 20, gallstones, gastric cancer, gastric resection, alarm symptoms for digestive or other diseases, type 2 diabetes, ulcerative colitis, Crohn's disease, coeliac disease, biliary cirrhosis, thyroid diseases and hepatitis B virus infection, severe psychiatric disorders) were included in this study. A ^{13}C-Urea breath test (Euroisotop, Germany) was performed to diagnose *H. pylori* infection. A detailed questionnaire containing information such as age, body mass index, history of various diseases and lifestyle habits was collected from each study participant.

A faecal sample was obtained from each patient enrolled in the study before initiating standard first-line *H. pylori* eradication therapy and two years later. Each patient was prescribed a medication regimen of *Esomeprazolum* 40 mg, *Clarithromycinum* 500 mg and *Amoxicillinum* 1000 mg, all administered twice daily for ten days. All procedures were performed following institutional ethical standards, and written informed consent was obtained from all participating patients before the commencement of the study. The samples were delinked and de-identified from the donors.

One hundred twenty samples were obtained within 30 minutes of defecation and transferred to an OC-Sensor tube (Eiken Chemical Co, Japan). Subsequent processing included immediate homogenization and storage at -80°C upon further processing.

Publication III: Abundance and prevalence of ESBL coding genes in patients undergoing first line eradication therapy for *Helicobacter pylori*

A total of 90 individuals with positive *H. pylori* infection who met the criteria (men and women aged 40 to 64 years; self-reported alcohol consumption 2-3 times a month or less; no history of colon or rectum polyps since age 20, gallstones, gastric cancer, gastric resection, alarm symptoms for digestive or other diseases, type 2 diabetes, ulcerative colitis, Crohn's disease, coeliac disease, biliary cirrhosis, thyroid diseases, hepatitis B virus infection, or severe psychiatric disorders) were included in this study. A ^{13}C-Urea breath test (Euroisotop, Germany) was used to diagnose *H. pylori* infection, and a detailed questionnaire was collected from each study participant, containing information such as age, body mass index, medical history (e.g., GIT diseases, viral infections, autoimmune diseases, and cancer), and lifestyle habits. Sixty individuals were used for the ESBL panel experimental group, with samples taken over two years, and the remaining 30 individuals were used for ESBL panel validation, with samples taken over one year (Figure 6).

A faecal sample was obtained from each patient enrolled in the study before initiating standard first-line *H. pylori* eradication therapy and one–two years after that. Each patient was

prescribed the following medication regimen for ten days: *Esomeprazolum* 40 mg, *Clarithromycinum* 500 mg and *Amoxicillinum* 1000 mg, all administered twice daily. All procedures were performed following institutional ethical standards, and written informed consent was obtained from all participating patients before the commencement of the study. The samples were delinked and de-identified from the donors.

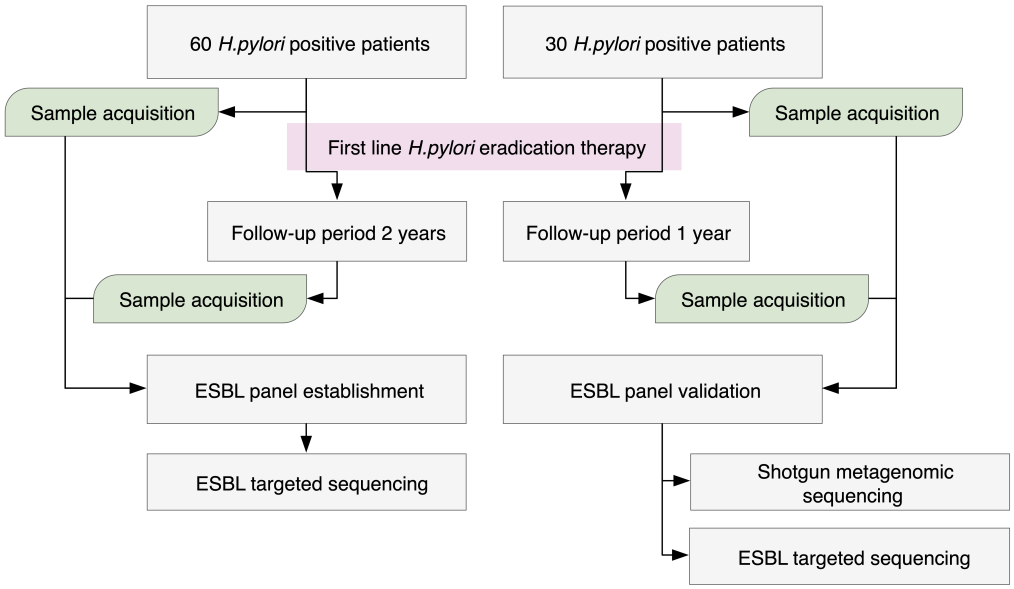

Figure 6. Study design. First-line *H. pylori* eradication therapy consisted of *esomeprazolum* 40 mg, *clarithromycinum* 500 mg and *amoxicillinum* 1000 mg, each twice daily for ten days.

One hundred eighty samples were obtained within 30 minutes of defecation and transferred to an OC-Sensor tube (Eiken Chemical Co, Japan). Subsequent processing included immediate homogenization and storage at -80°C upon further processing.

2.2 Isolation of microbial DNA

Faecal samples in the OC-Sensor tubes were partially suspended in a buffer and contained solid particles; hence, both liquid and solid fractions had to be collected. To achieve this, samples were mixed thoroughly to reduce particle size and extracted using a disposable syringe with the longest and widest needle available (0.8 × 120 mm). Following extraction, samples were transferred to pre-labelled 5 ml tubes.

Samples stored in OC-Sensor tubes were desolvated by freezing in liquid nitrogen and lyophilization in a Christ Alpha 1-2 LD Freeze Dryer (SciQuip Ltd., UK) overnight (approximately 15 hours). DNA was extracted from the samples using the FastDNA SPIN Kit for Soil (MP Biomedicals, USA). The amount of starting sample was adjusted as follows: 10 mg of samples not stored in OC-Sensor tubes were transferred to Lysing Matrix E tube;

lyophilised samples initially stored in OC-Sensor tubes were first diluted in 978 µL of Sodium Phosphate Buffer and then moved to Lysing Matrix E tube. Subsequent steps were performed according to the manufacturer's guidelines. DNA was eluted from SPIN™ Filters with 100 µL of DNase/Pyrogen-Free Water.

The concentration of extracted DNA was measured using a dsDNA High Sensitivity Assay kit on a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, USA). DNA quantity, average size and quality were assessed using electrophoresis in 1.2% agarose gels.

2.3 Sample preparation for sequencing analysis

2.3.1 Preparation of *16S rRNA* libraries for Ion Torrent PGM sequencing

PCR amplification of the V3 region of the *16S rRNA* gene was performed using primers specific for the bacterial domain Probio_Uni_R (5′-ATTACCGCGGCTGCT-3′) and Probio_Uni_F (5′-CCTACGGGRSGCAGCAG-3′) as previously described (Milani *et al.* 2013). Both primers were tagged with 10–11 bp unique barcode labels and the adapter sequence to allow multiple samples to be included in a single sequencing run.

PCR amplification was performed using Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific, USA) and a GeneAmp® PCR System 9700 (Thermo Fisher Scientific). The reaction mixture was prepared according to the manufacturer's recommendations, and the thermal conditions were as follows: 98°C for 30 seconds, 35 cycles of 98°C for 10 seconds, 67°C for 15 seconds, 72°C for 15 seconds with a final extension at 72°C for 7 minutes. The success of the reaction was then assessed by agarose gel electrophoresis, and the resulting libraries were purified using NucleoMag® NGS Clean-Up and a Size Select kit (Macherey-Nagel, Germany). For Publication I, amplicon quality and quantity were assessed using an Agilent DNA 7500 kit on an Agilent 2100 BioAnalyzer (Agilent Technologies, USA). For Publication II, amplicon quality and quantity were assessed using an Agilent High-Sensitivity DNA kit on an Agilent 2100 BioAnalyzer.

2.3.2 Library preparation and *16S rRNA* sequencing using Illumina MiSeq

Targeted sequencing samples corresponding to Publication I were sequenced using an Illumina MiSeq (Illumina Inc., USA) at The Clinical Genomics facility of the Karolinska Institute in Sweden. Samples were prepared according to Illumina´s protocol (Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System). *16S rRNA* gene V3-4 region sequencing was performed using the 341f-805r primers (Hugerth *et al.* 2014). After the initial amplification, a second PCR was performed to attach Illumina adapters and barcodes that allow for multiplexing. The generated raw sequences were 250 bp paired-end reads.

2.3.3 Library preparation and metagenomic sequencing using Illumina HiSeq 2000

Metagenomic samples corresponding to Publication I were sequenced using an Illumina HiSeq 2000 (Illumina Inc., USA) at The Clinical Genomics facility of the Karolinska Institute in Sweden. Ten samples were pooled into one lane with libraries prepared with an average fragment size of 300 bp. The generated raw sequences were 100 bp paired-end reads.

2.3.4 ESBL gene cluster primer design

ESBL gene cluster primer design for Publication III was performed by MSc Ivars Silamikelis from the Bioinformatics core facility, Latvian Biomedical Research and Study centre.

The known BL nucleotide sequences were obtained from NCBI GenBank (accession date: 02.01.2018). Primer design was performed separately for the TEM BLs, as they formed a homogenous group that was highly divergent from the rest of the BL sequences. MAFFT v.7.392 (Katoh *et al.* 2002) was used to generate multiple BL sequence alignments. The number of pairwise non-gapped mismatches between BL sequences was calculated, followed by hierarchical clustering with complete linkage based on the alignment. Clusters were defined with a distance cut-off of 0.1. For each cluster, contiguous conserved regions that varied by at least 10% nucleotides at each position in the alignment were identified using Shannon's index. Regions longer than 17 bases were used in subsequent experiments.

For each cluster, all possible pairs of conserved regions were calculated such that the interval between regions in a pair was no longer than 500 bp. Each pair of conserved regions was scored by summing their Shannon indices at each position and sorted by their scores in ascending order. Primers were then designed for these region pairs with PRIMER3 v.2.4.0, specifying PCR product sizes in the 200–500 bp range (Untergasser *et al.* 2012). The best primer pairs for each cluster were evaluated by identifying potential binding sites in the original list of BL sequences. A site was considered a possible binding site if there were no more than three mismatches between the primer sequence and the template. In addition, the last five nucleotides at the 3' end of the primer were not allowed to have mismatches with the template. The binding site algorithm was implemented using the SeqAn library (Reinert *et al.* 2017). The primer-bound regions were then aligned to the BL sequence database to assess possible off-target PCR products, that is, sequences mapped to multiple BL clusters. Primer pairs overlapping or forming PCR products shorter than 50 bp were identified and pooled into separate sequencing batches. The designed primers were synthesized by MetaBion.

2.3.5 Preparation of the ESBL targeted libraries for Ion Torrent PGM sequencing

Pools with equal molarities and volumes were prepared for primers targeting the ESBL-coding genes and primers for the normalization of ESBL counts- primer pair Probio_Uni-F/Probio_Uni-R targeting *16S rRNA* gene V3 region (Milani *et al.* 2013). PCR amplification of ESBL coding gene regions was performed using a 10 µM custom-designed primer pool, Phusion U Multiplex PCR Master Mix, and GeneAmp® PCR System 9700. The reaction mixture was prepared according to the manufacturer's recommendations, and the thermal conditions were set as follows: 98°C for 30 seconds; 35 cycles of 98°C for 10 seconds, 55°C for 30 seconds, 72°C for 15 seconds; with a final extension at 72°C for 7 minutes. Reaction success was then assessed by 1.2% agarose gel electrophoresis. 100 ng of the resulting amplicons were used for library generation using the Ion Plus Fragment Library Kit (Thermo Fisher Scientific, USA) and the NucleoMag® NGS Clean-Up and Size Select kit purification modules. The quality and quantity of the amplicons were assessed using an Agilent High Sensitivity DNA kit on an Agilent 2100 BioAnalyzer.

2.3.6 Library preparation for metagenomic sequencing

500 ng of DNA samples were sheared using a Covaris S220 Focused-ultrasonicator (Covaris, USA) to achieve an average fragment size of 300 bp. Libraries with an average insert size of 280 bp were prepared using MGIEasy Universal DNA Library Prep Set V1.0 (MGI Tech Co., China) under the manufacturer's recommendations. Quality control of the libraries was evaluated using the Qubit High Sensitivity dsDNA assay kit on a Qubit 2.0 instrument and the Agilent High Sensitivity DNA kit on an Agilent 2100 Bioanalyzer.

2.4 Sequencing analysis

2.4.1 *16S rRNA* library sequencing by Ion Torrent PGM

Publication I: Before clonal amplification, each library was diluted to 8 pM and pooled in up to 10 libraries per sequencing run. Template generation was performed using the Ion OneTouch™ 200 Template kit v2 DL (Life Technologies, USA) and the Ion OneTouch DL instrument (Life Technologies, USA). Sequencing was carried out with an Ion 316 v2 or Ion 318 v2 chip and Ion Torrent PGM using the Ion PGM 200 sequencing kit (Life Technologies, USA). All procedures were performed according to the manufacturer's instructions, and each run was expected to yield approximately 250'000 reads per sample. After sequencing, the individual reads were filtered by PGM software to remove low-quality reads. Sequences matching the PGM 3′ adaptor were automatically trimmed.

Publication II: Before clonal amplification, each library was diluted to 12 pM and pooled into up to 12 libraries per sequencing run. The Ion PGM™ Hi-Q™ View OT2 kit (Life Technologies, USA) and Ion OneTouch DL instrument were used for template generation. The sequencing was performed on an Ion 318 v2 chip and Ion Torrent PGM machine employing the Ion PGM™ Hi-Q™ View Sequencing kit (Life Technologies, USA). All procedures were carried out according to the manufacturer's instructions, and each run was expected to produce approximately 150'000 reads per sample. Following the sequencing procedure, the individual reads were filtered by the PGM software to remove low-quality reads. Sequences matching the PGM 3' adaptor were automatically trimmed.

2.4.2 ESBL targeted library sequencing by Ion Torrent PGM

Before emulsion PCR, each library was diluted to 12 pM and pooled for up to 18 libraries per sequencing run. An Ion PGM™ Hi-Q™ View OT2 kit (Life Technologies, USA) and an Ion OneTouch DL instrument (Life Technologies, USA) were used to generate templates. Sequencing was performed on an Ion 318 v2 chip and an Ion Torrent PGM using the Ion PGM™ Hi-Q™ View Sequencing kit (Life Technologies, USA). All procedures were performed according to the manufacturer's instructions, and each sample was expected to have at least 80'000 reads. After sequencing, the individual reads were filtered by PGM software to remove low-quality reads. Sequences matching the PGM 3′ adaptor were automatically trimmed.

2.4.3 Metagenomic sequencing by DNBSEQ

Libraries were pooled, diluted to 1 pM, and circularized using the MGIEasy Circularization Module (MGI Tech Co., China). The circularized libraries were then used as templates for preparing DNA nanoballs (DNB). DNBs were loaded onto the PE100 flow cell using an automated DNB loading system. The samples were sequenced using a DNBSEQ-G400 sequencer, utilizing a DNBSEQ-G400RS High-Throughput Sequencing Set (MGI Tech Co., China) following the standard protocol. Each sample was expected to have at least 20 million 100 bp paired-end reads.

2.5 Sequence analysis and statistics

Publication I: A widely used sampling device in colorectal cancer screening programmes allows for large-scale microbiome studies

16S rRNA data analysis and statistics

The following *16S rRNA* data analysis workflow corresponds to both Ion Torrent PGM and Illumina MiSeq generated sequence data. Sequencing data analysis was performed using

QIIME v.1.8.0. and UPARSE v.7.0.1001. workflow to quality filter and cluster *16S rRNA* V3 region sequences (Pylro *et al.* 2014). Quality control retained sequences with an average sequence quality score >20. Operational taxonomic units (OTUs) were built with 97% sequence identity with uclust (Edgar 2010). Taxonomic assignments were made to the lowest possible rank using the RDP (Wang *et al.* 2007) algorithm and the Greengenes (http://greengenes.secondgenome.com) reference dataset (gg_otus-13_8 release) (DeSantis *et al.* 2006). Alpha diversity (Shannon index) and beta diversity measures (weighted and unweighted UniFrac metrics) were computed by QIIME. OTU frequencies between storage conditions were compared using the Kruskal–Wallis test with false discovery rate (FDR), and Bonferroni corrected *p*-values within a QIIME environment.

Metagenomics data analysis and statistics

Metagenomics data analysis and statistics for Publication I was performed by PhD Saeed Shoaie from the Centre for Translational Microbiome Research, Department of Microbiology Tumor and Cell Biology at the Karolinska Institute in Stockholm, Sweden.

The raw reads from the initial metagenomics analysis were trimmed and filtered using the SolexaQA package (Cox *et al.* 2010). The quality of reads was assessed using FastQC. To remove reads of human origin, the reads were mapped to the human genome database (hg19) with SOAPAligner2 (Li *et al.* 2009). MetaPhlAn 2 (Truong *et al.* 2015) was used for taxonomic profiling and compilation of species abundances for each sample. Bray–Curtis dissimilarity indices were calculated to quantify similarities between samples, and Principal Coordinate Analysis (PCoA) was used to visualize results using the R package vegan.

Publication II: Lack of significant differences between gastrointestinal tract microbial population structure of *Helicobacter pylori*-infected subjects before and two years after single eradication event

16S rRNA data analysis

Sequence data analysis was performed using vsearch v.2.10.4 to quality-filter and cluster *16S rRNA* V3 sequences. Quality filtering was conducted on raw fastq files with an expected error of 0.75 for all read bases. Dereplication was performed at two levels, first for each sample and then for all. Sequences were pre-clustered at 97% identity, and *de novo* chimeric reads were removed. Reference chimera detection was conducted against the Silva gold bacterial reference database, and all dereplicated, non-chimeric and non-singleton sequences were used to construct OTUs at 97% identity. Taxonomic assignment was performed with the SINTAX (Edgar 2016) algorithm using RDP (Wang *et al.* 2007) training set v16.

16S rRNA data statistics

Statistical analysis was conducted on filtered feature abundance matrices using R v.3.5.2. Clustering samples into enterotypes was performed using the cluster package (Maechler *et al.* 2019) and clusterSim package (Dudek 2019). Samples were clustered based on their relative genus abundances by employing the Jensen Shannon distance and the Partitioning around medoids clustering algorithm. The optimal number of clusters was identified using the Calinski-Harabasz index. Enterotypes were visualized by PCoA utilising the ggplot2 package (Wickham 2016).

Measures of species richness (Observed, Chao1), alpha diversity (Shannon) and beta diversity (weighted and unweighted UniFrac metrics) were computed using the phyloseq package (McMurdie and Holmes 2013).

A correlation map for feature abundance matrices was produced using a Spearman rank correlation at the genus level. Sample counts were filtered only to retain those with at least 200 counts in each taxonomic assignment, and a log-relative transformation was applied. The correlation map was then plotted using the top 50 taxa. A Spearman correlation method was utilized to determine the ordinal association between treatment conditions and explanatory variables.

To identify significant taxonomic units across treatment states, differential expression analysis based on the negative binomial distribution was performed with the DESeq2 package (Love *et al.* 2014) implemented in R. Log-relative transformation was applied to normalise taxonomical counts, and significantly differentially abundant taxa (e.g., $p<0.05$) were visualized using the ggplot2 package.

A Redundancy Analysis (RDA) was conducted to estimate the variation present in the dataset that could be explained by the explanatory variables. Each variable was evaluated by Analysis of Variance (ANOVA). Then, a Monte Carlo permutation test was used to reduce the number of explanatory variables entering the analysis, based on the Akaike Information Criterion (AIC) and the associated *p*-values derived from comparing the variables.

Publication III: Abundance and prevalence of ESBL coding genes in patients undergoing first line eradication therapy for *Helicobacter pylori*

Data analysis of ESBL coding genes

ESBL sequence analysis for Publication III was performed by MSc Ivars Silamikelis, MSc Janis Pjalkovskis, and BSc Ilva Danenberga, all from the Bioinformatics core facility, Latvian Biomedical Research and Study centre.

The obtained raw reads were subjected to adapter trimming using Cutadapt v.1.16 (Martin 2011). The targeted sequencing data was aligned to a curated BL sequence database using Bowtie2 v.2.3.5.1 with a very sensitive pre-set (Langmead and Salzberg 2012). The host reads were removed from the metagenomic sequence data before mapping to BL sequences using Bowtie2. Quantification of *16S rRNA* was performed using SortMeRNA v.2.1 (Kopylova *et al.* 2012), and *16S rRNA* sequences obtained from RNA central v10 (The RNAcentral Consortium *et al.* 2017) were used with the search query 'rna_type: "rRNA" AND TAXONOMY: "9606" AND length: [19 TO 2000000000]'.

A classification scheme to assign sequencing reads to a given ESBL cluster was developed: a region was identified for each ESBL cluster where the formation of PCR product was expected. Each putative product was aligned to all BL sequences to assess whether the product was specific to a particular cluster. Putative products were considered specific if mapped to sequences from only one cluster. Clusters were combined if the putative PCR product was specific to a set of clusters and if such clusters were not discernible. If multiple PCR products were mapped to the same position within a cluster, an alignment score threshold was set as the minimum score from the set of the true positive alignments. An annotation table with details on reference sequence IDs, start and end coordinates of the primer product regions, and the alignment score thresholds were then created. Sequence reads were assigned to clusters if they overlapped with the coordinates in the annotation table and exceeded the alignment score threshold. Putative PCR products were identified using SeqAn, Pandas and Bowtie2. To quantify the number of reads in each cluster for each sample, an in-house built Python script reading sample binary alignment map files with the Pysam package were used. The number of reads of each BL cluster was normalized by the number of reads of the *16S rRNA* gene of a given sample.

Metagenome data analysis

The paired-end reads obtained were subjected to quality control and quality trimming using FastQC and Trimmomatic v0.39 (Bolger *et al.* 2014), respectively, with a quality threshold of 20 and a minimum read length of 36. The quality-filtered sequences were then aligned to the human genome reference GRCh37 (hg19, UID:2758) and sequences that matched the human genome were removed using Bowtie2 v.2.3.5.1. The taxonomic profile of the metagenomic dataset was assigned using Kraken2 v.2.0.8 (Wood *et al.* 2019) and RefSeq database release 98 (O'Leary *et al.* 2016). *De novo* read assembly into contigs was performed using the IDBA_UD (Peng *et al.* 2012) assembler with a *k*-mer length of at least 50. The

generated assembly was evaluated using metaQuast (Mikheenko *et al.* 2015). The assembly database and local alignment of input reads to the assembly were performed using Bowtie2. Open reading frame detection and subsequent annotation were conducted using PROKKA v.1.14.6 (Seemann 2014) with the manually curated Swiss-Prot UniProtKB (Consortium 2019) database (accessed 08.02.2021.). Contigs shorter than 250 nt, rRNA, and tRNA predictions were excluded during annotation. The coordinates of predicted protein-coding features (CDS) were quantified against the assembly database using HTSeq (Anders *et al.* 2015) and the intersection-nonempty resolution mode. Metagenomic read counts were standardized using the Transcripts Per Million method (Wagner *et al.* 2012) with an in-house built Python script. CRISPR annotations were removed from the corresponding annotation files, while contig IDs with the respective product information were retained using an in-house sed and awk scripts. The read counts were combined with filtered annotations by contig ID column for each sample separately. The Pandas library (The Pandas development, 2020) was used within the Python environment to combine all samples into a single dataset by annotation column.

Contigs obtained from the study subjects were utilized to predict the resistome profile using the Resistance Gene Identifier (RGI) v.5.1.1 along with the Comprehensive Antibiotic Resistance Database (CARD) (Alcock *et al.* 2020) and the DIAMOND (Buchfink *et al.* 2015) alignment tool. The results were obtained for each sample and visualized using a heatmap function of RGI. The resistance genes were organized based on the resistance mechanism and gene family. Hierarchical clustering was also performed to group the samples based on their similarity.

Statistical analysis of taxonomical data

The Kraken reports were initially processed using Pavian v.1.0.0 (Breitwieser and Salzberg 2016), wherein taxonomic entries with an assigned sequence count below 200 were removed. Next, SIAMCAT v.1.9.0 (Wirbel *et al.* 2020) was employed to investigate the association of microbial species between pre- and post-eradication states. This was done by separating the taxonomical entities into two groups, designated as “case” and “control” for pre- and post-eradication states, respectively. The relative abundance cut-off for the species was set to 0.001, and the Wilcoxon test was used to determine the association between the two groups at a significance level of $p<0.05$, with the FDR multiple hypothesis correction. Normalization was performed using log transformation, and the Area Under the Receiver Operating Characteristics Curve (AU-ROC) was used to measure the enrichment. All the acquired results

of the association between the two groups were visualized in the SIAMCAT built-in association plot.

The dataset was classified into four groups based on the treatment states, with F-post-erad representing subjects in the post-eradication group with ineffective *H. pylori* eradication, F-pre-erad representing subjects in the pre-eradication group with ineffective *H. pylori* eradication, S-post-erad representing subjects in the post-eradication group with successful *H. pylori* eradication and S-pre-erad representing subjects in the pre-eradication group with successful *H. pylori* eradication. Alpha diversity metrics, including Shannon, Chao1 and Observed, were computed and presented using the Phyloseq v.1.30.0 (McMurdie and Holmes 2013) package. Wilcoxon rank sum test and Holm P-value adjustment method were utilized for pairwise comparisons of alpha diversity metrics between the treatment states with the Vegan v.2.5-7 package. Additionally, non-metric multidimensional scaling was performed with Phyloseq.

Statistical analysis of ESBL amplicon data

The two-tailed paired t-test was employed from the Vegan v.2.5-7 package to compare the relative abundances of ESBL clusters between the two treatment states. The Kruskal-Wallis test was also used from the same package to evaluate the significance of differences in the abundance of individual ESBL clusters between pre- and post-eradication states. To explore the overlapping of ESBL clusters among datasets, cluster IDs that appeared at least once in a sample were extracted from all three datasets, and a Venn diagram was constructed using the ggVennDiagram (Gao *et al.* 2021) v0.1.9 within the R environment.

Statistical analysis of functional data

The summarized annotation dataset was processed by converting UniProtKB entry IDs to Gene Ontology (GO) IDs using the UniProt online Retrieve/ID mapping tool. The resulting dataset was filtered to remove UniProtKB entries without corresponding GO IDs. MaAsLin2 v1.8.0 (Mallick *et al.* 2021) was used to determine the association of the microbiome functional profile with the treatment state. The significance threshold was set to $q<0.05$, with a minimum abundance of 50 and a minimum percentage of samples of 25% for each GO term. Patient ID was selected as a random effect, and treatment state was set as a fixed effect in the model. The Benjamini-Hichberg multiple-testing correction method was applied to control the significance of the association.

2.6 Ethics

This study received ethical approval from the Biomedical Ethics Committee of the RECUH Support Foundation (approval No. 13-A/13) on October 3, 2013. Samples used in this thesis were acquired from the GISTAR project sample cohort, which received ethical approval from the International Agency for Research on Cancer (IARC) (approval No. IEC 12-36), the RECUH clinical support fund ethics committee (approval No. 14-A/13), and the Latvian Central Medical Ethics Committee (approval No. 01-29.1/11).

3 RESULTS

3.1 A widely used sampling device in colorectal cancer screening programmes allows for large-scale microbiome studies

Highlights:

- FIT buffer (OC-Sensor) effectively maintains the microbial community structure of faecal material for up to one week.
- Material stored within this container can be used for *16S rRNA* gene and metagenome sequencing analysis.
- Whole metagenome analysis revealed that the FIT buffer effectively maintained the integrity of the results at different levels of analysis.
- Analysis of faecal material collected within the scope of colorectal screening projects can provide valuable insight into the composition and function of the gut microbiome, which could have far-reaching implications for medical research.

Author contribution:

- Development of DNA extraction methodology from samples stored in FIT sample containers.
- Sample preparation for the Ion Torrent PGM sequencing analysis.
- Creation of *16S rRNA* sequencing and statistical data analysis pipelines.
- Interpretation of acquired data, preparation, and revision of the manuscript.

A widely used sampling device in colorectal cancer screening programmes allows for large-scale microbiome studies

We read with interest the article by Passamonti *et al*,[1] reporting the performance of two different faecal immunochemical tests (FITs) highlighting the importance of standardisation and validation of screening methodologies. Conventionally, laboratory-based FIT is the preferred approach in testing for occult blood in faeces, which includes colorectal cancer screening programmes.[2–4] The potential of preserving stable faecal samples in a widely used FIT buffer for microbiome research would enable prospective microbiome studies in generally healthy subjects undergoing colorectal cancer screening.

For this purpose, we evaluated faecal sample stability in the commonly used OC-Sensor (Eiken Chemical, Tokyo, Japan) under various storage conditions. Faecal samples from five healthy adult individuals were used for the analysis and exposed to 16 different conditions: immediately frozen at −86°C (with FIT (wFIT)/without FIT (woFIT)); immediately frozen at −20°C (wFIT/woFIT); wFIT stored at 4°C for 1, 2, 7, 14 days; stored at 20°C for 1, 2, 7, 14 days; stored at 30°C for 1, 2, 7 days and lastly stored at 4°C for 2 days and at 20°C for additional 2 days. Shotgun metagenomic analysis was performed by Illumina 2500, while taxonomic compositions were determined by 16S rRNA analysis employing Illumina MiSeq and Ion Torrent PGM.

Our first attempt to extract DNA from wFIT samples failed due to low amount of DNA obtained. Thus, lyophilisation for all of the wFIT samples, except for woFIT samples, was applied, increasing the yield of DNA up to 30 times.

As shown by the figure 1, the Shannon index in both platforms displayed a clear pattern of decreased diversity during prolonged storage. While testing for differences between samples we discovered that woFIT samples, wFIT samples stored at 4°C for 1 and 2 days, wFIT samples stored at 20°C for 1 day from the Illumina MiSeq data significantly differ from wFIT samples stored at 30°C for 7 days (one-way analysis of variance; $p<0.05$).

The similarity matrix using unweighted principal coordinate analysis (PCoA, figure 2A,C) demonstrated that samples collected from each individual clustered together and was consistent in data gained from both sequencing platforms, suggesting that the biological effect outweighed the effect of the sample handling. Further, the weighted PCoA (figure 2B,D) displays that native samples tend to cluster closer while samples stored for prolonged periods tend to be located further away from the native ones.

Comparing observed operational taxonomic units (OTUs) between samples that were stored within various storage conditions, we were unable to identify any specific OTU cluster across all taxonomical levels that would significantly differ (Kruskal-Wallis test) between any storage condition. Nevertheless, we found that the relative abundances of Gram-negative bacteria tended to decrease while the abundances for most of the Gram-positive bacteria tended to increase over time. However, it is possible that even within native samples certain bacterial OTUs could fluctuate

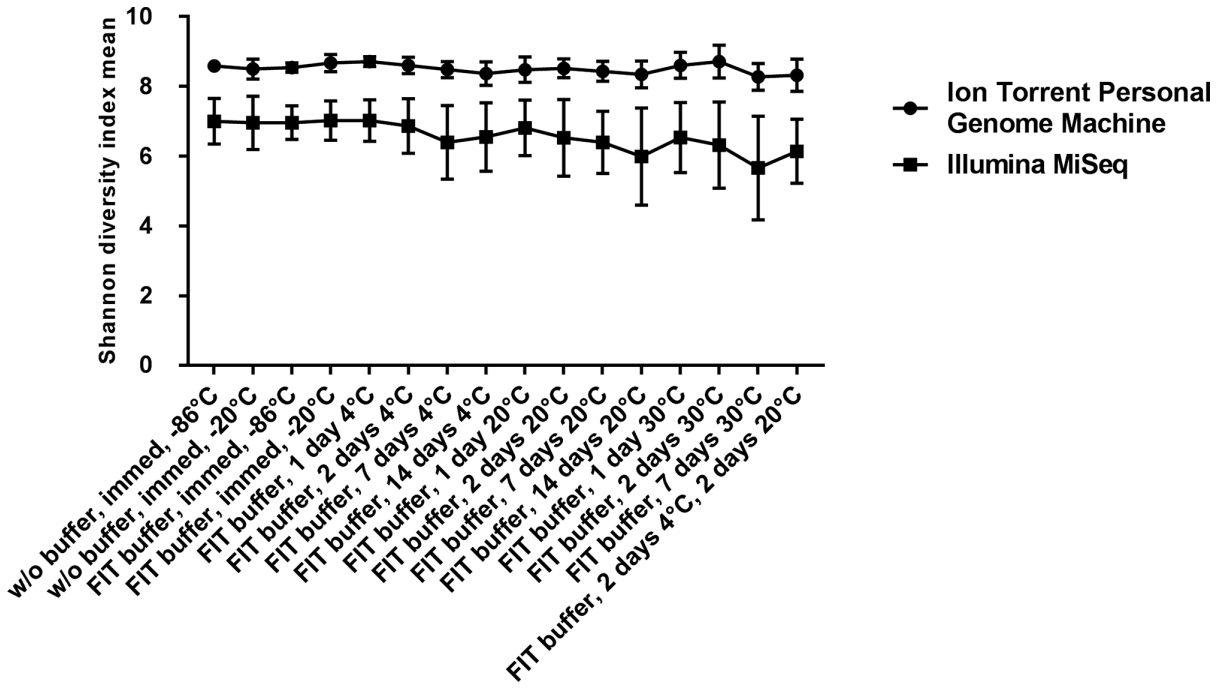

Figure 1 Mean Shannon diversity index values by storage conditions of faecal samples sequenced on Ion Torrent Personal Genome Machine and Illumina MiSeq. Error bars represent SEs. FIT, faecal immunochemical test.

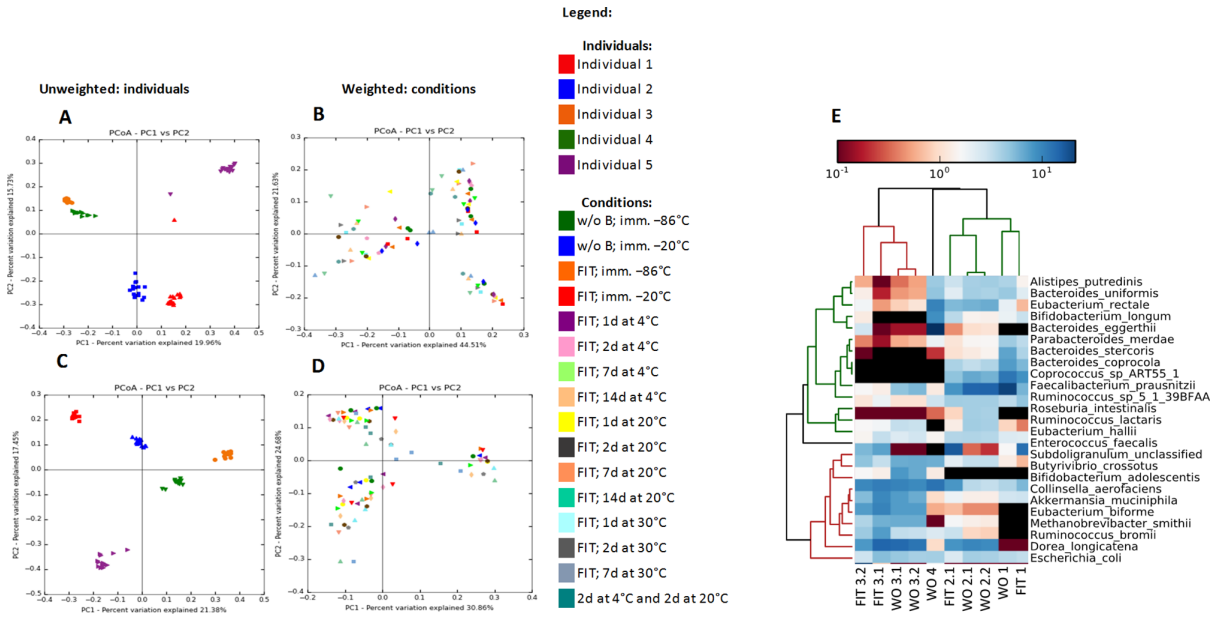

Figure 2 Beta diversity (panels A–D) analysis of GI tract bacterial communities between the individuals and storage conditions presented in a form of principal coordinate analysis (PCoA) plot of weighted and unweighted UniFrac distances. Panels A and C are coloured according to the individual, panels B and D are coloured according to the conditions that samples were exposed to. Panels A and B represent the UniFrac metrics obtained from sequencing on the Illumina MiSeq platform, whereas panels C and D represent UniFrac metrics obtained from sequencing on the Ion Torrent PGM platform. The results of the metagenomics analysis at the species level are presented within panel E in a form of the heat-map profile for the most abundant entities in the metagenome samples. According to upper dendrogram, there is a consistency within the samples composition as the samples from the same individual are clustering together. Samples WO1, WO2.1, WO2.2, WO3.1, WO3.2, WO4 were without faecal immunochemical test (woFIT) and immediately frozen at −86°C; while samples FIT1, FIT2.1, FIT2.2, FIT3.1, FIT3.2 and FIT4 were with FIT (wFIT) and stored at 4°C for 2 days and then at 20°C for additional 2 days. Samples FIT2.2 and FIT4 failed at the sequencing stage and therefore are not presented within the figure. Both sample groups include technical replicates for individuals 2 and 3 (eg, WO2.1. and WO2.2.; WO3.1 and WO3.2.; FIT2.1 and FIT2.2.; FIT3.1. and FIT3.2.).

regardless of the sequencing platform employed.

Shotgun sequencing was performed on 12 samples (figure 2E) from four individuals: six woFIT samples (WO1–4) that were immediately stored at −86°C and six wFIT samples from three individuals (FIT1–4), stored for 2 days at 4°C and for 2 days at 20°C to imitate the typical sample treatment procedure when the material is being transported to a central laboratory by standard mail delivery. In addition to access the reproducibility of acquired data, technical replicates in the form of DNA that was extracted from independently collected samples from individuals 2 and 3 were included within this analysis. However, two wFIT samples (eg, FIT2.2 and FIT4) failed during the sequencing stage and therefore were excluded from further analysis. A similarity matrix was built with relative abundances to calculate the PCoA. Figure 2E illustrates the similarity between species for two types of sampling showing that results are not biased to the sampling procedures. Results indicate that sample similarities tended towards samples from the same subject rather than the storage method.

In this study, we sequenced microbiome samples employing two different sequencing platforms—Ion Torrent PGM that allows to analyse one variable region (V3) and to verify acquired results and also Illumina MiSeq platform that enables the analysis of two variable regions (V3–V4) at a time. Analysing absolute OTU frequencies within the samples, we did not find a significant difference between immediately frozen samples, which coincide with previous studies.[5–8] Although previous study[9] has found lower FIT stability measures, we observed some limitations as they extracted DNA from a small proportion of sample, while in our study design lyophilisation procedure was included allowing to extract DNA from whole volume. However, we did not include technical replicates for 16S rRNA analysis. Another critical aspect in FIT-oriented studies is the variety of available FIT test tubes in the market. Each FIT buffer from various manufacturers might contain slightly different ingredients and concentrations,[10] which seems to be a trade secret and might alter the microbial composition. In our study, employed OC-Sensor is a reliable and convenient sampling device that can be used in large-scale microbiome studies on a population level even outside colorectal cancer screening programmes.

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Contributors Study concept and design was created by ML and LE. Data collection was performed by DG, HW, IR-S, RH, IK, IE, GS. Bioinformatics analysis performed by DG, SS, IS, RP. DG and SS wrote the manuscript. Funding was obtained by LE, DF, JK and ML. All authors approved the final version for publication.

Funding The work was supported by the ERDF project 'Long-term effects of H. pylori eradication on gastrointestinal tract microbiome and development of screening system for detection of extended-spectrum beta-lactamase coding genes within faeces samples' (project no: 1.1.1.1/16/A/272) and in part by the Swedish Research Council (project no: K2013-56X-10848) and the Söderbergs Foundation.

Competing interests None declared.

Patient consent Obtained.

Ethics approval Biomedical Ethics Committee of the Riga East University Hospital Support Foundation, approval no: 13-A/13.

Provenance and peer review Not commissioned; externally peer reviewed.

OPEN ACCESS

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DG and SS contributed equally.

To cite Gudra D, Shoaie S, Fridmanis D, *et al*. *Gut* 2019;**68**:1723–1725.

Received 9 February 2018
Revised 8 August 2018
Accepted 26 August 2018
Published Online First 21 September 2018

Gut 2019;**68**:1723–1725. doi:10.1136/gutjnl-2018-316225

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3.2 Lack of significant differences between gastrointestinal tract microbial population structure of *Helicobacter pylori*-infected subjects before and two years after single eradication event

Highlights:

- The most significant determinants of the overall composition of the GIT microbiome before and after the eradication therapy were individuals and their specific characteristics rather than the treatment itself.
- Microbial alpha and beta diversities on genus level did not differ significantly between *H. pylori* pre- and post-eradication states.
- The relative abundance of nine genera showed significant differences between the pre- and post-eradication states, suggesting minor differences between the treatment states when considering the long-term impact.

Author contribution:

- Sample preparation for the Ion Torrent PGM sequencing analysis.
- Ion Torrent PGM sequencing.
- Creation of *16S rRNA* sequencing and statistical data analysis pipelines.
- Interpretation of acquired data, preparation, and revision of the manuscript.

Received: 21 February 2020 | Revised: 13 July 2020 | Accepted: 14 July 2020

DOI: 10.1111/hel.12748

ORIGINAL ARTICLE

Helicobacter WILEY

Lack of significant differences between gastrointestinal tract microbial population structure of *Helicobacter pylori*-infected subjects before and 2 years after a single eradication event

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Funding information
European Regional Development Fund, Grant/Award Number: ERAF 1.1.1.1/16/A/272

Abstract

Background : According to recent estimates 80% of Latvian population is infected with *Helicobacter pylori* thus their susceptibility to numerous gastric tract diseases is increased. The 1st line *H. pylori* eradication therapy includes treatment with clarithromycin in combination with amoxicillin or metronidazole and a proton pump inhibitor. However, potential adverse events caused by such therapies to microbiome are insufficiently studied.

Objective: This study aimed to evaluate the long-term effect of *H. pylori* eradication on human gastrointestinal tract (GIT) microbiome.

Methods: The assessment of *H pylori* eradication impact on GIT microbiome was done by analyzing 120 samples acquired from 60 subjects. Each individual was prescribed the following 10-day eradication regimen: *Esomeprazolum* 40 mg, *Clarithromycinum* 500 mg, and *Amoxicillinum* 1000 mg, BID. Samples from each individual were collected before starting *H pylori* eradication therapy, and 2 years after the completion of the therapy in OC-Sensor (Eiken Chemical Co.) sample collection containers and stored at −86°C. Prior to DNA extraction, the samples were lyophilized, and total DNA was extracted using FastDNA Spin Kit for Soil. 16S V3 rRNA gene sequencing was done employing Ion Torrent PGM, and the obtained raw sequences were analyzed using vsearch and R (phyloseq, cluster packages).

Results: Alpha diversity measurements—observed OTUs, Chao1 and Shannon index did not differ significantly between the pre- and post-eradication states (two-tailed paired *t* test: $P = .95$; $P = .71$, $P = .24$, respectively). Unweighted and weighted UniFrac distances of beta diversity analysis indicated a non-specific pattern of sample clustering. Enterotype shift was observed for the majority of individuals comparing pre- and post-eradication study groups. Association analysis revealed that certain bacterial genera significantly correlated with age (eg, *Dialister*, *Paraprevotella*, *Bifidobacterium*), individual (eg, *Thermotunica*, *Streptomyces*, *Faecalibacterium*), and history of respiratory and/or allergic diseases (eg, *Colinsella*, *Faecalibacterium*). Redundancy analysis

Gudra and Pupola contributed equally.

confirmed that the individual was a significant determinant of the subject's microbial community composition (ANOVA, 999 perm., $P = .001$) with the further lower impact of subject-specific medical history (eg, medication used as prescribed: $P = .005$, history of cardiovascular diseases: $P = .005$, history of respiratory, and/or allergic diseases: $P = .015$) and physiological (eg, age: $P = .005$, gender: $P = .02$) parameters. In the post-eradication study group, number of influential genera (n = 260) was increased compared to the pre-eradication study group (n = 209).

Conclusion: Modest global differences at the community level exist between individuals before and after the eradication therapy; however, the microbiome structure is more related to the subject-specific parameters rather than by the eradication therapy itself.

KEYWORDS

16S sequencing, *Helicobacter pylori*, microbiome, OC-Sensor

1 | INTRODUCTION

Helicobacter pylori, a Gram-negative spiral-shaped microaerophilic bacterium found in the stomach, has proven to promote a variety of gastrointestinal (GIT) diseases, such as peptic ulcer disease[1] and gastric cancer.[2] The prevalence of *H pylori* ranges from 30% to 50% in developed countries and above 70% in developing countries.[3] Although regionally specific, several repetitive studies globally have shown that eradication rates decline while the prevalence of antibiotic resistance increases.[4-10]

Latvian population is characterized by low resistance to clarithromycin and by a high number of *H pylori*-infected individuals, which allows the use of clarithromycin-based triple therapy as a first-line treatment.[11] The estimated level of *H pylori* infections reaches 80% in a generally healthy population,[12] and the extensive level of infection has encouraged physicians and researchers to discuss and develop a strategy for treatment and management of *H pylori* infections. Recent published recommendations have suggested to employ the "screen-and-treat" strategy for *H pylori* infection in healthy asymptomatic adults from high-risk areas,[4,13] but they are accompanied by concern that such actions might lead to high antibiotic consumption in the general population and subsequent increased antibiotic resistance of microorganisms other than *H pylori*.[14,15] As endorsed by the Maastricht V/Florence Consensus report,[4] standard *H pylori* eradication therapy in geographical areas with low rates of resistance to clarithromycin (<15%) consists of a combination of a proton-pump inhibitor with clarithromycin, amoxicillin, or metronidazole for 10-14 days.

Antibiotic compounds have been found to alter the taxonomic, genomic, and functional capacity of the human gut microbiome with transient or long-lasting effect.[16] In the case of *H pylori*, several studies have been conducted to evaluate the potential perturbations caused by triple eradication therapy to the microbiome. However, since the prevalence of various *H pylori* strains,[3,17,18] resistance,[19] and global composition of the microbiome[20,21] is region-specific, there is still insufficient knowledge on the eradication therapy impact to the microbiome. Current evidence indicates that *H pylori* eradication therapy is associated with significant disturbances of the intestinal microbiota—a decrease in bacterial diversity, as well as alterations in the abundance of *Bifidobacteria*, *Enterococci*, *Lactobacilli*, *Actinobacteria*, butyrate producers, and detrimental bacteria, have been shown.[22-26] Additionally, one study reported that perturbations of the gut microbiota in some cases remain for up to 4 years after the treatment is completed.[27]

While the emphasis for majority of *H pylori* eradication—gut microbiome studies is on short-term impact of antimicrobial therapy, the potential adverse effects to microbiome in a long-term period are insufficiently studied. Therefore, this study aimed to investigate the impact of the first-line *H pylori* eradication therapy on the human gut microbiome by using 16S rRNA gene sequencing to determine whether antibiotic treatment resulted in a long-term shift of the gut microbial community profile.

2 | METHODS

2.1 | Study subjects, sample collection, and storage

Sixty generally healthy individuals aged 40-64 at inclusion were recruited following the general study protocol of the GISTAR pilot study.[13,28] Patients with alarm symptoms, with gastric cancer or gastric surgery in the past, were excluded. In accordance with the protocol of the GISTAR pilot study,[28] the initial testing for *H pylori* included serology (Biohit Plc.) was followed by histology during upper endoscopy (in a subgroup). The follow-up testing was performed at the time of repeated fecal microbiota test, that is, 2 years following the recruitment. ^{13}C-urea breath test (UBT) was used to control the effectiveness of eradication. More details on the testing within the GISTAR pilot are provided in Park et al.[28] A detailed questionnaire

data from each study participant were collected. It included information on previous medical history (eg, GIT diseases, viral infections, autoimmune diseases, cancer) and lifestyle, in addition, measurements, for example, for body mass index were performed.

From each recruited individual, fecal sample was acquired before starting the standard first-line *H pylori* eradication therapy and 2 years after (in average 728.36 ± 43.62 days, range: min. 651, max. 842 days). Each subject was prescribed the following eradication therapy for 10 days: *Esomeprazolum* 40 mg, *Clarithromycinum* 500 mg, and *Amoxicillinum* 1000 mg, all BID. All procedures were carried out according to the institutional ethical standards, and written consent was obtained from individuals before study participation. Samples were delinked and anonymized.

Study subjects were asked to use the OC-Sensor sample collection container (Eiken Chemical Co) for placing the fecal material collection immediately after the defecation and according to the instructions of the manufacturer. The samples were then brought to the study center as soon as possible, desirably on the same or the following day. All received material was frozen immediately for storage at −80°C. Sample handling and storage were carried out in concordance with previously validated storage conditions.[29]

2.2 | Sample withdrawal from the sampling bottles and DNA extraction

Since fecal samples were partially dispersed in the OC-Sensor sample collection container (originally designed for fecal immunochemical occult blood testing) buffer and contained solid particles, the collection of both: the liquid and solid fractions, was performed. To achieve this, samples were thoroughly mixed and extracted using a single-use syringe with the needle size of 0.8 × 120 mm and transferred to pre-labeled 5-mL tubes. The excess of the OC-Sensor tube solvent from the samples was removed by overnight lyophilization (around 15 hours) in a Christ Alpha 1-2 LD Freeze Dryer (SciQuip Ltd.). DNA from the dry remnant of OC-Sensor samples was isolated using FastDNA SPIN Kit for Soil (MP Biomedicals) according to manufacturer's guidelines.

2.3 | Polymerase chain reaction (PCR)

The PCR amplification of 16S rRNA V3 region was performed employing Probio_Uni_R (5′-ATTACCGCGGCTGCT-3′) and Probio_Uni_F (5′-CCTACGGGRSGCAGCAG-3′) primers, which as previously described by Ref.30 are specific to the domain bacteria. Both primers were tagged with 10-11 bp unique barcode labels along with the adapter sequence to allow multiple samples to be included in a single sequencing run.

PCR amplification was carried out using Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific) and GeneAmp® PCR System 9700 (Thermo Fisher Scientific). The reaction mixture was prepared according to manufacturer's recommendations, and the thermal conditions were set as follows: 98°C for 30 seconds, 35 cycles of 98°C for 10 seconds, 67°C for 15 seconds, and 72°C for 15 seconds with a final extension at 72°C for 7 minutes. The success of the reaction was then assessed by 1.2% agarose gel electrophoresis, and acquired libraries were purified using NucleoMag® NGS Clean-Up and Size Select kit (Macherey-Nagel). The quality and quantity of amplicons was assessed using Agilent High Sensitivity DNA kit and Agilent 2100 BioAnalyzer (Agilent Technologies).

2.4 | 16S rRNA gene sequencing

Prior to clonal amplification, each library was diluted to 12 pmol/L and pooled to up to 12 libraries per sequencing run. The Ion PGM™ Hi-Q™ View OT2 kit (Life Technologies) and Ion OneTouch DL instrument (Life Technologies) were used for template generation. The template-positive ISPs were enriched using Dynabeads MyOne™ Streptavidin C1 beads (Life Technologies) and Ion OneTouch ES module. ISP enrichment was confirmed using the Qubit 2.0 fluorometer (Life Technologies). The sequencing was performed on Ion 318 v2 chip and Ion Torrent PGM machine employing the Ion PGM™ Hi-Q™ View Sequencing kit (Life Technologies). All procedures were carried out according to manufacturer's instructions, and each run was expected to produce approximately 150 000 reads per sample. Following the sequencing procedure, the individual reads were filtered by the PGM software to remove low-quality reads. Sequences matching the PGM 3′ adaptor were automatically trimmed. All PGM quality-approved, trimmed, and filtered data were exported as fastq files.

2.5 | 16S rRNA gene sequencing data analysis

Sequencing data analysis was performed using vsearch v.2.10.4 to quality-filter and cluster 16S rRNA sequences. Raw fastq files were quality filtered with the total expected error 0.75 for all bases in the read. Dereplication was performed at two levels—first, for each sample and then across all samples. Sample sequences were pre-clustered at 97% identity, and de novo chimeric reads were removed. Then, reference chimera detection was performed against Silva gold bacteria reference database, and all dereplicated, non-chimeric, and non-singleton sequences were retained and used to build operational taxonomic units (OTUs) at 97% identity. Taxonomic assignment to the lowest possible rank was performed by SINTAX[31] algorithm using RDP[32] training set v16.

2.6 | Statistical analysis

Statistical analysis was conducted on filtered feature abundance matrices using R v.3.5.2. Clustering of the samples into enterotypes was performed using the cluster[33] and clusterSim[34] packages according to the instructions available on https://enterotype.embl.de. Samples

were clustered based on relative genus abundances using Jensen Shannon distance and the partitioning around medoids clustering algorithm. The Calinski-Harabasz index determined the optimal number of clusters. Enterotypes were visualized by principal coordinates analysis (PCoA) using the ggplot2[35] package.

Species richness (Observed, Chao1), alpha diversity (Shannon), and beta diversity measures (weighted and unweighted UniFrac metrics) were calculated using the phyloseq[36] package. To assess the significance of alpha diversity measurements between treatment groups, two-tailed paired *t* test was performed using the vegan package. ANOVA-like pairwise comparison permutation test was conducted to assess the significance of each treatment group within each of the UniFrac metric using the vegan package.

For correlation map using Spearman rank correlation at the genus level, feature abundance matrices were filtered to retain samples that contain at least 200 counts per taxonomic assignment. Following, log-relative transformation to the sample counts was applied, and the top 50 taxa were depicted to be displayed at the correlation map. Spearman correlation method was used to measure the ordinal association between treatment states and explanatory variables.

In order to determine significant taxonomic entities across treatment states, differential expression analysis based on the negative binomial distribution was performed by package DESeq2[37] implemented in R. Taxonomical counts were normalized by using log-relative transformation, and significantly differentially abundant taxa (eg, $P < .05$) were visualized using ggplot2 package.

Redundancy analysis (RDA) was used to extract and summarize the variation in a dataset that can be explained by the explanatory variables. Each variable was tested by ANOVA analysis, and further reduction in the number of explanatory variables entering the analysis was performed by Monte Carlo permutation test based on Akaike information criterion (AIC) and *P*-values for the comparison of the variable.

3 | RESULTS

Altogether, 60 study subjects (Table 1, Table S1) were included in the study and provided the initial and the follow-up samples. All the study subjects were defined as *H pylori* infected at the time of enrollment, 52 were *H pylori* negative, but eight remained *H pylori* positive at the follow-up investigation. The study subjects reported alcohol consumption not exceeding 2-3 times per week, and none of the study subjects had reported personal or family history of polyps at young age, type 2 diabetes, inflammatory bowel disease, celiac disease, biliary cirrhosis, thyroid disease, hepatitis B or severe psychiatric disease, that is, other diseases with potential impact on gut microbiome. All the group characteristics provided in Table S1 were included as explanatory variables for further statistical analyses.

Analysis of the stool microbiome was performed by NGS of the 16S V3 rRNA gene as described in Materials and Methods section. Following the sequencing, acquired data were subjected to quality filtering, which resulted in collection of more than 16.8 million reads, corresponding to a mean of 140 271 reads and 3423 OTUs per sample under the similarity threshold of 0.97.

TABLE 1 Characteristics of the study subjects

Physiological parameters	Value (men/women)
Gender (n, %)	25 (41.7%)/35 (58.3%)
Average age	54.5 ± 5.7/50.4 ± 5.7
Mean body mass index	28.1 ± 4.6/27.8 ± 4.2
Positive *H pylori* status as identified by ^{13}C-Urea breath test before eradication	25 (41.7%)/35 (58.3%)
H pylori status as identified by ^{13}C-Urea breath test after eradication	Positive: 2 (3.3%)/6 (10.0%) Negative: 23 (38.3%)/29 (48.3%)
Medication used as prescribed	100%: 20 (33.3%)/31 (51.7%) 90%-100%: 0/1 (1.7%) <90%: 5 (8.3%)/3 (5.0%)
Medical history	**Diagnosed**
Asthma	0/2 (3.3%)
Liver biliary disease	4 (6.7%)/10 (16.7%)
Duodenitis	2 (3.3%)/4 (6.7%)
Tuberculosis	3 (5.0%)/1 (1.7%)
Hepatitis A	3 (5.0%)/4 (6.7%)
Have experienced cardiovascular diseases	7 (11.7%)/6 (10.0%)
Have experienced respiratory tract or allergic diseases	1 (1.7%)/10 (16.7%)
Side effects of triple antibiotic therapy	9 (15.0%)/18 (30.0%)

3.1 | Microbiome structure analysis

To ensure a good estimation of bacterial diversity, we measured the proportion of total bacterial species represented in samples of each treatment group by the abundance-based coverage estimator (ACE). Estimated ACE coverage ranged from 1875.17 to 5712.12 in all pre-eradication samples (hereinafter referred to as *All-pre-erad)* and from 1553.41 to 6434.82 in all post-eradication samples (hereinafter referred to as *All-post-erad)*, suggesting that the 16S rRNA results from each library represent the majority of bacteria present in the gut.

The subject-specific profile of the microbial community at the genus level shown in Figure 1A indicates that microbial profiles of all analyzed samples are comparable with each other, which is further supported by the averaged taxonomic profile (Figure 1B) across all treatment states. In total, the most prevalent genera in *H pylori* -positive pre-eradication study group, excluding the *H pylori*-positive subject microbiomes in post-eradication state (hereinafter referred to as *S-pre-erad*), was *Oribacterium* (11.8%), *Bacteroides* (10.2%), *Prevotella* (5.7%), *Parasutterella* (5.3%), *Oscillibacter* (5.1%),

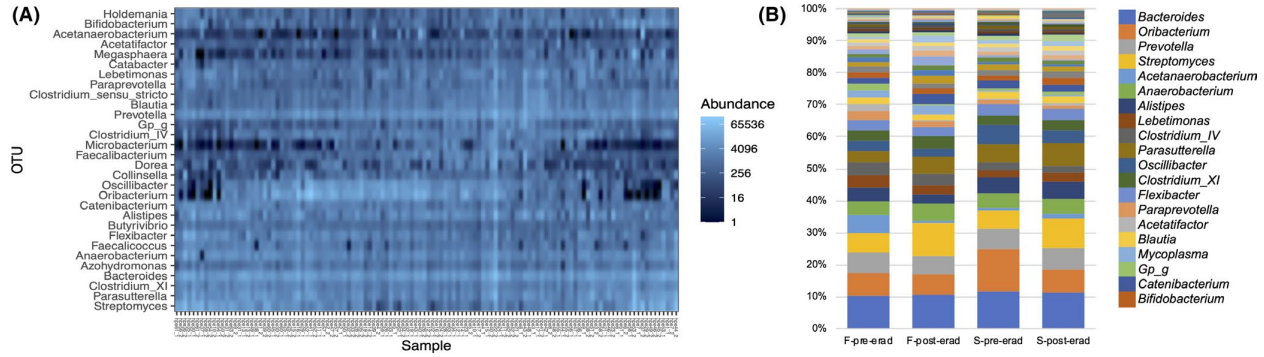
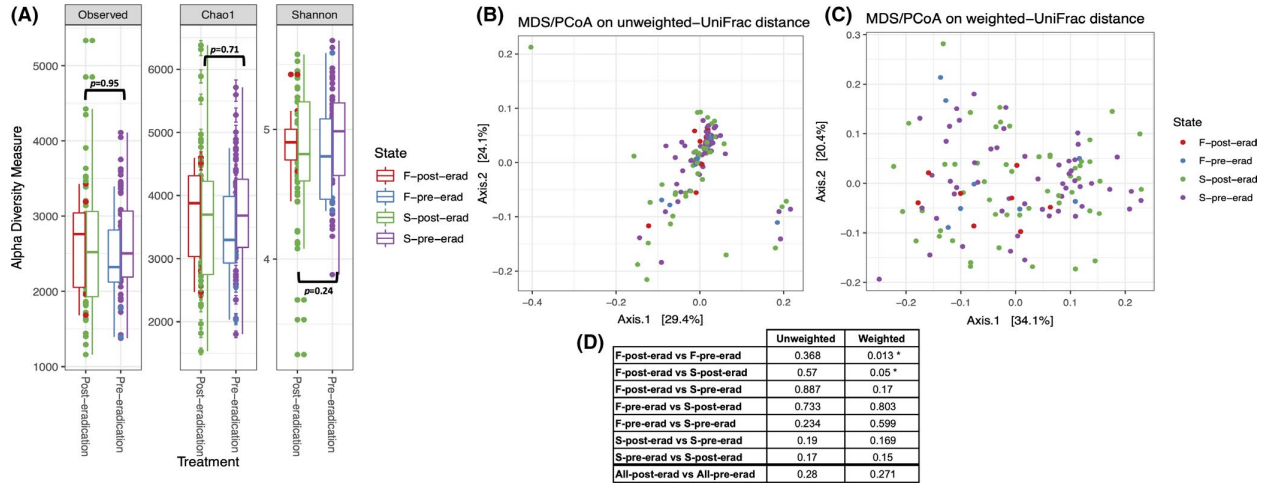


FIGURE 1 Panel A: Top 30 most abundant genera were depicted to make a heatmap using NMDS ordination and Bray-Curtis distance method. On the *x*-axis, samples are depicted, whereas on *y*-axis, OTUs or genus-level taxonomic identities are presented. Panel B: relative abundances of bacterial genera by treatment state. Each column represents bacterial community averaged from the fecal samples of all individuals sequenced. F-post-erad—samples of post-eradication state from subjects with ineffective eradication therapy, that is, *H pylori*-positive subjects; F-pre-erad—samples of pre-eradication state from subjects with ineffective eradication therapy; S-post-erad—*H pylori*-negative samples of the post-eradication state; S-pre-erad—*H pylori*-positive samples of pre-eradication state



	Unweighted	Weighted
F-post-erad vs F-pre-erad	0.368	0.013 *
F-post-erad vs S-post-erad	0.57	0.05 *
F-post-erad vs S-pre-erad	0.887	0.17
F-pre-erad vs S-post-erad	0.733	0.803
F-pre-erad vs S-pre-erad	0.234	0.599
S-post-erad vs S-pre-erad	0.19	0.169
S-pre-erad vs S-post-erad	0.17	0.15
All-post-erad vs All-pre-erad	0.28	0.271

FIGURE 2 Estimated alpha and beta diversity. Panel A represents averaged alpha diversity measurements by the global treatment state and colored by the sub-divided treatment states. Panels B and C represent beta diversity analysis of GIT bacterial communities between the treatment states in a form of principal coordinate analysis (PCoA) plot of weighted (Panel B) and unweighted (Panel C) UniFrac distances. Panel D represents results of ANOVA-like pairwise comparison permutation test to assess the significance of each treatment group. Value marked with an asterisk is considered as significant ($P < .05$). F-post-erad—samples of post-eradication state from subjects with ineffective eradication therapy, that is, *H pylori*-positive subjects; F-pre-erad—samples of pre-eradication state from subjects with ineffective eradication therapy; S-post-erad—*H pylori*-negative samples of the post-eradication state; S-pre-erad—*H pylori*-positive samples of pre-eradication state

and *Streptomyces* (4.8%), whereas in the *H pylori*-negative post-eradication group (hereinafter referred to as *S-post-erad*) *Bacteroides* (9.9%), *Streptomyces* (8.3%), *Oribacterium* (6.6%), *Parasutterella* (6.5%), *Prevotella* (6.0%), and *Alistipes* (4.9%). The most prevalent genera of ineffective *H pylori* eradication therapy in the pre-eradication state (hereinafter referred to as *F-pre-erad*) was *Bacteroides* (9.2%), *Oribacterium* (6.4%), *Prevotella* (5.9%), *Streptomyces* (5.4%), and *Acetanaerobacterium* (4.9%), in the post-eradication state (hereinafter referred to as *F-post-erad*)—*Bacteroides* (9.4%), *Streptomyces* (8.9%), *Oribacterium* (5.6%), *Prevotella* (5.2%), and *Anaerobacterium* (4.7%). The relative abundances of taxonomy entities at the genus level in pre-eradication and post-eradication study groups did not differ significantly (two-tailed paired *t* test, $P > .05$).

To further evaluate alterations in the microbiome structure between all samples of pre- and post-eradication states, we measured microbial alpha diversity (ie, within-sample diversity) and beta diversity (ie, diversity between samples).

For the alpha diversity estimation, we used the richness estimator—observed OTUs, the Shannon diversity index, and abundance estimator Chao1 index of individual microbiomes (Figure 2A). Observed OTUs ranged from 1378 to 4111 in All-pre-erad group and from 1160 to 5335 in All-post-erad group (two-tailed paired *t*

test, $P = .95$), Chao1 index ranged from 1802.81 to 5713.57 in All-pre-erad group and from 1531.61 to 6380.28 in All-post-erad group (two-tailed paired t test, $P = .71$). The mean value of the Shannon index of the All-post-erad group compared to the mean values of the Shannon index of All-pre-erad group samples was consistent (4.82 ± 0.49 vs 4.90 ± 0.42, respectively; two-tailed paired t test, $P = .24$).

In order to compare the global composition of the microbiome in the fecal samples, we calculated UniFrac distances. Both qualitative (unweighted UniFrac; Figure 2C) and quantitative (weighted UniFrac; Figure 2B) analyses indicated a non-specific pattern of sample clustering suggesting mutual similarity of the samples at the genus-level diversity and the incidence of microbial taxonomic groups regardless of the pre- or post-eradication states (permutation test for homogeneity, $P > .05$). Further on analyzing the four groups—F-pre-erad F-post-erad, S-pre-erad, and S-post-erad group, permutation test for homogeneity of multivariate dispersions (Figure 2D) revealed that weighted UniFrac distances significantly differed between F-pre-erad and F-post-erad state ($P = .013$), and between S-post-erad and F-post-erad state ($P = .05$). To further evaluate the global composition of the microbiome, we conducted redundancy analysis (RDA) to extract and summarize the variation in a dataset that can be explained by the explanatory variables (Table 1). By testing each variable, ANOVA analysis identified only "Individual" (999 permutations, $P = .001$) as a significant determinant of the subject's microbial community composition. Further on, we reduced the number of explanatory variables entering the analysis to optimize the variation explained by them. Using forward selection, individual ($P = .005$), age ($P = .005$), a medication used as prescribed ($P = .005$), history of cardiovascular diseases (n = 13, $P = .005$), respiratory and/or allergic diseases (n = 11, $P = .015$), gender ($P = .02$), tuberculosis (n = 4, $P = .03$), and wine (n = 28, $P = .035$) were considered as significant determinants in shaping microbial community composition. Results were confirmed by testing the parsimonious RDA model with the global model (999 permutations, $P = .001$).

3.2 | Influence of eradication therapy on the gut microbiome

Enterotype clustering analysis (Figure 3) revealed three dominant clusters for both global groups—All-pre-erad and All-post-erad. Enterotype cluster No. 1 was dominated by the following genera: *Prevotella*, *Clostridium sensus stricto*, *Gemmiger*, and *Slackia*; enterotype cluster No. 2 was dominated by *Acetothermia genus incertae sedis*, genus *Ercella*, and *Adhaeribacter*, while enterotype cluster No. 3 was dominated by the following genera: *Ruminococcus*, *Romboutsia*,

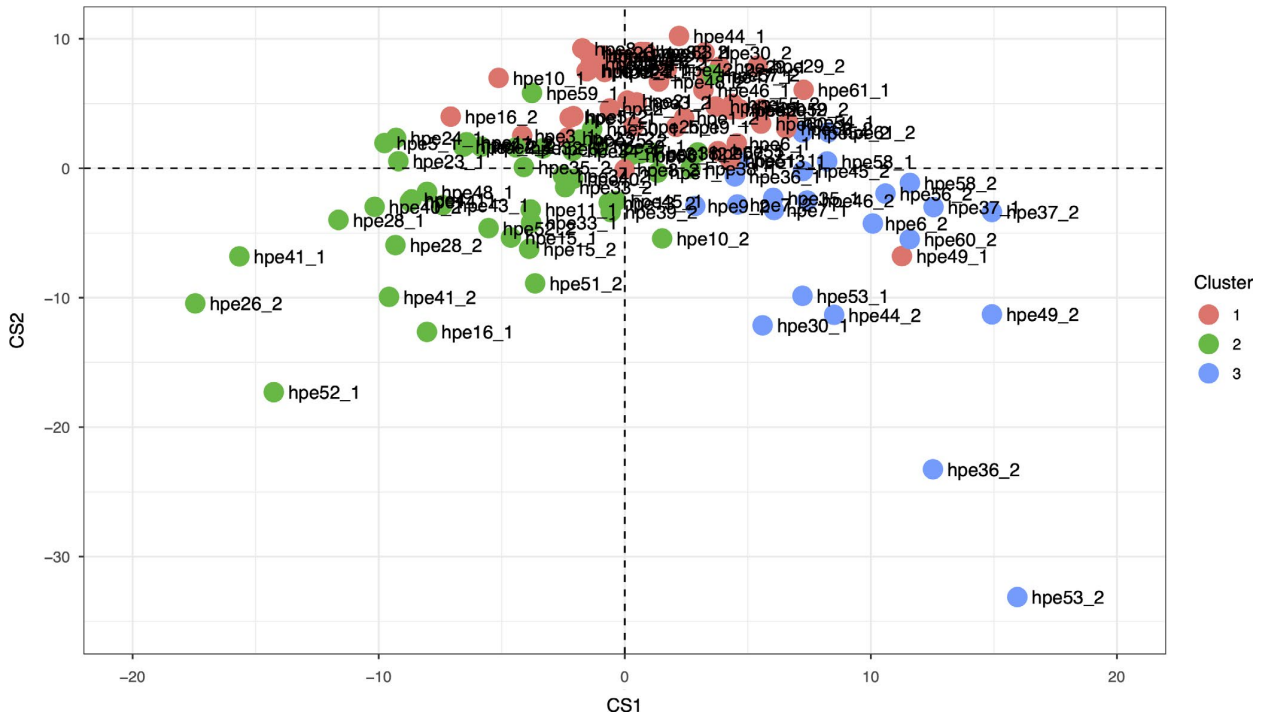


FIGURE 3 Between-class analysis, presenting enterotype clustering of the genus-level taxonomic composition in a form of principal coordinate analysis. Dominate genera of enterotype No.1: *Prevotella*, *Clostridium* sensu *stricto*, *Gemmiger*, *Slackia*, *Sinomicrobium*, *Porphyromonas*, *Photorhabdus* and *Garciella*; enterotype No.2: *Acetothermia genera incertae sedis*, *Ercella*, *Adhaeribacter*, *Streptacidiphilus*, *Hydrogenophaga*, *Ethanoligenens*, *Rhizobacter* and *Aeromonas*; and of enterotype No.3: *Ruminococcus*, *Romboutsia*, *Saccharofermentans*, *Roseburia*, *Peptococcus*, *Peptostreptococcus*, *Oribacterium*, and *Pseudobutyrivibrio*

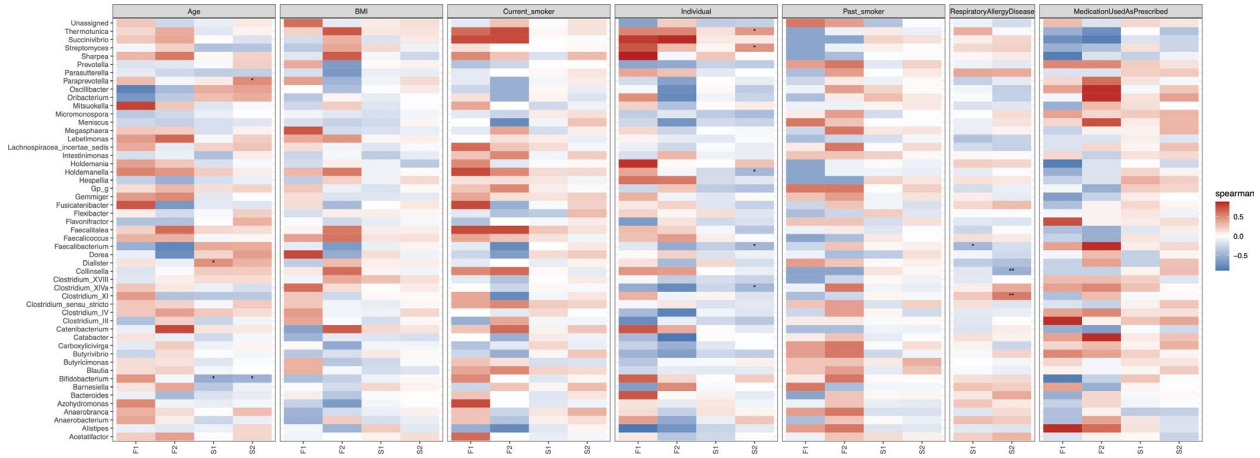


FIGURE 4 Correlation between genus-level phylogenetic groups and treatment state using the Spearman rank correlation coefficient. In the correlation map on the horizontal axis, treatment state is depicted, on vertical axis taxonomies are presented. Significance levels: "*" $P < .05$, "**" $P < .01$. F1—samples of pre-eradication state from subjects with ineffective eradication therapy; F2—samples of post-eradication state from subjects with ineffective eradication therapy; S1—*H pylori*-positive samples of the pre-eradication state; S2—*H pylori*-negative samples of post-eradication state

Saccharofermentans, and *Roseburia*. All three enterotypes were mainly dominated by phylum Firmicutes. We discovered enterotype shifting for 32 individuals comparing All-pre-erad and All-post-erad study groups, but no specific tendency was observed. Shifts between enterotypes were observed among all clusters: For 19 individuals, shift was observed between cluster No. 1 and No. 2; for four individuals, shift was observed between cluster No. 2 and No. 3; and for nine individuals, enterotype shift was observed between cluster No. 1 and No. 3.

Seven factors, individual, age, BMI, smoking status (current or past smoker), history of respiratory tract and/or allergic diseases, and survey information if the medication of the eradication therapy was used as prescribed, were included in a Spearman rank correlation analysis (Figure 4), to determine the association between various parameters and identified microbial taxonomic units. We found a statistically significant positive correlation between S-pre-erad and S-post-erad study groups in relation to age (genera *Dialister*, $P < .05$; *Paraprevotella*, $P < .05$), individual (genera *Thermotunica*, $P < .01$; *Streptomyces*, $P < .05$), experienced respiratory tract, and/or allergic diseases (genera *Clostridium XI*, $P < .01$). On the contrary, statistically significant negative correlation was found between the S-pre-erad and S-post-erad study groups in relation to age (genus *Bifidobacterium*, $P < .05$), individual (genera *Holdemanella*, $P < .05$; *Faecalibacterium*, $P < .05$; and *Clostridium XIVa*, $P < .05$), and history of respiratory and/or allergic diseases (genera *Faecalibacterium*, $P < .05$; *Collinsella*, $P < .01$). Graphical illustration of correlation analysis for all variables defined in Table S1 is included in Figure S1. Correlation between All-pre-erad and All-post-erad study groups (Figure S1) resulted in a slightly higher number of taxonomic entities that showed association with such parameters as if study participants followed the instruction of the prescribed eradication therapy (genus *Thermotunica*, $P < .05$ and genus *Oribacterium*, $P < .05$) and history of respiratory tract and/or allergic diseases (genera *Parasutterella*, $P < .05$; *Lebetimonas*, $P < .05$ and *Dialister*, $P < .05$). Hence, it seems that individuals with experienced non-effective *H pylori* eradication therapy might affect the composition and proportion of global relative abundances within the full dataset; however, due to the limited number of subjects (n = 8) within the scope of this research, we are unable to verify this conjecture. All other available information on subject medical history, physical activities, diet, lifestyle habits (eg, smoking, alcohol consumption), environmental factors, side effects of medication, and other did not yield a significant impact on GIT microbiome comparing pre- and post-eradication study groups.

Furthermore, differential abundance testing with DeSeq2 ($P_{adj} < .05$, $|\log_2(\text{fold change})| > 1.5$) (Figure 5) revealed eight genera that were differentially abundant (with $P < .05$) across the four treatment states: *Balneola* ($P = 0$), *Mesoplasma* ($P = 4E\text{-}05$), *Rhodospira* ($P = .00018$), *Geothermobacter* ($P = .00214$), *Finegoldia* ($P = .02085$), *Actinotignum* ($P = .03555$), *Glycocaulis* ($P = .03555$), and *Caminicella* ($P = .03555$). Performing differential analysis across All-pre-erad and All-post-erad states, nine genera were differentially abundant (with $P < .05$; Figure S2): *Lebetimonas* ($P = .02126$), *Paraprevotella* ($P = .02126$), *Lactonifactor* ($P = .02126$), *Armatimonadetes gp* ($P = .0212$), *Opitutus* ($P = .0215$), *Desulfosporomusa* ($P = .02301$), *Thermotunica* ($P = .02434$), *Microbacterium* ($P = .02598$), and *Mitsuokella* ($P = .02598$); however, due to the high *p*-value for individual genera, these results should be considered with caution.

We also calculated the genera of each of the All-pre-erad and All-post-erad treatment groups that contribute to the observed divergence among individual samples. In the All-pre-erad study group, we identified 52 genera and in the All-post-erad study group 60 genera that significantly correlated along the PCoA axes out of 865 genera (correlation cutoff ≥70; 999 permutations: $P < .001$). With slightly weaker significance, 53 genera in the All-pre-erad study group and 74 genera in the All-post-erad study group (correlation cutoff ≥70;

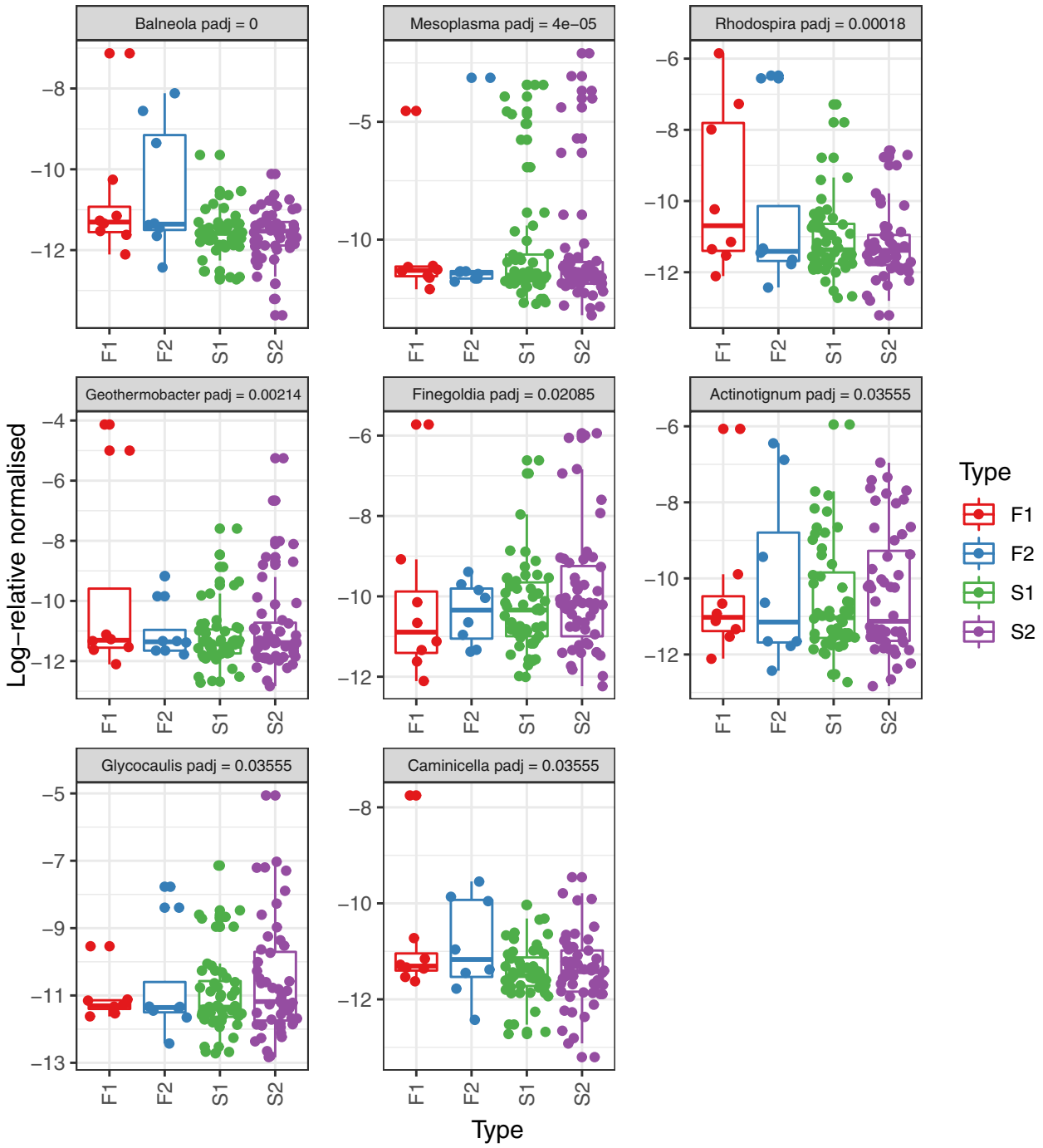


FIGURE 5 Differentially abundant genus-level entities across the treatment states at the significance cutoff of $P < .05$. F1—samples of pre-eradication state from subjects with ineffective eradication therapy; F2—samples of post-eradication state from subjects with ineffective eradication therapy; S1—*H pylori*-positive samples of the pre-eradication state; S2—*H pylori*-negative samples of post-eradication state

999 permutations: $0.002 < P > .009$) significantly correlated along the PCoA axes. Lastly, with slight significance 104 genera in the All-pre-erad study group and 126 in the All-post-erad study group correlated along the PCoA axes (correlation cutoff ≥70; 999 permutations: $0.01 < P > .05$). In total, 209 genera in All-pre-erad and 260 genera in the All-post-erad group are suggested as influential genera (Table S2).

Altogether, these data suggest modest global differences at the community level exist between individuals before and after the eradication therapy when considering the long-term impact.

4 | DISCUSSION

Microbiome imbalance caused by *H pylori* infection is considered as an initiator of such digestive tract diseases as chronic gastritis, peptic ulcers, gastric cancer, and other.[38] As worldwide concerns for *H pylori* infection are increasing, the importance of knowledge on prescribed therapy impact on the microbiome is rising, especially when broad-spectrum antibiotics are used as first-line therapy. Amoxicillin and clarithromycin are among the most commonly used broad-spectrum antibiotics, also used as combined antibacterial therapy in *H pylori* eradication schemes in Latvia.[39] In this study, we evaluated long-term influence of *H pylori* first-line triple eradication therapy on the human gut microbiome, focusing on microbial diversity and taxonomic composition. While results may not be generalizable to national level, this study reveals that differences at the community level exist between *H pylori* pre- and post-eradication states as determined by association analysis, redundancy analysis, and differential expression analysis. This study highlights the need for expanded research comprising different ethnic populations, where each subject shall provide full metadata repository—such as diet, medical history, and exercises, to better explain the microbial entity variation between the study subjects. These observations may have global implications in the use of broad-spectrum antibiotics as they alter the gut microbial population structure, leading to higher possibility of gut dysbiosis and promoted antibacterial resistance.

According to the exclusion/inclusion criteria, 60 *H pylori*-positive participants were recruited in the study from March 2017 until March 2019, where two samples were taken from each participant within a time interval of 2 years. Our study reveals some minor, but statistically insignificant changes in GIT microbiome taxonomic unit abundances. For instance, relative abundances were decreased in the All-post-erad study group for *Bacteroides*, *Oribacterium*, *Prevotella*, but increased for *Parasutterella* and *Streptomyces* when comparing most abundant taxonomical units at the genus level. Also, we identified nine differentially abundant genera, comparing All-pre-erad and All-post-erad sample sets, with a statistically significant difference ($P < .05$). Still, currently there is not enough evidence on the impact of those genera to the gut microbiome. *Bacteroides* and *Prevotella* are one of the most dominant bacterial groups in the healthy human gut microbiome, whose relative abundance scores could easily be changed by diet, while *Oribacterium* is defined as oral pathobiont, also linked to obesity and liver diseases.[40,41] The genus of *Parasutterella* has been defined as a core component of the human and mouse gut microbiota and has been correlated with various health outcomes. The potential role of *Parasutterella* in GIT could be bile acid and cholesterol metabolism maintenance.[42] The other increased genus—*Streptomyces* is previously described as a therapeutic target, suggesting that the members of this genus are producing antiproliferative agents and immunosuppressants that could prevent autoimmunity diseases, allergy, and colon cancer.[43] Altogether, it seems that revealed changes are rather human health-enhancing than damaging.

In our study, no significant changes were found comparing the global relative abundances of taxonomic entities, microbial alpha diversity, and beta diversity at the genus level between All-pre-erad and All-post-erad states. Similar studies indicate decreased bacterial richness, including *Bacteroidetes* and *Bifidobacterium spp*., and significant increase in *Firmicutes*, short-chain fatty acid (SCFA)-producing bacteria, and glucose-removing bacteria. These are reported as the most often gut microbiome modifications after the eradication therapy with both—immediate and in some cases with long-term consequences.[26,43,44] Recent studies showed that after the *H pylori* eradication gastric and intestinal microbiota gradually is restored similar to baseline levels before therapy, although recovery rates vary with regimens. Best results are shown for the first-line triple therapy where Liou et al[45] reported that alpha and beta diversities are restored within 8 weeks, while concomitant and quadruple therapy shows contradictory results indicating that diversity levels might not restore even within a year.[45,46] Few studies have indicated that already after 8 weeks certain metabolic parameters are improved, with a decrease in insulin resistance, triglycerides, LDL, and an increase in HDL and GLP-1 secretion, after the use of standard triple therapy.[23,47] Interestingly, recent report on the use of bismuth quadruple therapy indicates contradictory results, suggesting that *H pylori* eradication restores rather than disturbs the GIT microbiota by restoring the diversity of gastric microbiota with enrichment of beneficial bacteria, while other studies have shown alpha and beta diversity alterations.[45,48]

As it is nowadays widely accepted that the gut microbiome is affected by a wide variety of factors, which includes physiological peculiarities of individual, BMI, gut-related and unrelated diseases, age, diet, and many other. Number of studies suggest that all these factors together mediate the unique composition of individual gut microbiome and our findings strongly support this by identifying "Individual" as major gut microbiome structure affecting factor in both main study groups.[49-51] Other factors such as age, history of respiratory tract and/or allergic diseases, eradication therapy implementation level also showed both positive and negative correlation, supporting suggestions of other researchers.[51] Additionally, observed non-specific enterotype shifts could be explained by the participant individual microbiome, comparing the pre- and post-eradication study groups, where we were unable to identify any specific tendency.

As identified by the UBT, eight persons remained positive for *H pylori* infection after the eradication therapy. For those individuals, alpha diversity measurements were similar to the S-pre-erad and S-post-erad cases indicating that within-sample diversity was similar among all samples analyzed and was not impacted by the eradication therapy during the long-term observation. Evaluating beta diversity metrics, UniFrac distances differed significantly between the F-pre-erad and F-post-erad state, and between F-post-erad and S-post-erad state. Even more this observation is in concordance with the relative abundances of bacterial genera, where when comparing to the S-pre-erad state to F-pre-erad state samples, the later display lower abundances of such genera as

oral pathobiont *Oribacterium*[40] and contentious *Oscillibacter*—described as common gut microbiome habitant in generally healthy individuals[52] and opportunistic pathogen with a potential to cause bacteremia,[53] gut microbiome habitant of nonobese individuals *Allistipes*,[54] and common gut member *Paraprevotella*, when comparing to the S-pre-erad state. On the other hand, the same analysis also shows that samples from F-pre-erad state have higher abundances of such genera as common gut microbiome habitant *Acetanaerobacterium*[55] and thermophilic genus *Lebetimonas*. While there is no statistical justification to this observation, this might advert that there are differences in bacterial population structure between individuals with successful and unsuccessful *H pylori* eradication, indicating that in the case of certain gut microbiome composition standard triple eradication regimen might be ineffective. However, further analysis by deep metagenomic sequencing, which provides functional and taxonomical and functional annotation to the species and strain level, would be required to assess these differences qualitatively.

The impact of *H pylori* eradication therapy on human gut microbiome remains contradictory, especially when a number of therapeutic choices are available. One of the most widely studied *H pylori* eradication therapies within a context of a microbiome is standard triple therapy. Two complementary studies from Spain have reported that bacterial richness, evenness, and diversity were decreased after the *H pylori* eradication by standard triple therapy[23,26] along with improved carbohydrate metabolism during the 2-month observation.[26] Another study from Sweden reported that gut microbiota in some cases was perturbed for up to 4 years after the eradication treatment, with increased level of macrolide resistance gene during the long-term observation. However, this study was limited by a low number of subjects.[27] Comprehensive studies have been conducted in the Asia-Pacific region, where observed alterations of the alpha and beta diversities and increased resistance to a number of antibiotic compounds were restored 2 months after the *H pylori* eradication triple therapy and accompanied by improved levels of metabolic parameters.[47] However, eradication rates by triple therapy continue to decline in many Asian countries.[56,57] Alternatives to triple eradication therapy include bismuth quadruple therapy, concomitant therapy, sequential therapy,[4] and recently introduced vonoprazan therapy.[58,59] While concomitant and bismuth quadruple therapy have showed long-term perturbations of the gut microbiome,[47] vonoprazan therapy supplemented by probiotics has been shown to improve the rate of eradication and potentially aids the maintainment of species diversity within the gut microbiome.[60] Although a number of studies have identified vonoprazan as effective eradication strategy,[59-61] study evaluating the effect of vonoprazan monotherapy on the gut microbiome revealed stimulated lipopolysaccharide biosynthesis thus increasing the risk of enteric infection.[62] In general, adverse events are common in combinatorial therapies, thereof vonoprazan therapy accompanied by probiotics might be promising strategy in *H pylori* eradication, especially in regions with high resistance rates to clarithromycin.

In this study, a control group without *H pylori* infection was not introduced as during the preliminary analysis we did not observe significant differences of the global microbiome structure between pre- and post-eradication states. Thus, for the setup of the study we included 60 paired samples. Although larger group would be able to provide results of greater statistical power, current sample size has shown to be efficient in discriminating potentially insidious statistical results. From the recruited subjects, eight individuals remained *H pylori* positive after the eradication therapy. For those subjects, we can only speculate about the reasons for the lack of eradication therapy efficiency as the sample size is far too small to justify observed conjectures. However, this might be key issue in certain populations and would deserve further investigation in the future. Additionally, the design of this study does not allow addressing the gut resistome that potentially could be affected by the use of antibiotics; to address this issue additional studies are in being carried out.

In summary, our findings partially indicate similarity with other researchers, suggesting that the gut microbiome is restored, considering the long-term impact of *H pylori* eradication. Modest differences in the taxonomic composition exist between pre- and post- eradication study groups; however, the microbiome structure is more related to the subject-specific parameters, such as age or medical history, rather than by the eradication therapy itself.

ACKNOWLEDGEMENTS

The work was supported by the ERDF project No. 1.1.1.1/16/A/272 "Long-term effects of *H pylori* eradication on Gastrointestinal tract microbiome and development of screening system for detection of extended-spectrum beta-lactamase coding genes within feces samples." Additionally, authors would like to thank the GISTAR project for providing biological material.

ETHICAL APPROVAL

The study was approved by the Biomedical Ethics Committee of the Riga East University Hospital Support Foundation, approval No. 13-A/13 from October 3, 2013.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Gudra D, Pupola D, Skenders G, et al. Lack of significant differences between gastrointestinal tract microbial population structure of *Helicobacter pylori*-infected subjects before and 2 years after a single eradication event. *Helicobacter*. 2020;00:e12748. https://doi.org/10.1111/hel.12748

3.3 Abundance and prevalence of ESBL coding genes in patients undergoing first line eradication therapy for *Helicobacter pylori*

Highlights:

- The developed large-scale ESBL screening panel was effective in targeting ESBL coding genes within the GIT microbiome samples. Therefore, this panel can be used for accurate population screening and surveillance of ESBL genes in symptomatic and asymptomatic individuals.
- Whole metagenome sequencing revealed that the abundance and prevalence of ESBL coding genes in the GIT microbiome samples was very low.
- Acquired data indicated the patient-specific distribution profile of ESBL coding genes.
- Relative abundances of ESBL coding gene clusters remained similar between the pre- and post-eradication states. However, there were differences in the relative abundance of some ESBL genes, which tended to decrease comparing the pre- and post-eradication states.
- The applicability of the developed panel is not limited to the detection of ESBL coding genes in the GIT microbiome of *H. pylori*-infected individuals but can also potentially be applied to different samples, populations, and various infection cases.

Author contribution:

- Sample preparation for the Ion Torrent PGM and DNBSEQ-G400 sequencing analyses.
- Ion Torrent PGM sequencing.
- Creation of metagenome sequencing and statistical data analysis pipelines.
- Statistical analysis of ESBL-coding gene sequencing data.
- Interpretation of acquired data, as well as manuscript preparation and revision.

PLOS ONE

RESEARCH ARTICLE

Abundance and prevalence of ESBL coding genes in patients undergoing first line eradication therapy for *Helicobacter pylori*

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Citation: Gudra D, Silamikelis I, Pjalkovskis J, Danenberga I, Pupola D, Skenders G, et al. (2023) Abundance and prevalence of ESBL coding genes in patients undergoing first line eradication therapy for *Helicobacter pylori*. PLoS ONE 18(8): e0289879. https://doi.org/10.1371/journal.pone.0289879

Editor: Iddya Karunasagar, Nitte University, INDIA

Received: December 5, 2022

Accepted: July 28, 2023

Published: August 10, 2023

Data Availability Statement: Raw sequencing data were deposited in the European Nucleotide Archive under study accession No. PRJEB48983. URL: https://www.ebi.ac.uk/ena/browser/view/PRJEB48983?show=related-records All relevant data (docx, xlsx) are within the paper and its Supporting Information files.

Funding: The work was supported by the ERDF project No. 1.1.1.1/16/A/272 "Long term effects of H. pylori eradication on Gastrointestinal tract microbiome and development of screening system

Abstract

The spread of extended-spectrum beta-lactamases (ESBLs) in nosocomial and community-acquired enterobacteria is an important challenge for clinicians due to the limited therapeutic options for infections that are caused by these organisms. Here, we developed a panel of ESBL coding genes, evaluated the abundance and prevalence of ESBL encoding genes in patients undergoing *H. pylori* eradication therapy, and summarized the effects of eradication therapy on functional profiles of the gut microbiome. To assess the repertoire of known beta lactamase (BL) genes, they were divided into clusters according to their evolutionary relation. Primers were designed for amplification of cluster marker regions, and the efficiency of this amplification panel was assessed in 120 fecal samples acquired from 60 patients undergoing *H. pylori* eradication therapy. In addition, fecal samples from an additional 30 patients were used to validate the detection efficiency of the developed ESBL panel. The presence for majority of targeted clusters was confirmed by NGS of amplification products. Metagenomic sequencing revealed that the abundance of ESBL genes within the pool of microorganisms was very low. The global relative abundances of the ESBL-coding gene clusters did not differ significantly among treatment states. However, at the level of each cluster, classical ESBL producers such as *Klebsiella* sp. for bla_{OXY} ($p = 0.0076$), *Acinetobacter* sp. for bla_{ADC} ($p = 0.02297$) and others, differed significantly with a tendency to decrease compared to the pre- and post-eradication states. Only 13 clusters were common across all three datasets, suggesting a patient-specific distribution profile of ESBL-coding genes. The number of AMR genes detected in the post-eradication state was higher than that in the pre-eradication state, which could be attributed, at least in part, to the therapy. This study demonstrated that the ESBL screening panel was effective in targeting ESBL-coding gene clusters from bacterial DNA and that minor differences exist in the abundance and prevalence of ESBL-coding gene levels before and after eradication therapy.

for detection of extended-spectrum beta-lactamase genes within feces samples". The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

1. Introduction

Appropriate and inappropriate use of antimicrobials is a well-recognized driver of resistance [1–3] as it can favor the selection of resistant bacteria [4, 5] opening up an ecological niche in which resistant pathogens can flourish [6]. To prevent the spread of resistance and maintain the effectiveness of antibiotics, a number of strategies have been put forth. These included controlled antibiotic use in agriculture, development of disease prevention strategies, improved antibiotic use strategies, development of novel antimicrobials, and others [6, 7]. Despite these measures, new technologies and improved diagnostics are needed to ensure that antimicrobials are used only when necessary to prevent the spread of antibiotic resistance.

Beta-lactam antimicrobial agents, which contain a β-lactam ring in their molecular structure, are the most common treatment option for bacterial infections. However, bacteria that produce beta-lactamase (BL) enzymes can deactivate these antibiotic agents by hydrolyzing the amide bond in the β-lactam ring [8], thus compromising the efficacy of empiric treatment. This deactivation mechanism is employed by a variety of Gram-negative bacteria such as *Escherichia*, *Klebsiella*, *Pseudomonas*, *Acinetobacter*, *Citrobacter*, *Proteus*, and others [9–12]. Continuous exposure of bacterial strains to a multitude of beta-lactam antimicrobials has evoked dynamic and continuous production and mutations of BL in these bacteria, thereby expanding their activity [13]. These enzymes, referred to as extended-spectrum BLs (ESBL), confer multi-drug resistance to a wide range of beta-lactam antibiotics, including penicillin, amoxicillin, cephalosporin, and others [11, 14]. Notably, BL and ESBL genes are often carried by highly mobile plasmids or other types of mobile genetic elements, which can enable their clonal spread among other bacterial species, while retaining resistance genes from other species, thus further limiting treatment options for infections caused by ESBL-producing bacteria [8, 11]. Even common infections, such as infections of the urinary tract caused by ESBL-producing bacteria, necessitate more elaborate treatment. Patients with these infections may require hospitalization and intravenous administration of carbapenem antibiotics, which are typically used as a last resort [15]. Carbapenems have been identified as one of the few remaining antibiotics capable of treating ESBL infections. However, the prevalence of resistance enzymes capable of deactivating these antibiotics has been observed to increase as well [15–17]. Consequently, infections caused by ESBL producers are associated with poor outcomes [18] and increased mortality [19, 20].

ESBLs are widely distributed worldwide, and more than 1.5 billion people are estimated to be colonized with ESBL-producing bacteria [21, 22]. This has led to ESBL-producing bacteria being considered as one of the most pressing public health threats in terms of antimicrobial resistance [15]. The highest prevalence of ESBLs has been observed in the Western Pacific, Eastern Mediterranean, and Southeast Asian regions, while the Americas and Europe display the lowest rates [21, 23]. While developing countries have the highest burden of ESBL-producing bacteria, developed countries are also experiencing an increase in prevalence [22]. Accumulated evidence suggests that risk factors for ESBL colonization include recent antibiotic exposure [24–28], repeated urinary tract infections [26, 27] and use of urinary catheter [26, 27, 29], use of intubation tube [28], recent hospitalization with an emphasis on ICU [27, 30], history of surgical procedures [27, 28, 30, 31], previous colonization with an ESBL-producing bacteria [27, 30], and even drinking water, food, and interaction with domestic animals [32]. Furthermore, antimicrobial use has been reported to be an additional risk factor for ESBL-producing bacterial infections among international travelers [1, 24, 33–36]. This raise concerns in regard to the global spread of ESBLs, thus emphasizing the importance of understanding the distribution and dynamics of the various ESBL encoding genes in order to develop effective control measures and prevent transmission, as well as to decolonize carriers.

The prevalence of *Helicobacter pylori* infection in developing countries is as high as 70% [37], although the rate of infection varies regionally. *H. pylori* is a chronic gastric pathogen that frequently colonizes mucosal layers and causes dyspeptic symptoms of varying severity [38]. The current first-line treatment for *H. pylori* infection consists of a combination of proton pump inhibitor (PPI) and two antibiotics, among which are amoxicillin, clarithromycin, or metronidazole. They are consumed daily for 7–14 days [38, 39]. Within the eradication scheme, PPIs are used to increase intragastric pH to maintain *H. pylori* in a replicative vegetative phase [40], whereas the macrolide antimicrobial agent clarithromycin is used to inhibit bacterial protein synthesis [41] and the beta-lactam antimicrobial agent amoxicillin is used to inhibit bacterial cell wall biosynthesis [42]. In addition, because amoxicillin is a broad-spectrum antibiotic used to treat upper and lower respiratory tract, skin, and other infections [43], its consumption is rapidly increasing worldwide [44].

To date, numerous studies have focused exclusively on the prevalence of ESBL genes in clinical samples of major ESBL producers, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca* and others. In order to gain further insight into this issue, in the present study we developed a screening panel that targets ESBL genes and employed both targeted and shotgun sequencing methodologies to investigate the abundance and prevalence of ESBL-coding genes in samples from patients undergoing first-line triple antibacterial eradication therapy for *H. pylori*. Additionally, the resistome profile of all study participants was evaluated before and after *H. pylori* eradication therapy. This is the first study that introduces an ESBL screening panel that is capable of identifying a wide range of ESBL coding genes originating from various microbial groups.

2. Methods

2.1 Patients, sample collection and storage

In total, 90 individuals with positive *H. pylori* infection who met the following criteria were included in this study: men and women aged 40 to 64 years; self-reported alcohol consumption 2–3 times a month or less; no history of colon or rectum polyps since age 20, gallstones, gastric cancer, gastric resection, alarm symptoms for digestive or any other diseases, type 2 diabetes, ulcerative colitis, Crohn's disease, coeliac disease, biliary cirrhosis, thyroid diseases, hepatitis B viral infection or serious psychiatric disorders. A ^{13}C-Urea breath test (Euroisotop, Germany) was performed to diagnose *H. pylori* infection. A detailed questionnaire was collected from each study participant, which contained information such as age, BMI, medical history (e.g., gastrointestinal diseases, viral infections, autoimmune diseases, and cancer), and lifestyle habits. Samples from 60 individuals collected over two years were used for the ESBL panel experimental group to explore long term variation in the abundance of ESBL coding genes. Samples from the remaining 30 individuals collected over one year were used for validation of the ESBL panel to account for the abundance of ESBL-encoding genes over a shorter time period (Fig 1).

Fecal samples were acquired from each recruited patient before starting the standard first-line *H. pylori* eradication (HPE) therapy and one–two years after. Each patient was prescribed the following medications twice a day for ten days: *Esomeprazolum* 40 mg, *clarithromycinum* 500 mg, and *amoxicillinum* 1000 mg. All procedures conformed to institutional ethical standards, and written consent was obtained from all patients prior to enrollment in the study.

In total, 180 samples were obtained within 30 minutes of defecation. Samples were transferred to an OC-Sensor tube (approx. 0.06–0.1 g) (Eiken Chemical Co, Japan), immediately homogenized, and stored at -80˚C until further processing according to previously validated storage conditions [45].

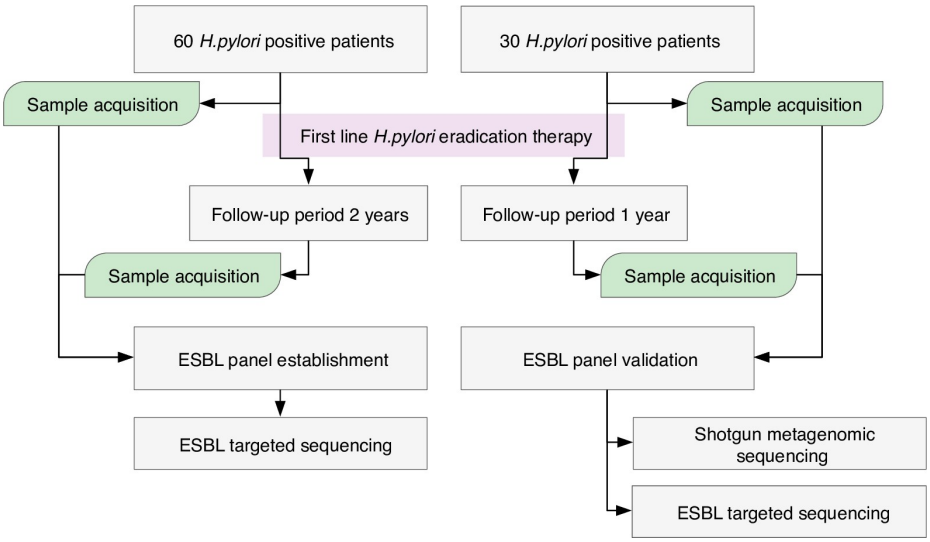

Fig 1. Study design. First line *H. pylori* eradication therapy consisted of *esomeprazolum* 40 mg, *clarithromycinum* 500 mg and *amoxicillinum* 1000 mg, each twice per day for ten days.

https://doi.org/10.1371/journal.pone.0289879.g001

2.2 DNA extraction

Fecal samples from the OC-Sensor tubes were extracted using a disposable syringe and transferred to pre-labelled 5 mL tubes. Excess OC-Sensor tube solvent was removed from the samples by lyophilization for approximately 15 h in a Christ Alpha 1–2 LD Freeze Dryer (SciQuip Ltd., UK). DNA from the dry remnants of OC-Sensor samples was isolated using the FastDNA SPIN Kit for Soil (MP Biomedicals, USA) according to the manufacturer's guidelines (S1 File).

2.3 ESBL gene cluster primer design

Known BL nucleotide sequences were obtained from the NCBI GenBank (accession date: 02.01.2018). Primer design was performed separately on TEM BLs, as they formed a homogenous group that was highly dissimilar from the rest of the BL sequences. Multiple alignments of the BL sequences were obtained using MAFFT v.7.392 [46]. The number of pairwise non-gapped mismatches between the BL sequences was calculated, and subsequent hierarchical clustering with complete linkage was performed on the distance matrix calculated from the alignment, using the number of non-gapped mismatches divided by the length of the alignment as the distance metric. Clusters were defined at a distance cut-off of 0.1. For each cluster, we identified contiguous conserved regions in the alignment using the Shannon index, for which at most 10% of the nucleotides would differ in each position. Regions with length 18 bases or longer were used in further experiments.

For each cluster, all possible pairs of conserved regions were computed such that the interval between regions in a pair was not longer than 500 bp. Each conserved region pair was scored by summing their Shannon indices at each position and sorted by their scores in ascending order. Primers were then designed on these region pairs with PRIMER3 v.2.4.0, by specifying PCR product sizes in the 200–500 bp range [47]. The best primer pairs for each cluster were evaluated by identifying the potential binding sites in the original list of BL sequences. A site was regarded as a potential binding site if there were up to three mismatches between the primer sequence and template. Additionally, no mismatches with the template were

allowed for the last five nucleotides at the 3' end of the primer. The binding site algorithm was implemented using the SeqAn library [48]. Regions bound by the primers were then aligned against the BL sequence database to evaluate potential off-target PCR products, that is, sequences that mapped against multiple BL clusters. Primer pairs that were overlapping (forming heterodimers estimated with primer3-py) or forming PCR products shorter than 50 bp were identified and pooled into separate sequencing batches. The designed primers were synthesized by MetaBion (Metabion International AG Ltd., Germany).

2.4 Sample preparation and targeted sequencing

Pools of primers with equal molarities and volumes were prepared for targeting the ESBL-coding genes and primers for the normalization of ESBL counts- primer pair Probio_Uni-F/Probio_Uni-R targeting *16S rRNA* gene V3 region [49]. PCR amplification of ESBL coding gene regions was performed using a 10 μM custom designed primer pool (S1 Table), Phusion U Multiplex PCR Master Mix (Thermo Fisher Scientific, USA), and GeneAmp® PCR System 9700 (Thermo Fisher Scientific, USA). The reaction mixture was prepared according to the manufacturer's recommendations, and the thermal conditions were set as follows: 98˚C for 30 s; 35 cycles of 98˚C for 10 s, 55˚C for 30 s, 72˚C for 15 s; with a final extension at 72˚C for 7 m. The success of the reaction was then assessed by 1.2% agarose gel electrophoresis. 100 ng of the acquired amplicons were used for library generation using the Ion Plus Fragment Library Kit (Thermo Fisher Scientific, USA) and the NucleoMag® NGS Clean-Up and Size Select kit (Macherey-Nagel, Germany) purification module. The quality and quantity of the amplicons were assessed using an Agilent High Sensitivity DNA kit on an Agilent 2100 BioAnalyzer (Agilent Technologies, USA).

Prior to emulsion PCR, each library was diluted to 12 pM and pooled for up to 18 libraries per sequencing run. The Ion PGM™ Hi-Q™ View OT2 kit (Life Technologies, USA) and Ion OneTouch DL instrument (Life Technologies, USA) were used for template generation. Sequencing was performed on an Ion 318 v2 chip and Ion Torrent PGM machine using the Ion PGM™ Hi-Q™ View Sequencing kit (Life Technologies, USA). All procedures were performed according to the manufacturer's instructions, and each run was expected to produce at least 80'000 reads per sample.

2.5 Sample preparation and shotgun sequencing

DNA samples for the shotgun metagenome analyses were normalized to an initial library input of 500 ng and sheared using a Covaris S220 Focused-ultrasonicator (Covaris, USA) to reach an average size of fragments 300 bp. Libraries with average insert sizes of 280 bp were prepared using MGIEasy Universal DNA Library Prep Set V1.0, (MGI Tech Co., China) according to the manufacturer's recommendations. Quality control of the libraries was assessed using the Qubit High Sensitivity dsDNA assay kit on a Qubit 2.0 instrument (Thermo Fisher Scientific, USA) and the Agilent High Sensitivity DNA kit on an Agilent 2100 Bioanalyzer.

Sequencing depth was calculated to achieve at least 20 million reads per sample (paired end, read length 100 bp). Libraries were sequenced using the DNBSEQ-G400 sequencer and a DNBSEQ-G400RS High-Throughput Sequencing Set PE100 (MGI Tech Co., China) according to the standard workflow.

2.6 Data analysis of targeted ESBL coding genes

A sequencing platform-specific adapter clipping of the obtained raw reads was performed with Cutadapt v.1.16 [50]. Targeted sequencing data were then aligned against the curated BL

sequence database using Bowtie2 v.2.3.5.1, pre-set at very sensitive [51]. Host reads from shotgun metagenomic sequencing data were filtered using Bowtie2 prior to mapping against BL sequences. *16S rRNA* was quantified with SortMeRNA v.2.1 [52] using *16S rRNA* sequences obtained from RNA central v10 [53] with search query 'rna_type: "rRNA" AND TAXONOMY: "9606" AND length: [19 TO 2000000000]'.

To classify sequencing reads into a specific ESBL cluster, we first created a classification scheme as follows: for each ESBL cluster, we identified regions where we expected the PCR product to form. Each putative product was aligned against all BL sequences to evaluate whether the product was specific to a particular cluster. We regarded putative products as specific if they mapped against sequences from only one cluster. Clusters were merged when a putative PCR product was specific to a set of clusters and if such clusters were not discernible. If multiple PCR products were mapped against the same position within a cluster, we established the alignment score threshold as the minimum score from a set of true positive alignments. An annotation table with cluster reference sequence IDs, start and end coordinates of the corresponding primer product regions, and alignment score thresholds were generated. Sequencing reads were assigned to clusters if overlapped with the coordinates in the annotation table and exceeded the alignment score threshold. Putative PCR products were identified using SeqAn, Pandas and Bowtie2. An annotation table and Python script reading sample binary alignment map files with the Pysam package (https://github.com/pysam-developers/pysam) were used to quantify the read count in each cluster for each sample. The read counts of each BL cluster were normalized to the read counts of the *16S rRNA* gene of a particular sample.

2.7 Metagenome data analysis

Quality control and quality trimming of the obtained paired-end reads were performed using FastQC and Trimmomatic v0.39 [54] with a quality threshold of 20 and a minimum read length of 36. Quality filtered sequences were then aligned to the human genome reference GRCh37 (hg19, UID:2758) and sequences matching the human genome were removed using Bowtie2 v.2.3.5.1. The taxonomical profile of the metagenomic dataset was assigned using Kraken2 v.2.0.8 [55] and RefSeq database release 98 [56]. *De novo* read assembling into contigs was performed using the IDBA_UD [57] assembler with the *k*-mer length of at least 50. Generated assembly was evaluated using metaQuast [58]. The assembly database and the local alignment of input reads to assembly was performed using Bowtie2. Open reading frame detection and subsequent annotation was performed using PROKKA v.1.14.6 [59] with the manually curated Swiss-Prot UniProtKB [60] database (accessed 08.02.2021.). During the annotation, predictions of rRNA and tRNA, as well as contigs below 250 nt were excluded. Coordinates of predicted protein-coding features (CDS) were used for quantification against the assembly database using HTSeq [61] and the intersection-nonempty resolution mode. According to previous studies [62–64], metagenomic read counts were standardized using the Transcripts Per Million method [65] with an in-house built python script. Subsequently, from annotation files, CRISPR annotation was removed while contig IDs with the respective product information were retained using in-house built sed and awk scripts. Read counts were joined with the filtered annotation by contig ID column for each sample separately. Next, all samples were merged into a single dataset by annotation column using the Pandas [66] library within the Python environment.

Contigs were used to predict the resistome profile of the study subjects using The Resistance Gene Identifier (RGI) v.5.1.1 along with the Comprehensive Antibiotic Resistance Database (CARD) [67] and the DIAMOND [68] alignment tool. Results were gathered for each sample

obtained using the heat map function of RGI, by organizing resistance genes based on the resistance mechanism and gene family. Additionally, hierarchical clustering was performed to cluster samples based on their similarity.

2.8 Statistical analysis of taxonomical data

Kraken reports were uploaded to the Pavian v.1.0.0 [69] package and taxonomic entries were filtered out if the sum of the assigned sequences for the taxonomic clade across the samples was below 200. Then, SIAMCAT v.1.9.0 [70] was used to evaluate the association of microbial species between pre- and post-eradication states. Briefly, the dataset of taxonomical entities was separated into two groups: pre-eradication (designated as case) and post-eradication (designated as control). The cut-off for the relative abundance of the species was set to 0.001. The association of microbial species between pre-and post-eradication states was determined using the Wilcoxon test at a significance level of $p<0.05$, with the False Discovery Rate multiple hypothesis correction method. In addition, log-transformed normalization was applied to the abundance matrix of microbial species and the Area Under the Receiver Operating Characteristics Curve (AU-ROC) was used as a non-parametric measure of the enrichment. All the acquired measures of association between the pre- and post-eradication groups were visualized in the SIAMCAT association plot.

Furthermore, the dataset was divided into four groups: F-post-erad denoted subjects of the post-eradication group with ineffective HPE; F-pre-erad denoted subjects of pre-eradication group with ineffective HPE; S-post-erad denoted subjects of post-eradication group with successful HPE and S-pre-erad denoted subjects of pre-eradication group with successful HPE. Alpha diversity metrics (Shannon, Chao1 and Observed) were calculated and visualized using Phyloseq v.1.30.0 [71]. Pairwise comparisons of alpha diversity metrics between the treatment states using the Wilcoxon rank sum test and the Holm P-value adjustment method were performed using the Vegan v.2.5–7 package. Non-metric multidimensional scaling was performed using Phyloseq.

2.9 Statistical analysis of amplicon data

The relative abundances of the ESBL clusters between the treatment states were compared using the two-tailed paired t-test in the Vegan v.2.5–7 package. The Kruskal-Wallis test was used to assess the significance in the abundance of individual ESBL clusters between the pre- and post-eradication states with the same package. To explore the clusters overlapping between datasets, cluster IDs which appeared at least once in a sample were extracted from all three datasets. A Venn diagram was constructed using the ggVennDiagram [72] v0.1.9 within the R environment.

2.10 Statistical analysis of functional data

UniprotKB entry IDs of the summarized annotation dataset were converted into Gene Ontology (GO) IDs using the UniProt online Retrieve/ID mapping tool (https://www.uniprot.org/uploadlists/). The UniProtKB entries which did not match any corresponding GO ID were removed from the dataset. Next, MaAsLin2 v1.8.0 [73] was used to determine the association of the microbiome functional profile with the treatment state. For the MaAsLin2 analysis, the q-value threshold for significance was set to 0.05, the minimum abundance for each GO term was set to 50, the minimum percentage of samples for which a GO term was detected at a minimum abundance was 25%, the random effect for the model was set to the patient ID, and the fixed effect for the model was treatment state. The significance of the association was controlled using the Benjamini-Hochberg multiple testing correction method.

2.11 Ethics Approval Statement

This study was approved by the Biomedical Ethics Committee of the Riga East University Hospital Support Foundation, approval No. 13-A/13 from October 3, 2013.

3. Results

Of the 180 samples, three failed during the ESBL-targeted amplicon PCR; therefore, these samples with their respective pairs were removed from further analysis. Thus, a total of 174 samples were sequenced (Table 1). Targeted ESBL quantification in samples from the experimental group resulted in acquisition of 56'418'406 Ion Torrent PGM sequence reads (n = 120, in average 454'987±131'587 reads per sample, two year interval between pre- and post-eradication), while the same analysis for samples from the validation group resulted in acquisition of 20'273'116 Ion Torrent PGM sequence reads (n = 54, in average 375'428 ±178'727 reads per sample, one year interval between pre- and post-eradication) and 1'522'622'154 DNBSEQ-G400 sequence reads (n = 54, in average 28'196'706±3'943'687 sequences per sample, one year interval between pre- and post-eradication).

3.1 Experimental setting: Targeted ESBL analysis

The ESBL panel was designed to reflect the prevalence and abundance of the most common ESBL types. A total of 245 gene clusters encoding evolutionarily related ESBLs were targeted by designed primer pool, and primers targeting V3 region of the *16S rRNA* gene were added to the pool to normalize the number of ESBL gene cluster counts (cluster names and respective primer sequences are shown in S1 Table). Analysis of the obtained raw sequence data resulted in 1'787 annotated microbial sources containing strain or clinical isolate IDs with respective BL gene groups, classes and gene names (S2 Table, sheet full annotation). Clusters with identical IDs were merged, and the lowest common ancestor was indicated as the microbial source (S2 Table, sheet merged annotation). Thus, from the 245 ESBL clusters targeted, results were obtained for 265 ESBL clusters, of which twenty clusters most likely represented sequence homology with similar BL gene clusters and thus were assigned to multiple clusters. In the pre-eradication subgroup, 89 ESBL clusters, while in the post-eradication subgroup 106 ESBL gene clusters were not detected in any of the study subject's samples.

Table 1. Descriptive summary of the patient-specific parameters.

	Experimental ESBL quantification (n = 60, men/women)	Validation group (n = 30, men/women)
Gender (n, %)	26 (43.3%) / 34 (56.7%)	10 (33.3%) / 20 (66.6%)
Average age	52.48 ± 6.26	52.2 ± 6.97
Mean Body Mass Index	28.387 ± 4.69	29.06 ± 4.75
Positive *H.pylori* status as identified by ^{13}C-Urea breath test before eradication	26 (43.3%) / 34 (56.7%)	10 (33.3%) / 20 (66.6%)
***H.pylori* status as identified by ^{13}C-Urea breath test after eradication**	Positive: 2 (3.3%) / 6 (10.0%)	Positive: 1 (3.3%) / 4 (13.3%)
	Negative: 23 (38.3%) / 29 (48.3%)	Negative: 9 (30%) / 16 (53.3%)
Asthma	0 / 2 (3.3%)	0 / 2 (6.7%)
Experienced Duodenitis	3 (3.3%) / 4 (6.67%)	0 / 0
Tuberculosis	1 (1.67%) / 1 (1.67%)	0 / 0
Hepatitis A	3 (3.3%) / 4 (6.67%)	0 / 3 (10.0%)

https://doi.org/10.1371/journal.pone.0289879.t001

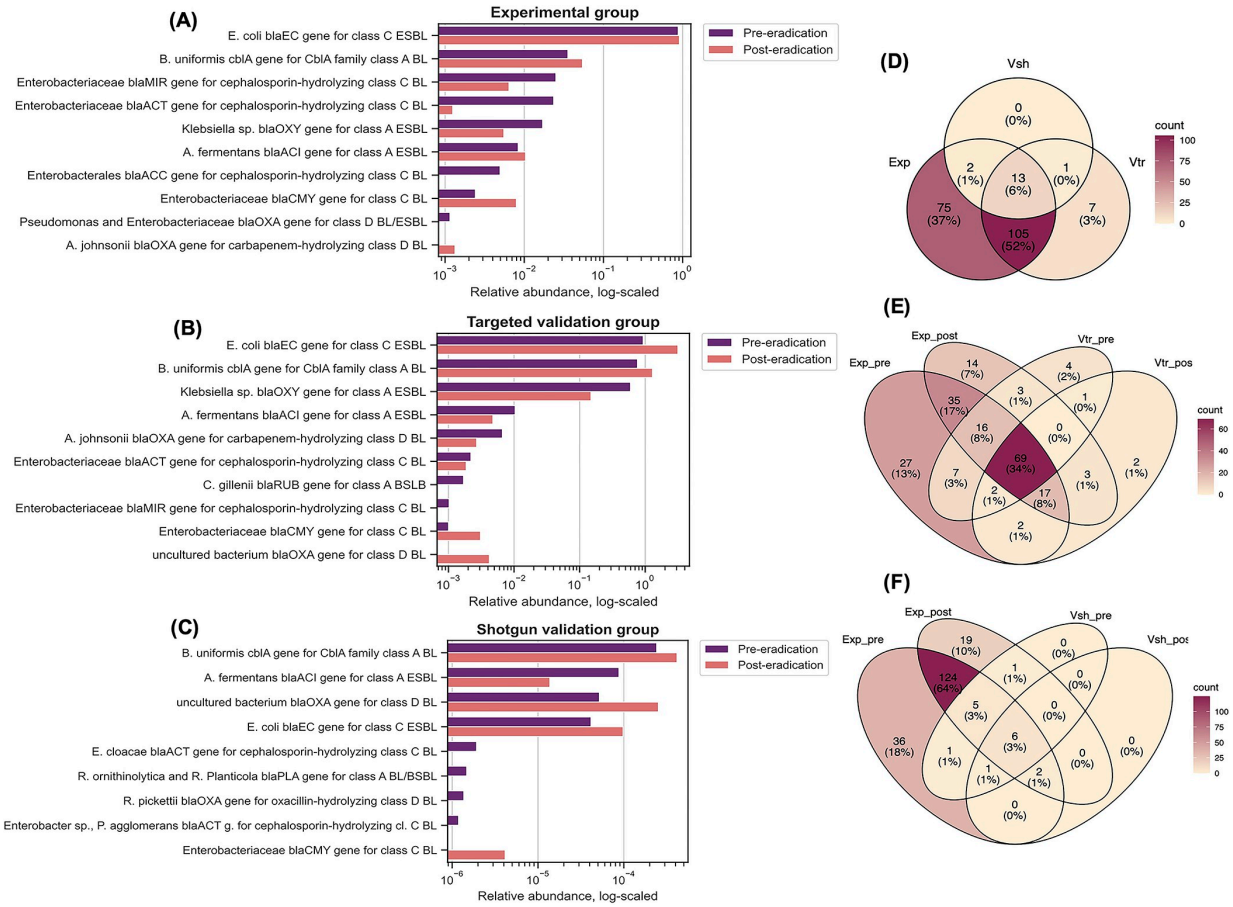

Fig 2. Relative abundance and proportion of common ESBL coding genes between the experimental, validation-targeted, and validation-shotgun groups. Section A-C: relative ESBL coding gene prevalence profile in the pre- and post-eradication samples of the analyzed datasets. For each dataset ESBL counts were normalized against *16S rRNA* gene V3 region bacterial counts and summarized by treatment state. For visualization purposes, for the experimental (A) and targeted validation (B) groups, the abundance threshold was set to 0.1%, and for the shotgun validation (C) group it was 0.0001%. Section D-F: Venn diagram of the ESBL gene cluster counts observed in the experimental group and the validation group of shotgun and targeted sequencing. Only gene clusters that appear at least once in the respective sample group were included in the analysis. Abbreviations: ESBL–extended spectrum beta lactamases, BL–beta lactamases, Exp–experimental group, Vtr–targeted validation group, Vsh–shotgun validation group, Exp-pre–pre-eradication sample set of experimental group, Exp-post–post-eradication sample set of experimental group, Vtr-pre–pre-eradication sample set of targeted validation group, Vtr-post–post-eradication sample set of targeted validation group, Vsh-pre–pre-eradication sample set of shotgun validation group, Vsh-post–post-eradication sample set of shotgun validation group.

https://doi.org/10.1371/journal.pone.0289879.g002

Assessing the presence of ESBL gene clusters in each sample in both treatment states, most clusters were absent, while the presence of a particular ESBL cluster in each sample was mostly dispersed. Examples of disperse clusters include bla_{PER}, bla_{MSI}, bla_{CMH}, bla_{L1}, bla_{GES}, bla_{TMB}, bla_{VIM}, bla_{E}, bla_{PDC}, and others. As a result, each sample was represented by 23 different ESBL gene clusters in the pre-eradication study group and 22 different ESBL gene clusters in the post-eradication study group.

Comparing pre- and post-eradication subgroups, the most prevalent clusters (Fig 2A) were the bla_{EC} gene group for class C ESBL with an annotated source of *Escherichia coli* (pre = 87.98%, post = 91.11%), cbl_{A} gene group for class A BL with annotated source of *Bacteroides uniformis* (pre = 3.56%, post = 5.44%), bla_{MIR} gene group for class C BL with annotated source of *Enterobacteriaceae* (pre = 2.52%, post = 0.64%), and bla_{ACT} gene group for class C

Table 2. Significant distribution of BL genes among annotated bacterial sources from patients comparing the pre- and post-eradication states.

	No.	Annotated taxonomical source	Cluster number	Gene group	Type of beta-lactamase	Embedded beta-lactamase gene	Normalized average relative abundance		*p*-value
							Pre-eradication	Post-eradication	
Experimental set	1.	*Klebsiella* sp.	69	bla_{OXY}	Class A ESBL	OXY-4-1, OXY-6-2, OXY-6-3, OXY-6-1, OXY-6-4, OXY-5-1, OXY-5-2, OXY-1-4, OXY-1-6, OXY-1-2, OXY-1-1, OXY-1-3	4572.625	1558.578	0.0076
	2.	*Nocardia farcinica*	85	bla_{FAR}	Class A ESBL	FAR-1	1.203125	1.25	0.00999
	3.	*Acinetobacter* sp.	166	bla_{ADC}	Class C BL	ADC-83, ADC-84	0.15625	0	0.02297
	4.	*Streptomyces albus*	86	*bla*	Exo family class A BL	-	0.09375	0	0.02297
	5.	Uncultured bacterium	58	bla_{LRG}	Class A ESBL	LRG-1	0.0234375	0.0625	0.02298
	6.	Uncultured bacterium	150	bla_{LRA}	Subclass B3 metallo-BL	LRA-17	0.609375	0.28125	0.04219
	7.	*Pseudomonas* sp. and *A. baumannii*	269	bla_{IMP}	Subclass B1 metallo-BL	IMP-44, IMP-41, IMP-11, IMP-21, IMP-16, IMP-22, IMP-58	0.46875	1.4375	0.04239
	8.	Uncultured bacterium	151	bla_{LRA}	Subclass B3 metallo-BL	LRA-19	0.21875	0.0625	0.04506
Validation set by PGM targeted sequencing	9.	*Achromobacter xylosoxidans*	130	bla_{OXA}	Class D BL	OXA-114g, OXA-114c, OXA-114f, OXA-114a, OXA-114e, OXA-114b, OXA-114d	0.0002929	0.00003792	0.01174
	10.	*Stenotrophomonas maltophilia*	125	bla_{L1}	Subclass B3 metallo-BL	-	0.00016589	0.000088825	0.01491
	11.	*Ralstonia pickettii*	135	bla_{OXA}	Class D BL	OXA-22	0.000017415	0.001294	0.01547
	12.	*Pseudomonas aeruginosa*	97	bla_{PME}	Class A ESBL	PME-1	0.0001257	0.000056175	0.02113
	13.	*Chromobacterium piscinae*	77	bla_{CRP}	Class A ESBL	CRP-1	0.0000030419	0.000020348	0.03198
	14.	*Ralstonia mannitolilytica*	136	bla_{OXA}	Class D BL	OXA-443	0.000030144	0.0011566	0.03428
	15.	*Nocardia farcinica*	85	bla_{FAR}	Class A ESBL	FAR-1	0.0000016024	0.000018579	0.03942
	16.	*Bacillus clausii*	100	bla_{BCL}	Class A BL	BCL-1	0.000024638	0	0.03967
	17.	*Pseudomonas* sp., *A. baumannii*	269	bla_{IMP}	Subclass B1 metallo-BL	IMP-44, IMP-41, IMP-11, IMP-21, IMP-16, IMP-22, IMP-58	0.000033418	0	0.03967
	18.	*Serratia marcescens*	6	bla_{SRT}	Class C BL	SST-1, SRT-1, SRT-2	0	0.000056522	0.03967

https://doi.org/10.1371/journal.pone.0289879.t002

BL with an annotated source of *Enterobacteriaceae* (pre = 2.35%, post = 0.12%). The normalized averaged relative abundance of ESBL genes was not significantly abundant between pre- and post-eradication ($p = 0.5467$). Next, we assessed if non-averaged relative abundance of any particular BL cluster differed significantly across treatment states. Thus, we identified eight clusters that were significantly different between pre- and post-eradication subgroups (Table 2, $p<0.05$). Among these dominated the cluster of class A bla_{OXY} group ESBL gene with the annotated origin from 12 different *Klebsiella oxytoca* strains, which had a higher relative abundance in the pre-eradication subgroup than in the post-eradication subgroup ($p = 0.0076$). Similarly, abundance of another strain-rich (n = 4) cluster of the B1 subclass bla_{IMP} group metallo-BL genes with annotated origins from *Pseudomonas aeruginosa*, *P. fluorescens*, *P. putida* and *Acinetobacter baumannii* was identified as a significantly different between the treatment states. However, the relative abundance of this one was noticeably

lower than that of the previous one. The relative abundance of the B1 subclass bla_{IMP} group metallo-BL genes was significantly higher ($p = 0.0424$) in the post-eradication subgroup than in the pre-eradication subgroup.

3.2 ESBL coding gene quantification using the validation group

The efficiency of ESBL panel was validated through targeted and shotgun metagenomic sequencing of DNA from another independent sample group (54 samples from 27 individuals), which also included pre-and post-eradication subgroups to validate the ESBL gene quantification experimental group. To provide results that are comparable to those of experimental group, we used the same ESBL reference database as before. As in the previous group, in the case of targeted sequencing data analysis, we used the number of reads of the V3 region of *16S rRNA* gene to normalize ESBL quantification data between samples, while in the case of the shotgun metagenomic sequencing, the total number of *16S rRNA* sequences were used for normalization.

Overall, sequencing and data analysis results for the validation group by targeted sequencing were similar to those of the experimental group. A total of 254 ESBL clusters were targeted, but data analysis resulted in identification of 265 ESBL clusters, of which twenty showed sequence homology and thus were assigned to multiple clusters. In total, we identified reads from 101 ESBL gene clusters in the pre-eradication subgroup samples and from 95 ESBL gene clusters in the post-eradication subgroup samples. Considering the arithmetic mean of each ESBL gene cluster within the respective eradication subgroup (Fig 2B), there were only seven clusters that exceeded the 0.05% abundance threshold, from which the most abundant ones were the C ESBL class bla_{EC} group genes (pre = 40.16%; post = 68.3%), the A BL class CblA family cbl_A group genes (pre = 32.86%; post = 27.94%), and the A ESBL class bla_{OXY} group genes (pre = 25.76%; post = 3.24%). The normalized average relative abundance of ESBL gene groups was not significantly different between pre- and post-eradication subgroups ($p = 0.32464$).

In contrast, analysis of shotgun metagenomic sequencing data from the validation group samples revealed that only 15 ESBL gene clusters were found in all 54 samples taken together. These included bla_{R1}, bla_{CMY}, cbl_{A}, bla_{ACI}, bla_{PLA}, bla_{CKO}, bla_{EC}, bla_{SHV}, bla_{OXY}, three of bla_{ACT}, and three of bla_{OXA}. However, their distribution was mostly dispersed among the samples. In the shotgun dataset, each sample was represented by 13 different ESBL gene clusters in the pre-eradication subgroup and by 8 different ESBL gene clusters in the post-eradication subgroup. Considering the arithmetic mean of all identified ESBL gene clusters in the pre- and post-eradication study subgroups (Fig 2C), 11 in the pre-eradication study subgroup and only five in the post-eradication study subgroup exceeded the 0.1% abundance threshold and the most abundant ones were the A BL class CblA family cbl_A group genes (pre = 56.04%, post = 53.0%), A ESBL class bla_{ACI} group genes (pre = 20.35%, post = 1.75%), D BL class bla_{OXA} group genes (pre = 12.02%, post = 32.13%), and C ESBL class bla_{EC} group genes (pre = 9.62%, post = 12.46%). The normalized average relative abundances of ESBL gene groups did not differ significantly between the pre- and post-eradication subgroups ($p = 0.20919$).

Further we assessed if the relative abundance of individual BL gene clusters differed significantly across the treatment states. Thus, within the targeted dataset, we identified 10 BL gene clusters that were significantly different between the pre- and post-eradication subgroups (Table 2, $p<0.05$). These included a cluster of various A ESBL class bla_{OXA} group genes with the annotated origin of *Achromobacter xylosoxidans*, a subclass B3 metallo-BL L1 family bla_{L1} group gene with the annotated origin of *Stenotrophomonas maltophilia*, and an oxacillin-

hydrolyzing D BL OXA-443 class bla_{OXA} group gene with the annotated origin of the *Ralstonia picketii* strain PIC-1. However, within the metagenomic dataset, we were unable to identify any specific BL cluster that differed significantly between treatment states and the most likely reason for that was the low number and low abundance of identified ESBL gene clusters ($p>0.05$).

We also quantified the number of ESBL gene clusters that overlapped between the experimental, targeted validation, and shotgun validation groups (Fig 2D–2F). A global comparison, for example, without considering the pre- and post-eradication subgroups, revealed that all three groups contained 13 clusters (Fig 2D) (cbl_A, bla_{PLA}, bla_{EC}, bla_{ACI}, bla_{OXY}, bla_{CMY}, bla_{R1}, three of bla_{OXA}, and three of bla_{ACT}). The highest number of shared ESBL gene groups was found between the experimental and targeted validation datasets (Fig 2E). Conversely, the shotgun dataset contained a notably lower amount of ESBL gene groups, resulting in lower coverage compared to the experimental dataset (Fig 2F). Thus, 69 ESBL gene groups were found between the experimental and targeted validation datasets, and only 6 shared ESBL gene groups were found between the experimental and shotgun validation datasets.

3.3 Taxonomical characterization

As our analyses included metagenomic sequencing, we also explored the taxonomic and functional composition of patient samples before and after *H. pylori* eradication therapy. A detailed taxonomic analysis of samples from the experimental group (n = 120) is described in our previous publication (Gudra et al., 2020). However, to be brief, we found that the dominant genera in the pre-eradication study subgroup were *Bacteroides* (10.3%), *Oribacterium* (9.08%), *Prevotella* (6.16%) and *Parasutterella* (4.87%), whereas in the post-eradication study subgroup–*Bacteroides* (9.75%), *Streptomyces* (7.75%), *Oribacterium* (7.41%) and *Prevotella* (5.81%). Alpha and beta diversities did not differ significantly between the pre-and post-eradication subgroups ($p>0.05$). Moreover, despite receiving eradication therapy, eight of the 60 individuals continued to test positive for *H.pylori*, and the available data show that there were no instances of recurrence.

In the current validation group (n = 27), 98.68±1.24% of taxonomic entries belonged to bacteria, 0.33±0.48% to viruses and 0.0026±0.003% to fungi and protozoa combined. A detailed summary of the taxonomy is provided in S1 File, but the most prevalent bacterial genera in the pre-eradication subgroup were *Bacteroides* (37.73%), *Faecalibacterium* (11.86%) and *Bifidobacterium* (6.67%), whereas in the post-eradication subgroup dominated *Bacteroides* (44.95%), *Faecalibacterium* (12.43%), and *Alistipes* (4.73%). We also found that at the species level, the most prevalent bacteria in the pre-eradication subgroup were *Faecalibacterium prausnitzii* (13.45%), *Bacteroides vulgatus* (10.38%), *Bifidobacterium adolescentis* (4.89%) and *Bacteroides uniformis* (4.53%), whereas in the post-eradication subgroup *Faecalibacterium prausnitzii* (14.21%), *Bacteroides vulgatus* (13.35%), *Bacteroides dorei* (6.12%) and *Bacteroides uniformis* (6.11%). Furthermore, we also tested if there was an association between microbial species and treatment state, and according to the acquired results, 12 microbial species were found to be differentially abundant between the pre- and post-eradication subgroups (Fig 3). Of these, two had increased relative abundance (*E. bolteae*, $p_{adj.} = 0.049$; *E. lenta*, $p_{adj.} = 0.003$), and 10 had decreased relative abundance (*S. faecalis*, $p_{adj.} = 0.042$; *A. intestini*, $p_{adj.} = 0.048$; *Olsenella* sp. GAM18, $p_{adj.} = 0.01$; *A. fermentans*, $p_{adj.} = 0.015$; *E. hirae*, $p_{adj.} = 0.003$; *B. angulatum*, $p_{adj.} = 0.013$; *C. aerofaciens*, $p_{adj.} = 0.042$; *T. succinifaciens*, $p_{adj.} = 0.013$; *M. funiformis*, $p_{adj.} = 0.009$; *S. dextrinosolvens*, $p_{adj.} = 0.022$) in the post-eradication subgroup compared to the pre-eradication subgroup.

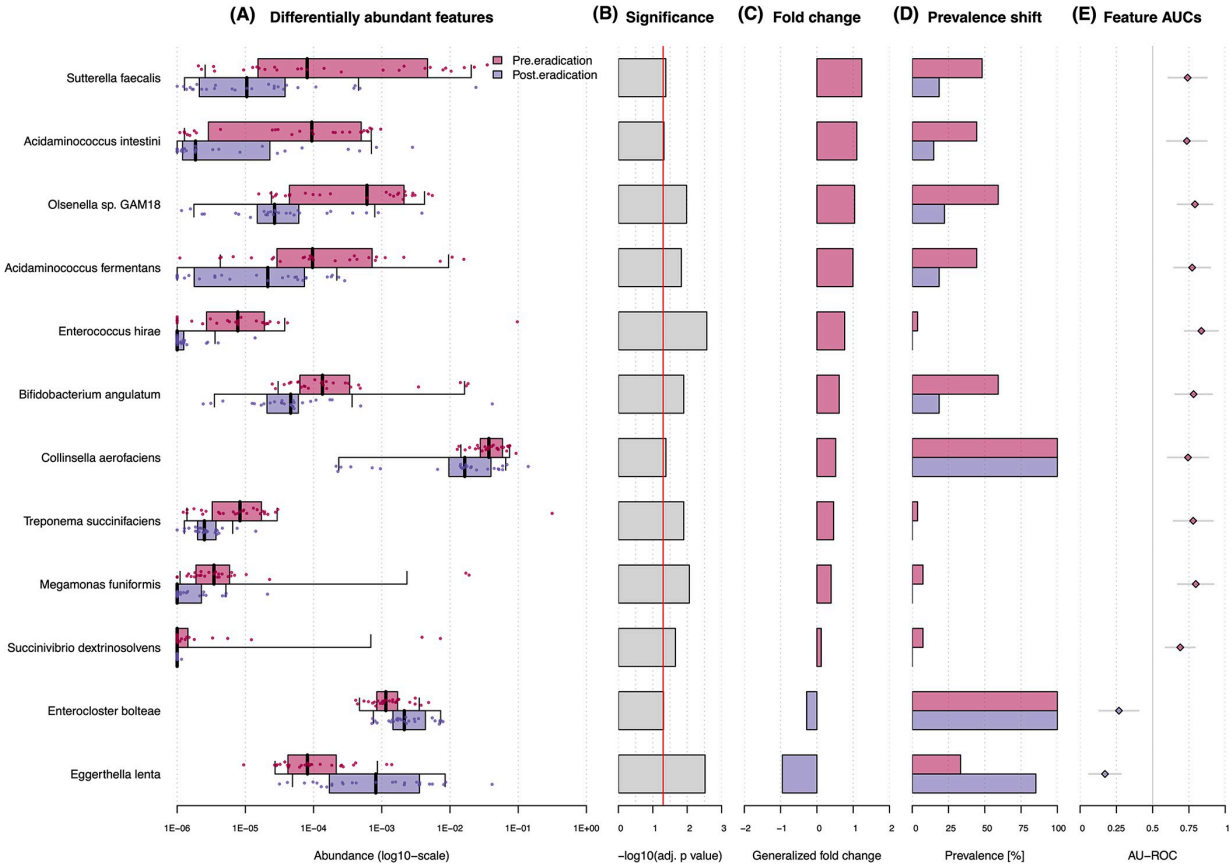

Fig 3. Significantly associated microbial species between the pre- and post-eradication states (at a significance level $p<0.05$). Section A–differentially abundant microbial species between pre- and post-eradication states; Section B–the significance of the enrichment calculated by a Wilcoxon test with FDR multiple hypothesis correction; Section C–generalized fold change of each significantly associated microbial species; Section D–the prevalence shift of each significantly associated microbial species; Section E–the Area Under the Receiver Operating Characteristics Curve (AU-ROC) as a non-parametric measure of the enrichment.

https://doi.org/10.1371/journal.pone.0289879.g003

Given that the majority of ESBLs detected during this work originated from the *Enterobacteriaceae* family, we decided to compare the abundance of enterobacteria between treatment states in the metagenomic dataset. Our ESBL targeted sequencing data showed that the bla_{EC} gene group with an annotated source of *E. coli* predominated in both treatment states, but in our metagenomic data, the incidence of *E. coli* was only 0.129% before and 0.338% after the eradication therapy. However, although this change in abundance is indeed low, it represents 2.6-fold difference ($p>0.05$). The relative abundance of entire *Enterobacteriaceae* family was 1.29% in the pre-eradication state and 2.44% in the post-eradication state, representing a 1.9-fold difference in favor of the post-eradication state ($p>0.05$). Further on assessing BL genes with significant distribution (Table 2) among the treatment states, *Klebsiella* sp. was one of the dominant annotated sources of ESBLs (Fig 3A, 3B). In metagenomic data, relative abundance of *Klebsiella* sp. was 0.062% in the pre-eradication state samples and 0.022% in the post-eradication state samples, which represents a 2.9-fold difference and agrees well with the targeted sequencing results. Bacterial species such as *Pseudomonas aeruginosa* (pre = 0.005%, post = 0.006%), *Ralstonia picketii* (pre = 0.014%, post = 0.004%) and *Serratia marescens*

(pre = 0.0001%, post = 0.0011%) were at very low abundance in all samples, whereas *A. xylosoxidans*, *S. maltophilia*, *C. piscinae*, *R. mannitolilytica*, *N. farcinica*, *B. clausii* and *A. baumannii* were not found in the metagenomic dataset. When comparing the outcomes of metagenomic sequencing with targeted sequencing, the observed variance may be due to differences in applied wet-lab and data analysis methodologies, as well as to insufficient sequencing depth to detect very low abundance microorganisms.

Assessing Shannon and Chao1 indices and the observed OTUs (S1 File), only the Shannon index differed significantly between the pre- and post-eradication subgroups ($p_{adj.} = 0.019$). Furthering, non-metric multidimensional scaling was applied to assess sample-to-sample variability (S1 File). The analysis revealed non-specific clustering of samples at the species level, suggesting mutual sample similarity irrespective of gender and treatment state. In addition, four of the 27 study subjects continued to test positive for *H.pylori* after receiving the eradication medication, and the data that are available indicate that there were no incidences of recurrence.

3.4 Functional analysis of the metagenomic profile

The functional profiles of the samples were very similar across treatment states, with the predominant gene products being tyrosine recombinase XerC (in both pre- and post-eradication subgroups: 0.59%), TonB-dependent receptor P3 (pre = 0.53%, post = 0.48%), adaptive response sensory kinase SasA (pre = 0.4%, post = 0.45%), the sensor histidine kinase RscC (pre = 0.32, post = 0.37%) and the TonB-dependent receptor SusC (pre = 0.32%, post = 0.25%) (Fig 4). Furthermore, the resulting UniProt IDs were converted to Gene Ontology (GO) annotations, and the original annotations that did not have the corresponding GO IDs were removed from the dataset. Thus, in total 1'993 unique GO IDs were obtained. To assess the relationship between functional profile and treatment state, association analysis was performed revealing 18 GO ID entries that were significantly different between pre- and post-eradication subgroups (S1 File). Significant associations between GO IDs and treatment state were found for entities related to molecule (GO:0035442, q-value = 0.012; GO:0034219, q-value = 0.012) and ion transport (GO:0006811, q-value = 0.005; GO0042777, q-value = 0.012; GO:0015693, q-value = 0.013; GO:0006817, q-value = 0.043), several biosynthetic processes (GO:0019242, q-value = 0.032; GO:0045226, q-value = 0.043), including cobalamin (GO:0009236, q-value = 0.032) and dTMP (GO:0006231, q-value = 0.044) biosynthesis, DNA restriction-modification system (GO:0009307, q-value = 0.012), metabolic processes (GO:0019568, q-value = 0.044; GO:0006541, q-value = 0.044; GO:0019243, q-value = 0.032), DNA-templated transcription and initiation (GO:0001123, q-value = 0.032), ribosomal small subunit biogenesis (GO:0042274, q-value = 0.012), adenine salvage (GO:0006168, q-value = 0.044) and cellular phosphate ion homeostasis (GO:0030643, q-value = 0.044). Notably, most of the abundances of significant GO IDs entities were increased in the post-eradication subgroup (n = 15), while only a few were increased in the pre-eradication subgroup (n = 3). GO entities that exhibited increased abundance in the pre-eradication subgroup and decreased abundance in the post-eradication subgroup were related to ribosomal small subunit biogenesis, DNA-templated transcription and initiation, and adenine salvage.

The diversity of antimicrobial resistance (AMR) genes found in the metagenomic sample set was revealed by additional mapping to the CARD database. Thus, we identified 54 gene families associated with AMR, conferring five different mechanisms of resistance (S3 Table). A total of 1'080 AMR genes were detected in the pre-eradication subgroup, of which 125 had 100% sequence similarity and 955 had strictly significant ($\geq$95% identity) sequence similarity to the CARD reference database. We detected 1'265 AMR genes in the post-eradication

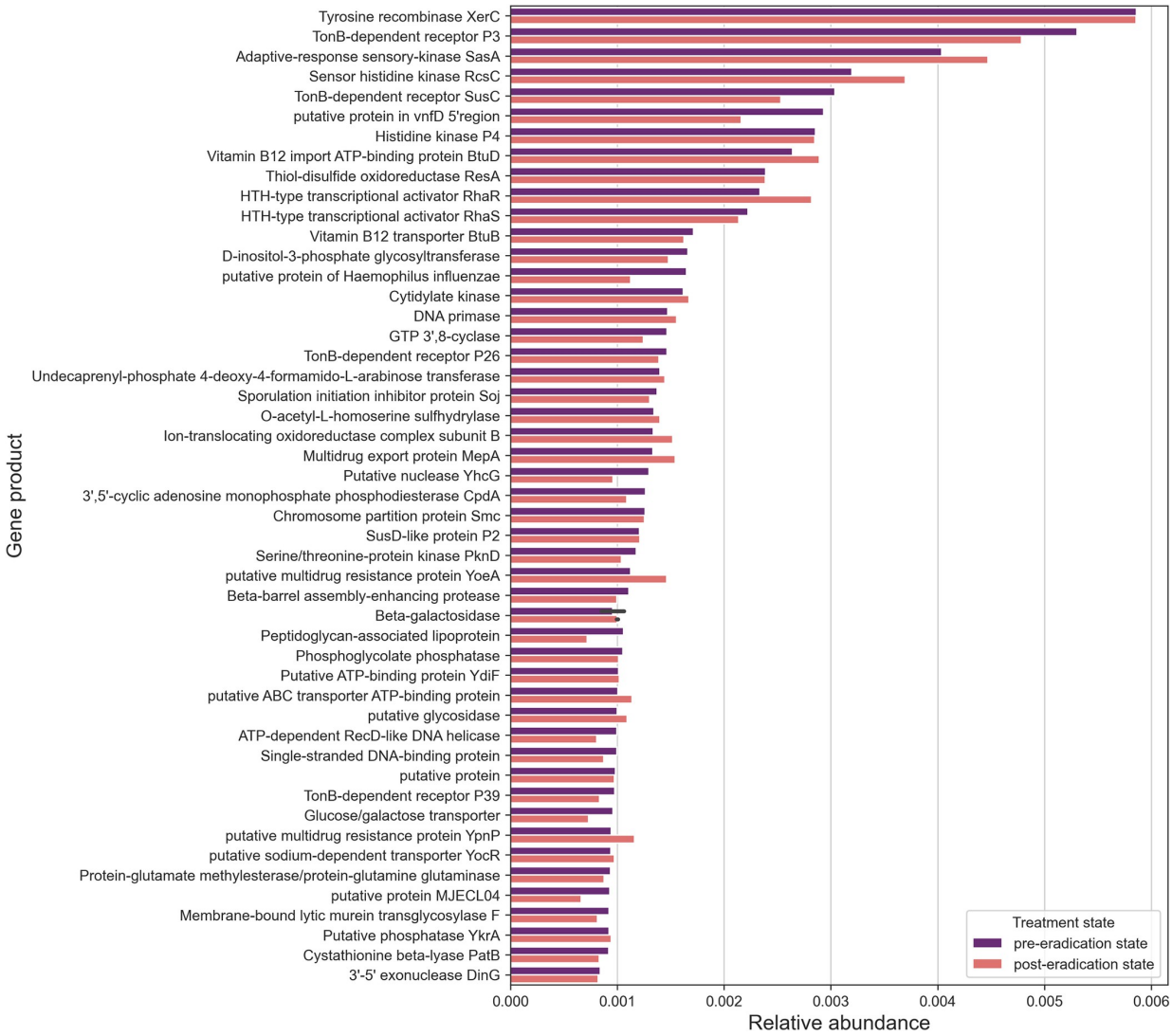

Fig 4. Top 50 most abundant gene products obtained by shotgun metagenomic sequencing between the pre- and post-eradication states.

https://doi.org/10.1371/journal.pone.0289879.g004

subgroup, of which 179 had 100% sequence similarity and 1'086 had strict (≥95% identity) sequence similarity to the CARD reference database. AMR genes present in at least 70% of samples in each treatment state (n = 20) were the antibiotic target alteration genes *ErmB* and *ErmF*; antibiotic inactivation genes chloramphenicol acetyltransferase, *aadS* and *lnuC*; antibiotic efflux genes *Mef(En2)*, *tet(40)*, and *adeF*; antibiotic target alteration gene *rpoB*; antibiotic target protection genes *tet(W/N/W)*, *tet32*, *tetM*, *tetO*, *tetQ*, and *tetW*; and the antibiotic target replacement gene *dfrF*. AMR genes present in all patient samples in the pre-eradication subgroup were antibiotic efflux gene *adeF*, antibiotic target protection gene *tetQ*, and antibiotic target replacement gene *dfrF*. In contrast, AMR genes present in all patient samples of the post-eradication study subgroup were the antibiotic target alteration gene *ErmF*; antibiotic efflux genes *tet(40)* and *adeF*; antibiotic target protection genes *tetO*, *tetQ*, and *tetW*; and the

antibiotic target replacement gene *dfrF*. After applying a hierarchical clustering analysis, 12 samples collected from different individuals and within a sample cluster showed a similar reservoir of detected AMR genes. Only two study subjects had pre- and post-eradication subgroup samples, respectively. The identified gene groups included rifamycin-resistant beta-subunit of RNA polymerase (*rpoB*) genes, resistance-nodulation-cell division antibiotic efflux pump genes, *pmr* phosphoethanolamine transferase genes, multidrug and toxic compound extrusion transporter genes, major facilitator superfamily antibiotic efflux pump genes, macrolide phosphotransferase genes, *ampC*-type BL genes, general bacterial porins with reduced permeability to BL genes, ATP-binding cassette antibiotic efflux pump genes, and others.

4. Discussion

The misuse of antibiotics has led to a rapid increase in antimicrobial resistance among clinically relevant microorganisms, particularly Gram-negative bacteria [74–78]. Resistance to beta-lactam antibiotics is an emerging problem in healthcare due to extremely limited therapeutic options. Additionally, resistance to this group of antimicrobials is often associated with resistance to other drugs [78, 79]. The emergence of ESBL enzymes highlights the importance of understanding how the associated genes could alter the abundance in gastrointestinal microbiome under prolonged antibiotic pressure. As this phenomenon is difficult to study using culturomics-based approaches and subsequent detection of phenotypes, due to unknown cultivation requirements of most members of the gastrointestinal microbiome, the only alternative is to employ molecular biology approach. Therefore, we developed a panel for detection and long-term abundance and prevalence assessment of BLs in *H. pylori*-infected patients before and after a single eradication event.

We were able to identify the majority of targeted ESBL clusters in samples from the experimental cohort. However, there were also some BL gene clusters that were not detected by our panel. Although the most obvious explanation for this would be the absence of a target in the pool of extracted bacterial DNA, it is also possible that, in some cases, the primer bound primarily to the off-target region due to high sequence homology or failed to bind entirely to intended target due to such wet-lab-related aspects as incompatibility with the annealing temperature used. However, detailed examinations involving qPCR or even digital PCR would be necessary to assess the validity of these speculations. Although some studies have used a *16S rRNA* gene copy normalization method to improve the estimation of the actual relative abundance of taxonomic groups, these attempts have so far been unsuccessful because bacterial taxa can have variable numbers of *16S rRNA* gene copies [80, 81]. However, the list of genetic elements in Bacteria that would display sufficient sequence conservation across all taxa is limited, therefore, in this work, *16S rRNA*-based normalization was performed to standardize sequencing results between samples.

The results of this study showed that the majority of BLs originated from Gram-negative bacteria from the genus *Enterobacteriaceae*. This finding is consistent with the results of several studies that demonstrated that the prevalence of ESBL-producing *Enterobacteriaceae* is increasing, even in healthy asymptomatic individuals [82, 83], where it is thought to serve as a reservoir for the spread of ESBLs. The most abundant and prevalent ESBLs in the experimental group were bla_{EC} from carbapenem-targeting class C ESBLs [84], the cephalosporinase gene cbl_A from class A BL targeting ESBLs [85] and bla_{ACI} from class A ESBLs [84]. As infections caused by ESBL producers are associated with increased mortality, length of hospital stays and increased treatment costs [86–88], the widespread identification of ESBLs in our patient samples suggests, that it is indeed a growing problem in today's society. To date, a large proportion of ESBL types have been poorly characterized and the focus is mainly on those of clinical

relevance. Therefore, decoding the effects of ESBLs in the host or environment in the context of our study is challenging. However, large-scale identification of ESBL genes in patient samples by the most sensitive molecular methods can still provide valuable information about their epidemiology.

Considering the abundance of ESBL clusters at the level of each sample, we found that most were absent in the individual samples of all three datasets, with the lowest number of clusters found in samples from the metagenomic dataset. While this might seem a striking result, we also observed that there are several overlapping clusters between the datasets, possibly reflecting the core population of ESBLs characteristic for our patient group. Four of these clusters originated from class A, three from class C, one from class D, and one from an unknown BL class. Furthermore, the number of ESBL clusters differed between treatment states. In all three groups (experimental, validation-targeted, and validation-metagenomic)–the number of ESBL gene clusters in the pre-eradication state was higher than in the post-eradication state, suggesting that some ESBL producers could be eliminated during the eradication therapy, resulting in an overall decrease in BL producers. Given that abundant bacterial species (e.g., the core microbiome) are relatively stable over time, statistically significant changes mainly affect bacteria with low abundance. Thus, it is plausible that these ESBL producers belonged to low-prevalence bacteria such as *Enterococcus hirae*, which has been reported to be tolerant to beta-lactam antibiotics [89, 90], and *Megamonas funiformis*, which in multiple cases has been reported as a carrier of various beta-lactamase genes in its genome; for both bacteria, their abundance was significantly reduced in the post-eradication state compared to the pre-eradication state [91]. On the other hand, the abundance of *Enterocloster bolteae* and *Eggerthella lenta* was increased in the post-eradication group. *E. bolteae* has been shown to be capable of producing beta-lactamases [92, 93], as was *E. lenta* [94]. Generally, monotherapy is used to treat common infections such as pneumonia [95] and urinary tract infections [96], while dual antimicrobial treatment is used for *H. pylori*. Therefore, it is plausible that the use of two types of antimicrobial agents may have a synergistic effect that contributes to the reduction of BL producers [97]. If this is true, it is possible that one way to limit the spread of antimicrobial resistance would be to use multiple antimicrobial agents at the same time, not only for *H. pylori* infection, but also for other infectious diseases.

In this study, we were able to detect several ESBL clusters whose relative abundances differed significantly between pre- and post-eradication states in both targeted datasets. The annotated taxonomic source indicated that these ESBLs originate from both–Gram-positive and Gram-negative bacteria, such as *Klebsiella* sp., *Pseudomonas* sp., *Acinetobacter* sp., *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia* and others. Most of these are known ESBL producers, suggesting gastrointestinal carriage and asymptomatic colonization of these organisms. Although the top abundant BL groups were common between the treatment states, the annotated taxonomic source of BL differed between the experimental and targeted validation groups, indicating that resistance genes in the post-eradication group were uptaken from the environment during the reestablishment of gut microbiome. These results were further supported by taxonomic profiles of the metagenomic dataset, which showed that some bacterial species whose ESBL clusters differed significantly between treatment states were increased in the post-eradication state (e.g., *Enterobacteriaceae* family, *Pseudomonas aeruginosa* and *Serratia marescens*), while for some the trend was opposite (e.g., *Escherichia coli*, *Klebsiella* sp. and *Ralstonia picketii*). Considering that these were long-term evaluations and that the mean age of the current patient group was 52 years, and that our previous study [98] uncovered stronger gut microbiome associations with patient-specific characteristics such as age, individual, gender, and medical history, it is plausible that the reservoir of BLs and possibly other AMR genes changes more dynamically than the bacteria themselves between both–Gram-positive and

Gram-negative bacteria. Therefore, our findings suggest that in the future greater emphasis should be placed on the development of novel probiotic products and procedures for the controlled gut microbiome reestablishment. These products should ensure that patients' guts are colonized by as few resistant microorganisms as possible, thus mitigating the situation where our microbiota serves as a reservoir of ESBLs. In addition, healthy lifestyle choices must be added to control the restoration of a non-resistant gut microbiome. Various living conditions, fluctuating lifestyles, diet, physical and/or outdoor activities, mental stress and other factors have been shown to contribute to the health of microbiome and host [99–101], therefore, during the period of microbiome restoration, reinforced attention should also be paid to appropriate lifestyle choices.

Considering the prevalence of ESBL clusters, the highest number of clusters was found in the experimental and targeted validation groups. There were only a few ESBL gene clusters in the metagenomic dataset, suggesting that ESBL abundance is at a very low level. Accurate analysis of the presence or absence of each cluster is possible by amplifying the perspective BLs or by increasing the target read count in metagenomic analyses. However, the amplification step in ESBL panel-based sequencing library preparation may lead to biases in the abundance of some ESBL gene clusters, especially those that share sequence homology.

In this study, we also evaluated the effect of *H. pylori* eradication therapy on the taxonomic composition of the gut microbiome using data obtained from shotgun metagenomic sequencing. We found that the microbiome diversity rate was significantly higher in the pre-eradication state than in the post-eradication state. This observation is consistent with literature data, which show that the gut microbiota is significantly altered immediately after eradication therapy and gradually restores to the baseline parameters over time; however, certain alterations may persist up to a year after completion of eradication therapy[98, 102–104]. Although we observed that diversity in the post-eradication state was lower than in the pre-eradication state, we did not observe significant changes in the beta diversity analysis, suggesting that the global composition of microbial communities was highly similar between the pre- and post-eradication states. Indeed, the relative abundance of bacterial species was highly similar between the eradication states and were dominated by *Faecalibacterium prausnitzii*, *Bacteroides vulgatus*, *B. dorei* and *B. uniformis*. Previous studies have suggested that some *F. prausnitzii* genogroups might contain class A BL [105], which was also the dominant BL class in the shotgun validation group, while *Bacteroides* sp. is a well-known group of BL-producing bacteria [106, 107]. In addition, cbl_A gene of class A BL with an annotated taxonomic source of *B. uniformis* was the predominant BL gene in the shotgun validation group, and its abundance was higher in the post-eradication group. In addition, during the association analysis, the abundance of certain microbial species varied between the pre- and post-eradication states. For instance, in the pre-eradication subgroup, we observed increased levels of *Acidaminococcus intestinalis*, previously shown to be elevated in overweight adults [108]. Similarly, we detected increased levels of *Collinsella aerofaciens* and *Treponema succinifaciens* in the pre-eradication subgroup. The former has been associated with low dietary fiber intake and anti-inflammatory effects on the intestinal epithelium [109, 110], while the later was enriched in traditional rural populations [111]. Altogether, minor differences exist in the composition of the microbiome between the pre- and post-eradication states, although these differences may be more related to the diet and general state of health than to the eradication therapy itself. However, in-depth studies involving the reconstruction of bacterial genomes from metagenomic data would be necessary to correlate bacterial abundance with ESBL gene abundance.

In addition to evaluating and validating the ESBL screening panel, we investigated the functional profile and resistome of the microbiome. Thus, we were able to show that the abundance of several genes was increased in the post-eradication state. These genes have a role in the

transport of molecules and ions; biosynthetic processes of cobalamin and extracellular polysaccharides; and methylglyoxal, arabinose, and glutamine metabolism. All the above-mentioned processes have a positive and beneficial effect on the human host, most profoundly in the case of cobalamin and extracellular polysaccharides. Additionally, our data also suggests that the abundance of several genes was decreased in the post-eradication state. These genes have a role in the DNA restriction-modification system, DNA-templated transcription and initiation, as well as in the biogenesis of ribosomal small subunits. Their increased levels may be related to the active reproduction of bacteria and the protection of their genome against the invasion of foreign DNA. In addition, since we detected minor differences in the abundance and prevalence of ESBL gene clusters between treatment states, we also evaluated the entire resistome profile. The number of AMR genes detected in the post-eradication subgroup was higher than in the pre-eradication subgroup. Thus, it is apparent that AMR gene diversity has increased under the pressure of antimicrobial therapy. All samples from the pre-eradication subgroup contained three AMR genes–the resistance-nodulation-cell division antibiotic efflux pump gene *adeF*, the tetracycline-resistant ribosomal protection protein gene *tetQ*, and the trimethoprim resistant dihydrofolate reductase dfr gene *dfrF*, while four were detected in all samples from the post-eradication subgroup: Erm 23S rRNA methyltransferase gene *ErmF*, the major facilitator superfamily antibiotic efflux pump gene *tet(40)*, and the tetracycline-resistant ribosomal protection protein genes *tetO* and *tetW*. Furthermore, AMR genes conferring macrolide resistance increased in the post-eradication subgroup. In one study subject, we were able to detect *Chlamydia trachomatis* 23S rRNA with mutations conferring resistance to macrolide antibiotics such as clarithromycin, which was prescribed to study participants in the current study. Although *C. trachomatis* is commonly associated with sexually transmitted diseases [112], it has also been shown that the human gastrointestinal tract might be a site of persistent infection with this pathogen [113, 114]. Moreover, in the post-eradication state, we observed an increase in AMR gene families, macrolide esterase and macrolide phosphotransferase, both of which contribute to the inactivation of macrolide antibiotics. This observation might be of increased importance because other studies have shown that previous macrolide use (even 10–12 years ago) correlates with low *H. pylori* eradication rates with clarithromycin-based triple antibiotic therapy [115, 116], thus emphasizing the ability of resistance genes to persist in the intestinal tract. In addition, functional analysis confirmed that the relative abundance of gene products involved in the spread of resistance, such as tyrosine recombinase, and gene products involved in signal transduction pathways, such as sensor histidine kinase, was increased in post-eradication state. Given that a sensor histidine kinase can sense the presence of antibiotics and activate transcription of AMR genes, these results, together with elevated levels of potential multidrug resistance proteins, highlight the need for increased attention to AMR gene distribution and dynamics using multi-omics approaches. To date, some studies have reported alteration in the number of AMR genes after *H. pylori* eradication therapy [117, 118], however, little is known about the functional mechanisms of gut microbiome dynamics after antibiotic treatment in the long term.

Despite all this wealth of knowledge, this study had several limitations. First, some targeted ESBL-coding gene clusters were absent in all patient samples, and the reason behind their absence remained ambiguous. Therefore, the designed primers that targeted ESBL-coding gene clusters should be further validated using methods such as RT-qPCR or digital PCR. Subsequently, although the validation group was able to mimic the experimental group, greater patient involvement is needed to increase the resolution of the diversity and abundance of genes encoding ESBLs and AMRs, particularly in the metagenomic dataset. In this study, we also observed that some individuals remained *H. pylori*-positive after eradication therapy, but the sample size was too small to confirm this observation. Lastly, this study did not assess

resistance to the prescribed antibiotics, amoxicillin and clarithromycin, and did not include *H. pylori* genomic characterization. However, considering the high prevalence of *H. pylori* in the Latvian population, we believe that such studies are of paramount importance and should be addressed in the near future.

Our study suggests that an NGS-based large-scale screening panel of ESBL-encoding genes can be used for accurate population screening and surveillance of ESBL genes in symptomatic and asymptomatic infections. The applicability of the currently developed methodology is not limited to the detection of ESBL-encoding genes in the gut microbiome of *H. pylori*-infected patients, but can also potentially be applied to different samples, populations and various infection cases facing increased resistance to cephalosporins, amoxicillin, penicillin and other. In addition, these results suggest BL recolonization during restoration of the gut microbiome, implying that greater microbiome control would be necessary after antibiotic treatment. In conclusion, we believe that the ESBL screening panel is suitable for screening changes in the prevalence of ESBL coding genes, and in-depth research of the resistome is required to better understand the AMR gene reservoir in relation to antibacterial therapy, which could aid clinicians in choosing antibacterial therapy in the future.

Supporting information

S1 File. Appendix of statistical data.
(DOCX)

S1 Table. Primer sequences with the respective targeted ESBL clusters.
(XLSX)

S2 Table. Cluster IDs and NCBI derived annotations.
(XLSX)

S3 Table. Antimicrobial resistance genes, gene families and resistance mechanisms of the metagenomic sample set as detected by RGI and CARD reference database.
(XLSX)

Acknowledgments

Authors would like to thank the GISTAR project for providing biological material.

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4 DISCUSSION

With the rapid development of molecular biology technologies, new knowledge is accumulated daily. These technologies have greatly enhanced our understanding on the complex relationships between bacteria and their hosts, different bacterial species, as well as bacteria and viruses at genomic, transcriptomic, proteomic, metabolomic, and taxonomic levels. While many aspects of these relationships have been uncovered, there is still much left to explore, especially concerning antimicrobial resistance. However, uncovering the most complex of the relationships requires access to a large number of samples, while one of the prerequisites for a successful microbiome study implementation is employment of validated of sampling methods. Therefore, while developing this thesis I, in collaboration with colleagues from Latvian Biomedical Research and Study centre, Institute of Clinical and Preventive Medicine, and Karolinska Institutet, evaluated the applicability of FIT test containers in microbiome studies, which was followed by the evaluation of standard triple *H. pylori* eradication therapy impact on the GIT microbiome and ESBL coding gene levels.

4.1 Microbiome sample stability in faecal immunochemical test containers

When we started the study on validation of FIT sample containers for use in microbiome studies, it was already evident that various sample storage conditions significantly influenced microbiome profiles (Choo *et al.* 2015). Thus, it became apparent that the development of uniform techniques, standards and protocols was necessary to minimize this bias. Particularly, there was a need for sampling methods that were applicable to large cohorts (Tedjo *et al.* 2015), yet also used within nation-wide screening programs.

Due to challenges in arrangement of immediate sample freezing and adequate transportation, the use of frozen faecal material in large-scale microbiome research is impractical and/or expensive. Therefore, most studies rely on various preservatives. Some years ago, RNAlater (Thermo Fisher Scientific, USA) was the most commonly used sample preservation solution in human microbiome studies. Although it has generally been demonstrated to preserve nucleic acids effectively, some studies have indicated that this media may reduce the yield and purity of the bacterial DNA and, after 72 hours of storage, alter the abundance of several bacterial phyla (Fu *et al.* 2016). However, in the context of microbiome research, there were only a few studies that were dedicated to the comparison of sample stability within FIT containers (Hundt 2009; van Rossum *et al.* 2009; De Girolamo *et al.* 2016), and none of them involved metagenome analysis.

Therefore, in the first study, we compared faecal sample stability within the FIT sample collection containers and compared the results using the two most frequently used sequencing platforms at that time – Ion Torrent PGM and Illumina MiSeq. During initial attempts, we performed DNA extraction from both the FIT buffer suspension and the solid particles in the samples. These approaches either yielded low amounts of DNA or showed significant variations in bacterial community composition among samples from the same individual. Upon re-evaluating our sample collection procedure, we identified two key factors. First, due to the collection rod's design, the collected sample's total mass was smaller than 10 mg. Second, the composition of the storage buffer possibly caused more readily lysis of certain bacterial types. Consequently, we decided to perform the extraction from the entire samples using a lyophilisation procedure. This adjustment resulted in obtaining up to 30 times more DNA than extraction from the FIT buffer suspension alone. Interestingly, a recent study with a design similar to ours indicated that concentrating samples using a lyophilisation procedure had no significant impact on alpha and beta diversity or the taxonomic profile compared to fresh-frozen or immediately frozen samples stored in FIT sample containers (Masi *et al.* 2020). Given that they also used bead-beating-based DNA extraction combined with targeted sequencing, it might be worthwhile to re-evaluate and further develop our proposed methodology for DNA extraction from the FIT tube sample containers. This could potentially facilitate the DNA extraction procedure and reduce sample processing time.

While sequencing by the Illumina MiSeq platform allows the analysis of two variable regions and thus identifies a proportionally larger number of OTUs, a higher microbial diversity was achieved by the Ion Torrent PGM, but the most likely explanation to that is a tenfold higher acquisition of sequence reads. Further on, while testing the absolute OTU abundances within the samples, we did not find a statistically significant difference between immediately frozen samples (−86°C and −20°C without buffer and with FIT), which is in accordance with results from other studies (Cardona *et al.* 2012; Carroll *et al.* 2012; Hale *et al.* 2015; Song *et al.* 2016; Masi *et al.* 2020; Zouiouich *et al.* 2023).

The bacterial community structures and taxonomical distribution of faecal samples remained relatively stable across different storage conditions in the FIT buffer, retaining their composition even after 14 days at 20°C or seven days at 30°C. One of the storage conditions in our study protocol was aimed to mimicking the typical sample treatment procedure during transportation to a central laboratory using standard mail delivery. It involved storing samples for two days at 4°C followed by an additional two days at 20°C. Our results indicated that

under these conditions, the faecal microbiota remained stable in both *16S rRNA* and metagenome analysis. However, our acquired data also showed a gradual increase in the relative abundance of Gram-positive bacteria over time, accompanied by a decrease in the abundance of Gram-negative bacteria. This conjecture might be attributed to the presence of the FIT buffer, primarily designed to release and preserve haemoglobin in faecal samples. As described in Patent US5198365A (Grow and Shah 1993), the FIT buffer might contain antimicrobial agents like penicillin and streptomycin, proteolytic activity inhibitors, enzymatic activity inhibitors/deactivators like formaldehyde, and other protective agents. These substances could potentially affect the taxonomical composition by arresting the growth of microorganisms with thinner cell membranes. Consequently, we speculate that the FIT buffer has a minor effect on bacterial composition during long-term storage, which might introduce some inaccuracy in results regarding the relationship between Gram-positive and Gram-negative bacteria. This trend has also been observed in other studies, where the relative abundance of such Gram-positive genera as *Blautia* and *Bifidobacterium* tended to increase, while such Gram-negative genera as *Parabacteroides* and *Bacteroides* tended to decrease during room temperature storage in FIT sample containers for multiple days (Masi *et al.* 2020). However, further investigation is required since the overall microbial composition generally varies by individual rather than by the storage condition or employed sequencing platform. Additionally, the relationship between Gram-positive and Gram-negative bacteria in other studies remains unclear (Krigul *et al.* 2021; Zouiouich *et al.* 2022; Brezina *et al.* 2023).

In summary, although the "Eiken Chemical" FIT buffer contains a chemical reagent mixture optimized for detecting hidden blood in faeces, we found no significant influence on bacterial composition. Therefore, our results prove that faecal material stored for up to seven days in FIT sampling bottles, whether in a household fridge, at room temperature, or within a hot climate, remains suitable for *16S rRNA* and metagenome-based microbiome research. This validity depends on following an appropriate protocol for faecal sample treatment and DNA extraction and, therefore, is applicable to large-scale epidemiology studies. To conclude, this was the first study assessing the wide range of storage conditions for samples collected in FIT sample collection containers for use in microbiome research. We believe that this study has encouraged the validation and employment of various other types of FIT collection containers in microbiome studies.

4.2 Long-term impact of the first-line *Helicobacter pylori* eradication therapy on the gastrointestinal tract microbiome and resistome

To decrease the incidence of gastric cancer, current international guidelines and expert working groups encourage the employment of "test-and-treat" strategy for *H. pylori* infection. This approach is expected to yield the highest benefits in countries with an increased incidence of gastric cancer and a high prevalence of *H. pylori* (Leja *et al.* 2017; Malfertheiner *et al.* 2022). The rationale behind this strategy is that 1-3% of infected individuals eventually develop gastric cancer (Wroblewski *et al.* 2010; Zhang *et al.* 2017). Moreover, as early as 1994, the IARC classified *H. pylori* infection as a class I carcinogen (IARC 1994).

In countries where the "test-and-treat" strategy would be most beneficial, this would mean offering an eradication regimen for most of the population. For instance, in Latvia, this would require antibiotic treatment for approximately 80% of the population (Leja *et al.* 2012). As a consequence, this would change the current status of our country from a low antibiotic-consumption country to an average consumption country (Leja and Dumpis 2020). However, the potential adverse effects of such therapies on the microbiome are still a subject of debate and ongoing investigation.

At the time when I and my colleagues were working on our second manuscript, which was aiming at elucidation of the *H. pylori* eradication therapy's long-term effects on the GIT microbiome, there were already reports of several studies that also investigated these aspects. However, due to the wide range of *H. pylori* treatment approaches that are used in various geographical regions and the previously observed variations in the GIT microbiome response to different antibiotics (Chen *et al.* 2022), only a limited number of those studies evaluated GIT microbiome changes following first-line *H. pylori* eradication therapy, which involves PPIs in combination with clarithromycin, amoxicillin, or metronidazole. Thus, one research group conducted two complementary studies within a two-month period to evaluate the impact of eradication therapy on the GIT microbiome. They observed that in comparison to healthy individuals, *H. pylori* infection and subsequent eradication therapy influenced bacterial richness and composition. In addition, these studies suggested that following the eradication therapy the GIT microbiota enhanced glucose, carbohydrate and SCFA metabolism, involving genera such as *Megamonas*, *Butyricimonas*, *Bifidobacterium* and *Lachnobacterium* (Cornejo-Pareja *et al.* 2019; Martín-Núñez *et al.* 2019). Another study that aimed at evaluation of long-term GIT microbiome changes at six, 12 and 18 months following a first-line *H. pylori* eradication regimen revealed insignificant differences in microbial richness and diversity at

these time points compared to the pre-eradication state. However, they did note variations in low-abundant genera between the pre- and post-eradication states, for instance, there was an increase of genera *Ruminococcus* at six months, *Dialister* at 12 months, and *Helicobacter* at 18 months (Yap *et al.* 2016). Nevertheless, it's important to note that all these studies included relatively small sample sizes and thus were subject to insufficient statistical power; therefore, the assessment of long-term *H. pylori* eradication impact on the GIT microbiome remained largely unexplored.

Evaluating the long-term impact of the *H. pylori* eradication regimen on the GIT microbiome, we did not observe a substantial change in bacterial richness between pre- and post-eradication states, which is consistent with other studies (Yap *et al.* 2016; Hsu *et al.* 2019; Liou *et al.* 2019; Zhou *et al.* 2021). Therefore, it is not surprising that the individual itself was the most significant factor contributing to the GIT microbiome composition. However, at the genus level, we did observe some differences in the relative abundance between the pre- and post-eradication states. These differences, however, were on the edge of statistical significance. Most of such genera were present at very low abundances, and only the thermophilic *Lebetimonas* and the common GIT microbiome member *Parapretovella* exceeded a relative abundance of 1%. Specifically, the relative abundance of the *Paraprevotella* genus was enriched, while that of *Lebetimonas* was depleted in the post-eradication state. Recent studies have positively associated *Paraprevotella* with circulating lipid levels, neuromodulation (Bourdeau-Julien *et al.* 2023), and the inflammatory factor IL-6 in obese children (Yuan *et al.* 2021). In contrast, the genus *Lebetimonas* has not been described in the context of the human GIT. Therefore, although the enrichment of *Paraprevotella* appears to have potential health-enhancing benefits as a long-term consequence of the eradication therapy, it is challenging to evaluate its true enrichment due at least for three reasons. First, we did not perform comparisons to healthy individuals; second, the statistical significance of the differential expression analysis was relatively high; and third, examining consecutive time points within the two years would aid in explaining the observed differences and their significance. Similar observations regarding the low-abundant genera were also reported in other studies (Hsu *et al.* 2019; Liou *et al.* 2019); however, these changes might also be linked to dietary patterns, physiological factors, lifestyle, and environmental changes during the long-term assessment, and therefore, they remain largely unexplored.

In third publication of my thesis, we also conducted a superficial assessment of taxonomic differences in the GIT microbiome before and after the first-line eradication therapy,

however at this time we used the shotgun metagenomic sequencing approach. Although the number of participants was half of that we had in our second publication, the comparison between pre- and one-year post-eradication datasets revealed a reduction in species richness. Our results contradict those acquired by other researchers that studied changes within the same timeframe, using the targeted sequencing, because according to their observations the species richness was restored within one year after the first-line eradication treatment (Liou *et al.* 2019; Zhou *et al.* 2021). However, two factors should be considered here – first, metagenomic sequencing may provide enhanced taxonomic resolution, potentially reflecting truer fluctuations of species abundancies (Oh *et al.* 2016); second, there are significant differences in the number of individuals recruited for each study, which, in turn, affects statistical power. Furthermore, we identified 12 microbial species that were differentially abundant between the pre- and post-eradication states. Similar to previous research studies, the relative abundance of predominant taxa remained similar between the pre- and post-eradication states (Yap *et al.* 2016; Hsu *et al.* 2019; Chen *et al.* 2022). Among the differentially abundant species, only *Collinsella aerofaciens* exceeded a relative abundance of 1%, while the others were present at very low abundances. In our study, its abundance was lower in the post-eradication state compared to the pre-eradication state. *C. aerofaciens* has been described as a pathobiont in the human GIT, and its presence has been shown to induce a pro-inflammatory reaction by the host within the GIT, leading to increased gastrointestinal permeability (Chen *et al.* 2016; Kalinkovich and Livshits 2019). Consequently, shotgun metagenomic taxonomic analysis extends previously described observations regarding the potential health-beneficial consequences of eradication therapy.

In general, taxonomy-based studies provide evidence of an immediate reduction in species richness and diversity after the *H. pylori* eradication therapy (Yanagi *et al.* 2017; Hsu *et al.* 2019; Liou *et al.* 2019; Guillemard *et al.* 2021; Chen *et al.* 2022; Wang *et al.* 2022). Nevertheless, over the long term, the GIT microbiome composition tends to return to its initial state, suggesting that the eradication therapy of *H. pylori* has a minimal interfering effect on it (Yap *et al.* 2016; Hsu *et al.* 2019; Liou *et al.* 2019; Gudra *et al.* 2020, 2023). However, when it comes to the GIT resistome, the landscape is slightly different.

In our third study, we extended taxonomic analyses by investigating the GIT resistome of *H. pylori* infected individuals before and after the eradication treatment. Here we observed a long-term decrease in the abundance of ESBL-encoding genes. This finding suggests that some bacteria containing ESBL genes in their genome may be eliminated during the eradication

therapy. Given the global rise in the prevalence of ESBL-producing bacteria (Bezabih *et al.* 2021), the use of two broad-spectrum antibiotics may reduce the levels of ESBL producers within the GIT. In contrast, earlier studies reported an increase in the relative abundance of BL genes within the GIT microbiome within a few days (Olekhnovich *et al.* 2019) and one month (Oh *et al.* 2016) after quadruple and triple *H. pylori* eradication therapy, respectively. However, both studies focused on short-term observations; therefore, it is plausible that further shifts in the abundance of BLs might appear during the long-term observation. While the diversity of ESBLs decreased, the opposite trend was observed for other ARGs. Although we did not determine the relative abundance of ARGs, it was evident that the number of different ARGs was higher in the post-eradication state, suggesting that bacteria under antibiotic pressure expanded their functional capabilities.

Only a few studies have evaluated the shifts in ARGs during *H. pylori* eradication therapy. One study reported that similar to taxonomic changes, ARGs also exhibited significant shifts in their abundance soon after the eradication therapy, but their abundances returned to baseline levels within six months. At the same time, they also observed that subjects with a higher diversity of ARGs at baseline, especially the abundance of the *ErmF* gene, were more likely to fail the first-line *H. pylori* eradication therapy. Additionally, patients who had previously experienced unsuccessful *H. pylori* eradication therapy showed elevated levels of the *ErmF* gene at baseline (Wang *et al.* 2022). This finding was intriguing, as in our patient cohort that underwent metagenomic sequencing, four patients remained *H. pylori* positive after the triple eradication therapy. The *ErmF* gene was detected in all those subjects and in the majority of the other patients (n=21). Furthermore, by the end of the follow-up period, all individuals had acquired the *ErmF* gene, suggesting that the failure of the eradication therapy is not limited to only the *ErmF* gene, but might involve a more complex response of the bacterial population, which involves activation of various ARGs. This is further supported by research conducted by Olekhnovich *et al.* (2019), who explored short-term alterations in the GIT resistome following a quadruple eradication regimen for *H. pylori*. By reconstructing bacterial genomes, they discovered that multiple ARGs were localized near each other in some bacterial species, and certain ARGs, such as 23S rRNA methyltransferases, had acquired new mutations to ensure their survival upon antibiotic exposure (Olekhnovich *et al.* 2019). Therefore, these results collectively indicate persistent increase of antibiotic-resistant microorganisms within the GIT microbiome after the *H. pylori* eradication.

In summary, taken together, it is possible that at the level of the individual, observed changes in the GIT microbiome following the first-line eradication therapy are not vitally important; however, from the global society health viewpoint, these changes might significantly impact the dissemination of ARGs and in turn, the efficiency of broad-spectrum antibiotics that are used to combat bacteria-induced infections.

5 CONCLUSIONS

1) Faecal Immunochemical Test (FIT) sample container maintains the microbial community structure of faecal material for up to one week.
2) Material stored in a FIT sample container, along with appropriate sample treatment prior to DNA extraction, can be used for *16S rRNA* gene and metagenome sequencing analysis.
3) The overall gastrointestinal taxonomic composition did not differ significantly between the pre- and post-eradication states of *H. pylori*. Certain low-abundant genera differed between the treatment states; however, they tend to have health-enhancing effects.
4) The most significant determinant of the gastrointestinal microbiome composition between the pre- and post-eradication states was the individual and its specific characteristics, not the eradication therapy.
5) The abundance of extended-spectrum β-lactamase (ESBL) genes within the pool of gastrointestinal microorganisms is very low; therefore, amplification of ESBL coding genes is a cost-effective approach to increase the resolution of their abundance and prevalence.
6) Relative abundances of ESBL coding genes remained similar between the pre- and post-eradication states; however, the relative abundance of some classical ESBL producers, such as *Klebsiella* spp. bla_{OXY} and *Acinetobacter* spp. bla_{ADC} tended to decrease.
7) Developed ESBL coding gene panel may be used as a cost-effective population or environmental screening method.
8) The diversity of antimicrobial resistance genes, other than β-lactamases, increased during the long-term assessment of *H. pylori* eradication therapy.

6 THESIS

1) The Faecal Immunochemical Test (FIT) used in national colorectal cancer screening programs can be used in gastrointestinal microbiome research.
2) Short-term first-line *H. pylori* eradication therapy does not cause significant long-term shifts in the taxonomic composition of the gastrointestinal microbiome.
3) Short-term first-line *H. pylori* eradication therapy reduces the richness of genes encoding extended-spectrum β-lactamases.
4) Short-term first-line *H. pylori* eradication therapy does not alter the composition of other antimicrobial resistance genes.

7 PUBLICATIONS

I. Gudra D, Shoaie S, Fridmanis D, Klovins J, Wefer H, Silamikelis I, Peculis R, Kalnina I, Elbere I, Radovica-Spalvina I, Hultcrantz R, Skenders G, Leja M, Engstrand L. (2019). A widely used sampling device in colorectal cancer screening programmes allows for large-scale microbiome studies. *Gut* **68**: 1723-1725. doi: 10.1136/gutjnl-2018-316225

II. Gudra D, Pupola D, Skenders G, Leja M, Radovica-Spalvina I, Gorskis H, Vangravs R, Fridmanis D. (2020). Lack of significant differences between gastrointestinal tract microbial population structure of *Helicobacter pylori*-infected subjects before and 2 years after a single eradication event. *Helicobacter* **25**: e12748. doi: 10.1111/hel.12748

III. Gudra D, Silamikelis I, Pjalkovskis J, Danenberga I, Pupola D, Skenders G, Ustinova M, Megnis K, Leja M, Vangravs R, Fridmanis D. (2023). Abundance and prevalence of ESBL coding genes in patients undergoing first line eradication therapy for *Helicobacter pylori*. *PLOS ONE* **18**(8): e0289879. doi: 10.1371/journal.pone.0289879

8 APPROBATION OF RESEARCH

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9 ACKNOWLEDGMENTS

This study was supported by the European Regional Development Fund grant No. 1.1.1.1/16/A/272 "Long-term effects of *H. pylori* eradication on gastrointestinal tract microbiome and development of screening system for detection of extended-spectrum beta-lactamase coding genes within faeces samples" by the European Social Fund grant No. 8.2.2.0/20/I/006 "Strengthening of the capacity of doctoral studies at the University of Latvia within the framework of the new doctoral model" and in part by the Swedish Research Council (grant No. K2013-56X-10840) and the Söderbergs Foundation. I would also like to thank BioSan Ltd and the Foundation of the University of Latvia for financial support during my PhD studies.

I sincerely thank my supervisor, PhD Dāvids Fridmanis, for involvement in the research and support throughout my doctoral studies. I am grateful for our fruitful and inspiring discussions and his encouraging advice in various challenges that arose during the research. I would also like to thank the rest of the group, especially MSc Maija Ustinova, for the successful teamwork.

I also express my gratitude to Prof. MD Mārcis Leja and Prof. MD Lars Engstrand for their suggestions and help with the preparation of manuscripts. I thank MSc Darta Pupola and PhD Saeed Shoaie for their successful collaboration during the research and preparation of manuscripts for the first two publications.

My deepest gratitude also goes to GISTAR project implementors for their longitudinal national-level work to reduce gastric cancer mortality in Latvia.

I also extend my gratitude to the Latvian Biomedical Research and Study Centre, the Genome Centre, and the Bioinformatics Core Facility for providing the infrastructure, services, and knowledge necessary for conducting this research. I also acknowledge Riga Technical University's HPC Center for providing access to its computing infrastructure.

Last but not least, the warmest thank you goes to my husband, family, and friends for their support, encouragement, and understanding during the long nights and weekends of work.

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