Development and use of an ESBL coding gene panel in patients undergoing first-line eradication therapy for *Helicobacter pylori*

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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. The epidemiology of these infections is complex and combines the expansion of mobile genetic elements with clonal spread. Insufficient empirical therapy for serious infections caused by these organisms is independently associated with increased mortality. Here, we developed an ESBL coding gene panel, evaluated the abundance and prevalence of ESBLs encoding genes in patients undergoing *H. pylori* eradication therapy, and summarized the effect of eradication therapy on gut microbiome functional profiles. To assess the repertoire of known beta lactamase (BL) genes, we divided them in clusters according to their evolutionary relation, designed primers for amplification of cluster marker regions and assessed efficiency of this amplification panel on 120 fecal samples acquired from 60 patients undergoing *H. pylori* eradication therapy. In addition, fecal samples from additional 30 patients were used to validate the detection efficiency of designed 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 across the 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 among all three datasets, suggesting a patient-specific prevalence 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 at least partly might be attributed to the therapy. This study demonstrated that the ESBL screening panel was efficient for 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.

1. Introduction

Appropriate and inappropriate use of antimicrobials is a well-established driver of resistance (Arcilla et al., 2017; El Salabi et al., 2013; Oo et al., 2022) because it can facilitate the selection of resistant bacteria (Mulder et al., 2021; von Wintersdorff et al., 2016) by opening an ecological niche where resistant pathogens can flourish (Colson et al., 2021). Several steps have been suggested to slow down the emergence of resistance and preserve antimicrobial efficiency. These included controlled usage of antibiotic agents in farming, development of strategies to prevent illnesses, improved antibiotic usage strategies, development of novel antimicrobial agents, and others (Alghamdi, 2021; Colson et al., 2021). Therefore, new technologies and improved diagnostics are required to tackle at least some of the aforementioned issues and ensure that antimicrobial agents are only used when necessary.

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 inactivate these antibiotic agents by hydrolyzing the amide bond in the β-lactam ring (Shaikh et al., 2015), thus compromising the effectiveness of empirical treatment. This inactivation mechanism is employed by a variety of gram-negative bacteria, from such genus as *Escherichia, Klebsiella*, *Pseudomonas*, *Acinetobacter, Citrobacter, Proteus* and others (Aldrazi et al., 2019; Iredell et al., 2016; Ouchar Mahamat et al., 2019; Xia et al., 2016). Constant exposure of bacterial strains to a multitude of beta-lactam antimicrobials has evoked dynamic and continuous production and mutation of BL in these bacteria, thus expanding their activity (Paterson and Bonomo, 2005). These enzymes are known as extended-spectrum BLs (ESBL), and they confer multi-drug resistance to an extended range of beta-lactam antibiotics, including penicillin, amoxicillin, cephalosporin, and others (Aldrazi et al., 2019; Sanjit Singh et al., 2017). BL and ESBL genes are often carried by highly mobile plasmids or other types of mobile genetic elements. The clonal spread of these elements' harbors resistance genes to other bacterial species, further limiting the treatment options for infections caused by ESBL-producing bacteria (Aldrazi et al., 2019; Shaikh et al., 2015). Infections caused by ESBL producers are associated with poor outcomes (Lindblom et al., 2019) and increased mortality (Schwaber et al., 2006; Tumbarello et al., 2006).

ESBLs are widespread worldwide, and it has been estimated that more than 1.5 billion people have been colonized with ESBL-producing bacteria (Doi et al., 2017; Woerther et al., 2013) with the highest rates in Western Pacific, Eastern Mediterranean and Southeast Asia and lowest in America and Europe(Schoevaerdts et al., 2012; Woerther et al., 2013). In general terms, the highest prevalence of this burden is in developing countries, but the spread of ESBL-producing microorganisms is also increasing in developed countries (Doi et al., 2017). Furthermore, antimicrobial use has been reported as an additional risk factor for infections by ESBL-producing bacteria among international travelers (Arcilla et al., 2017; Hassing et al., 2015; Reuland et al., 2016; Ruppé et al., 2018; Wuerz et al., 2020) paving the way for further global spread of the ESBLs. Therefore, understanding the dynamics and prevalence status of the various ESBL encoding genes is crucial for the development of effective control measures, including the prevention of ESBL transmission and decolonization of carriers.

Helicobacter pylori is a chronic gastric pathogen that frequently colonizes the mucosal layers and causes dyspeptic symptoms of variable severity (Malfertheiner et al., 2017). The prevalence of *H. pylori* amounts up to 70% in developing countries (Hooi et al., 2017), although the incidence of infection varies regionally (Malfertheiner et al., 2017). The current first line treatment for *H. pylori* infection consists of a proton pump inhibitor (PPI) and a combination of two antibiotics, among which are amoxicillin, clarithromycin, or metronidazole. These are consumed daily for 7–14 days (Malfertheiner et al., 2017; Suzuki et al., 2010). Within the eradication scheme PPI is used to increase intragastric pH to keep *H. pylori* in a replicative vegetative phase (Ierardi et al., 2019), clarithromycin is a macrolide class antimicrobial agent used to inhibit bacterial protein synthesis(Hassounah et al., 2016) and amoxicillin is a beta-lactam class antimicrobial agent used to inhibit the bacterial cell wall biosynthesis(Karaman, 2015). In addition, since amoxicillin is a broad-spectrum antibiotic used to treat upper and lower respiratory tract, skin and other infections (Castle, 2007), its global consumption rate is increasing rapidly (Browne et al., 2021).

To date, most of the studies have focused exclusively on the main producers of ESBLs, such as *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca* and others to explore the prevalence of ESBL genes in clinical samples. Therefore, in this study we developed a screening panel targeting ESBL genes and used targeted and shotgun sequencing methodologies to investigate the abundance and prevalence of ESBL-coding genes in samples from patients undergoing *H. pylori* first-line triple antibacterial eradication therapy. In addition, we estimated the entire resistome profile of the study participants before and after *H. pylori* eradication therapy. To our knowledge, this is the first study that presents an ESBL screening panel that can identify a broad spectrum of ESBL coding genes that stems from a variety of microbial groups.

2. Methods

2.1 Patients, sample collection and storage

In total, 90 individuals with positive *H. pylori* infection that met the following criteria were included in this study: 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 the age of 20 years, 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 virus infection, or serious psychiatric disorders. To diagnose *H. pylori* infection, the ^{13}C-Urea breath test (Euroisotop, Germany) was performed. A detailed questionnaire containing information such as age, BMI, history of diseases (e.g., gastrointestinal diseases, viral infections, autoimmune diseases, and cancer), and lifestyle habits was collected from each study participant. Samples of 60 individuals, collected over two years, were used for the experimental group of the ESBL panel, and samples of the remaining 30 individuals, collected over one year, were used for the ESBL panel validation (Fig. 1).

Fecal samples were acquired from each recruited patient before starting the standard first-line *H. pylori* eradication (HPE) therapy as well as 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 followed institutional ethical standards, and written consent was obtained from all patients before enrolling in the study.

In total, 180 samples were obtained within 30 minutes after defecation. Samples were transferred to the OC-Sensor tube (Eiken Chemical Co, Japan), immediately homogenized, and stored at -80°C after further treatment according to previously validated storage conditions (Gudra et al., 2019).

2.2 DNA extraction

Fecal samples from the OC-Sensor tubes were extracted using a single-use syringe and transferred to pre-labelled 5 mL tubes. The excess OC-Sensor tube solvent from the samples was removed 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.

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 (Katoh et al., 2002). The number of pairwise non-gapped mismatches between the BL sequences was calculated, and subsequent hierarchical clustering with complete linkage was performed on the alignment. 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 least 10% of the nucleotides would differ in each position. Regions longer than 17 bases 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 (Untergasser et al., 2012). 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 (Reinert et al., 2017). 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 that were overlapping 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 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 (Supplementary 1), 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. One hundred nanograms 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 modules. 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 (Agilent Technologies, USA).

Sequencing depth was calculated to achieve at least 20 million reads per sample (paired end, read length 100 bp). Pooled and circularized libraries were used as templates for DNA nanoball (DNB) preparation. DNBs were loaded onto the PE100 flow cell using an automated DNB loading system. Libraries were sequenced using the DNBSEQ-G400 sequencer and a DNBSEQ-G400RS High-Throughput Sequencing Set (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 (Martin, 2011). Targeted sequencing data were then aligned against the curated BL sequence database using Bowtie2 v.2.3.5.1, pre-set at very sensitive (Langmead and Salzberg, 2012). 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 (Kopylova et al., 2012) using *16S rRNA* sequences obtained from RNA central v10 (The RNAcentral Consortium et al., 2017) 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 (Bolger et al., 2014) 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 (Langmead and Salzberg, 2012). The taxonomical 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 assembling into contigs was performed using the IDBA_UD (Peng et al., 2012) assembler with the *k*-mer length of at least 50. Generated assembly was evaluated using metaQuast (Mikheenko et al., 2015). 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 (Seemann, 2014) with the manually curated Swiss-Prot UniProtKB (Consortium, 2019) 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 (Anders et al., 2015) and the intersection-nonempty resolution mode. Read counts were standardized based on the Transcripts Per Million method (Wagner et al., 2012) using 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 (The pandas development, 2020) 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) (Alcock et al., 2020) and the DIAMOND (Buchfink et al., 2015) 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 (Breitwieser and Salzberg, 2016) 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 (Wirbel et al., 2020) 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 (McMurdie and Holmes, 2013). 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 (Gao et al., 2021) 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 (Mallick et al., 2021) 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

Out of the 180 samples, three failed during the ESBL-targeted amplicon PCR; therefore, these samples with their respective pairs were discarded from further analysis. Thus, in total 174 samples (Table 1) were sequenced. Targeted ESBL quantification on samples from 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 years apart between pre- and post-eradication), while the same analysis on samples from 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 apart 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 apart 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 widely distributed ESBL types. In total, 245 gene clusters encoding evolutionary related ESBLs-were targeted by designed primer pool, and for the normalization of ESBL gene cluster counts, primers targeting the *16S rRNA* gene V3 region were added to pool (cluster names and respective primer sequences are shown in Supplementary 1). After analysis of the obtained raw sequence data, 1’787 annotated microbial sources containing strain or clinical isolate IDs with respective BL gene groups, classes, and gene titles were obtained (Supplementary 2, sheet full annotation). Clusters with identical IDs were merged, and the lowest common ancestor was designated as the microbial source (Supplementary 2, sheet merged annotation). Thus, out of 245 targeted ESBL clusters, results were obtained for 265 ESBL clusters, from which twenty clusters most likely represented sequence homology to similar BL gene clusters and were thus assigned to multiple clusters. In the pre-eradication subgroup 89 ESBL clusters, while in the post-eradication subgroup 106 ESBL gene clusters were not found in any of the study subject samples.

Considering the abundance of ESBL gene clusters in each sample, most clusters were absent, while the presence of a particular ESBL cluster in each sample was mostly dispersed. On average each sample was represented by 23 different ESBL gene clusters in the pre-eradication study group and by 22 different ESBL gene clusters in the post-eradication study group.

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

2.52%, post = 0.64%), and the *bla*$_{ACT}$ gene group for class C BL with the annotated source of *Enterobacteriaceae* (pre = 2.35%, post = 0.12%). The normalized averaged relative abundances of ESBL genes between pre- and post-eradication subgroups did not differ significantly ($p = 0.5467$). Next, we assessed if non-averaged relative abundances of any specific BL cluster differed significantly across the treatment states. Thus, we identified eight clusters that differed significantly between the pre- and post-eradication subgroups (Table 2, $p < 0.05$). Among these dominated the cluster of class A *bla*$_{OXY}$ group ESBL genes with an 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 an annotated origin from *Pseudomonas aeruginosa*, *P. fluorescens*, *P. putida* and *Acinetobacter baumannii* was identified as a significantly different between the treatment states. However, for this one the relative abundance was noticeably lower than for previous. 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 the pre-and post-eradication subgroups to validate the experimental group of ESBL gene quantification. To provide results that are comparable to those of experimental group, we used the same ESBL reference database as previously. Just like with previous group, in the case of the targeted sequencing data analysis, we used read count of *16S rRNA* gene V3 region to normalize ESBL quantification data between samples, while in the case of the shotgun metagenomic sequencing, the total count of *16S rRNA* sequence counts was used for the normalization.

Overall, sequencing and data analysis results for the validation group by targeted sequencing were similar to those of the experimental group. In total, 254 ESBL clusters were targeted, but data analysis resulted in identification of 265 ESBL clusters, from which twenty showed sequence homology and thus were assigned to multiple clusters. In total within the samples from pre-eradication subgroup we identified reads from 101 ESBL gene clusters, while within the samples from post-eradication subgroup there were reads from 95 ESBL gene clusters. Considering the arithmetic mean of each ESBL gene cluster within the respective eradication subgroup (Fig. 2-B), 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 *cblA* 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 the ESBL gene groups between the pre- and post-eradication subgroups did not differ significantly ($p = 0.32464$).

In contrast, the analysis of data from shotgun metagenomics sequencing of validation group 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 allocation to different samples was mostly dispersed. Within 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. 2-C), 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 between the pre- and post-eradication subgroups did not differ significantly ($p = 0.20919$).

Further we assessed if the relative abundances of individual BL clusters differed significantly across the treatment states. Thus, within the targeted dataset, we identified 10 BL gene clusters that displayed significant difference between the pre- and post-eradication subgroups (Table 2, $p < 0.05$). These included cluster of various A ESBL class bla_{OXA} group genes with the annotated origin of *Achromobacter xylosoxidans*, subclass B3 metallo-BL L1 family bla_{L1} group gene with the annotated origin of *Stenotrophomonas maltophilia*, and the oxacillin- hydrolyzing D BL OXA-443 class bla_{OXA} group gene with the annotated origin of the *Ralstonia picketii* PIC-1 strain. However, within the metagenomic dataset, we were unable to identify any specific BL cluster that significantly differed between the treatment states and the most likely reason for that was the low number and low abundancy 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-F). Global comparison, for example, without considering the pre- and post-eradication subgroups, revealed that 13 clusters were present in all three groups (Fig. 2-D) (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. 2-E). Conversely, the shotgun dataset contained notably lower amounts of ESBL gene groups, resulting in lower coverage compared to the experimental dataset (Fig. 2-F). Thus, 69 ESBL gene groups were found in between the experimental and targeted validation datasets, and only 6 common ESBL gene groups were found between the experimental and shotgun validation datasets.

3.3 Taxonomical characterization

Since our analyses involved metagenomic sequencing, we also explored the taxonomical and the functional composition of patient samples before and after *H. pylori* eradication therapy. Detailed taxonomic analysis of the experimental group samples (n = 120) is described in our previous publication (Gudra et al., 2020), but to be brief we uncovered 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 ($p > 0.05$).

In the current validation group (n = 27) metagenomic data, 98.68 ± 1.24% of the taxonomical entries belonged to bacteria, 0.33 ± 0.48% to viruses, and 0.0026 ± 0.003% to fungi and protozoa combined. A detailed taxonomic summary is provided in Supplementary 3.1., 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 the microbial species and treatment state and according to acquired results 12 microbial species were found to be differentially abundant between the pre- and post-eradication subgroups (Fig. 3). Out of these, two had increased relative abundance (*E. bolteae,* $p_{adj.}$=0.049; *E. lenta,* $p_{adj.}$=0.003), and 10 displayed a 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 that in the pre-eradication subgroup.

Assessing Shannon and Chao1 indices and observed OTUs (Supplementary 3.2-A, Supplementary 3.3), only the Shannon index differed significantly between the pre- and post-eradication subgroups ($p_{adj.}$=0.019). Furthermore, to assess the sample-to-sample variability, non-metric multidimensional scaling was applied (Supplementary 3.2-B). Analysis revealed non-specific sample clustering at the species level, suggesting mutual sample similarity irrespective of gender and treatment state.

3.4 Functional analysis of the metagenomic profile

Functional profiles of samples across the treatment states were highly similar. The prevalent gene products were tyrosine recombinase XerC (in both the 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%), sensor histidine kinase RscC (pre = 0.32, post = 0.37%), and TonB-dependent receptor SusC (pre = 0.32%, post = 0.25%) (Fig. 4). Furthermore, the UniProt IDs obtained were converted into Gene Ontology (GO) annotations, and initial annotations that did not have the respective GO IDs were removed from the dataset. Thus, in total 1’993 unique GO IDs were obtained. To assess the relationship between the functional profile and treatment state, an association analysis was carried out, revealing 18 GO ID entries that differed significantly between the pre- and post-eradication subgroups (Supplementary 3.4).

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, the 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 significant GO IDs entity abundances identified were increased in the post-eradication subgroup (n = 15), while only a few were increased in the pre-eradication subgroup (n = 3). The 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.

To reveal the diversity of antimicrobial resistance (AMR) genes found in the metagenomic sample set, additional mapping against the CARD database was performed. Thus, we identified 54 gene families associated with AMR conferring five different resistance mechanisms (Supplementary 4). In total, 1’080 AMR genes were detected in the pre-eradication subgroup, from which 125 had 100% sequence similarity and 955 had strictly significant (≥95% identity) sequence similarity with the CARD reference database. We detected 1’265 AMR genes in the post-eradication subgroup, from which 179 had 100% sequence similarity and 1’086 had strict (≥95% identity) sequence similarity with the CARD reference database. AMR genes present in at least 70% of samples per each treatment state (n = 20) were 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 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 antibiotic target replacement gene *dfrF*. After applying a hierarchical clustering analysis, 12 samples collected from different individuals and within the cluster of samples showed a similar reservoir of detected AMR genes. Only two study subjects had respective samples of the pre- and post-eradication subgroups. The detected 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 resulted in a rapid increase in antimicrobial resistance among clinically relevant microorganisms, particularly gram-negative bacteria (Al-Tawfiq et al., 2020; Koulenti et al., 2019; Maraki et al., 2020; Wang et al., 2017; Yusef et al., 2018). Resistance to beta-lactam antibiotic is an emerging problem in health care due to the extremely limited therapeutic options. Additionally, resistance to this group of antimicrobials is often associated with resistance to other drugs (Koulenti et al., 2019; Tooke et al., 2019). The emergence of ESBL enzymes highlights the critical importance of understanding how associated genes could change abundances in gastrointestinal microbiome under prolonged antibiotic pressure. Since it is difficult to study this phenomenon using culturomics based approaches and subsequent detection of phenotypes, because the cultivation requirements for majority of gastrointestinal microbiome representatives are not known, 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.

Within the samples of experimental sample group, we were able to identify majority of the targeted ESBL clusters. However, there were also some BL gene clusters that were not detected by our panel. Although the most obvious explanation to this would be the absence of target within the pool of extracted bacterial DNA, it is also possible that in some cases the primer was primarily binding to 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 employed annealing temperature. However, to evaluate the validity of these speculations it would be necessary to perform detailed examinations involving qPCR or even digital droplet PCR.

Acquired results revealed that most of BLs originated from gram-negative bacteria from genus *Enterobacteriaceae*. This finding is in agreement with results of several studies, which demonstrated that the prevalence of ESBL-producing *Enterobacteriaceae* is increasing, even in healthy asymptomatic individuals (Higa et al., 2019; Karanika et al., 2016), where it is believed to serve as a reservoir for the spread of ESBLs. The most abundant and prevalent ESBLs within the experimental group were bla_{EC} from carbapenem-targeting class C ESBLs (Ur Rahman et al., 2018), the cephalosporinase gene cbl_A from class A BL targeting ESBLs (Smith et al., 1994) and bla_{ACI} from class A ESBLs. (Ur Rahman et al., 2018)As infections caused by ESBL producers are associated with increased mortality, length of hospital stay, and increased treatment cost (Ahn et al., 2017; Coque et al., 2008; Sianipar et al., 2019), the widespread identification of ESBLs in our patient samples indicates that this is indeed a growing problem in modern society. To date, a large proportion of ESBL types have been poorly characterized and the focus is mainly on the clinically relevant ones. Therefore, decoding the effect of ESBLs in the host or environment in the context of our study is challenging. However, large-scale ESBL gene identification in patient samples by the most sensitive molecular methods can still provide valuable information on 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 main population of ESBLs typical 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 the treatment states. In all three groups (experimental, validation-targeted, and validation-metagenomic)–the number of ESBL gene clusters was higher in the pre-eradication state than in the post-eradication state, suggesting that during eradication therapy a proportion of ESBL producers might be eliminated, resulting in an overall decrease in BL producers. Generally, monotherapy is used to treat common infections such as pneumonia (Rossio et al., 2015) and urinary tract infections (Dowson et al., 2020), 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 favors the reduction of BL producers (Schmid et al., 2019). If true, it is possible that one of the ways to limit the spread of antimicrobial resistance would be the simultaneous use of several antimicrobial agents, not only in the case of *H. pylori* infection, but also in the case of other infectious diseases.

In this study, we were able to detect several ESBL clusters whose relative abundance differed significantly between the pre- and post-eradication states in both targeted datasets. The annotated taxonomic source indicated that these ESBLs originated from *Klebsiella* sp. (Sarojamma and Ramakrishna, 2011), *Pseudomonas* sp. (Laudy et al., 2017), *Acinetobacter* sp. (Abdar et al., 2019) *Achromobacter xylosoxidans* (El Salabi et al., 2012), *Stenotrophomonas maltophilia* (Adegoke et al., 2017) and others. Most of these are known ESBL producers, suggesting gastrointestinal carriage and asymptomatic colonization by these organisms. While the top abundant BL groups were common between the treatment states, the annotated taxonomic source of BLs 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. If true, our findings suggest that in the future greater emphasis should be paid to the development of novel products and procedures for controlled gut microbiome reestablishment. These products should ensure that patients' gut is colonized by non-resistant microorganisms thus mitigating the situation where our microbiota serves as an ESBL reservoir.

Considering the prevalence of ESBL clusters, the highest number of clusters was found in the experimental and targeted validation groups. The metagenomic dataset contained only a few ESBL gene clusters, suggesting that ESBLs abundances are at very low level. A precise 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 within ESBL panel-based sequencing library preparation might induce bias in the abundance of certain 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 the shotgun metagenomic sequencing analysis. The

microbiome diversity rate was significantly higher in the pre-eradication state than in the post-eradication state. This observation agrees with literature data, which suggests that the gut microbiota is significantly altered immediately after eradication therapy and gradually restores to baseline parameters over the time; however, certain alterations may persist up to a year after completing an eradication therapy (Gudra et al., 2020; Liou et al., 2019; Martín-Núñez et al., 2019; Yap et al., 2016). While we observed that diversity was lower in the post-eradication state 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 between the pre- and post-eradication states was highly similar. However, 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*, which has been previously shown to be increased in overweight adults (Palmas et al., 2021). 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 (Gomez-Arango et al., 2018), whereas the later was enriched in traditional rural populations (Angelakis et al., 2019). Altogether, minor differences exist in the composition of the microbiome between the pre- and post-eradication states, although these differences could be more related to the diet and general state of health and not to the eradication therapy itself. In our previous study (Gudra et al., 2020) we evaluated the long-term effects of *H. pylori* eradication on the gut microbiome using patient samples from the current experimental group and analyzed them by sequencing the V3 region of the *16S rRNA* gene. In that study we found that the gut microbiome was stable over two years and was more associated with subject-specific parameters, such as age and medical history than to the eradication therapy itself. These results are comparable to the current study concluding that in the long-term gut microbiome recovers after single antibiotic intervention.

In addition to the evaluation and validation of the ESBL screening panel, we investigated the functional profile and resistome of the microbiome. Thus, we were able to show that abundancy of several genes was increased in the post-eradication state. These genes have a role in molecule and ion transport; cobalamin and extracellular polysaccharide biosynthetic processes; and methylglyoxal, arabinose, and glutamine metabolic processes. All the above-mentioned processes have a positive and beneficial impact on the human host, most profoundly in the case of cobalamin and extracellular polysaccharide synthesis. Additionally, our data also suggests that abundance of several genes were decreased in the post-eradication state. These genes have a role in the DNA restriction-modification system, DNA-templated transcription and initiation, and in ribosomal small subunit biogenesis. Their increased levels might be associated with active bacterial reproduction and their genomic defense from invading foreign DNA. In addition, since we detected minor differences in the abundance and prevalence of ESBL gene clusters between the treatment states, we also evaluated the entire resistome profile. The number of AMR genes detected in the post-eradication subgroup was higher than that detected in the pre-eradication subgroup. Thus, it is apparent that the diversity of AMR genes has increased under the pressure of antimicrobial therapy. There were three AMR genes that were present in all samples of the pre-eradication subgroup – resistance-nodulation-cell division antibiotic efflux pump gene *adeF*, tetracycline-resistant ribosomal

protection protein gene *tetQ*, and trimethoprim resistant dihydrofolate reductase dfr gene *dfrF*, while four were detected in all samples of the post-eradication subgroup: Erm 23S rRNA methyltransferase gene *ErmF*, major facilitator superfamily antibiotic efflux pump gene *tet(40)*, and tetracycline-resistant ribosomal protection protein genes *tetO* and *tetW*. Furthermore, the number of AMR genes that confer resistance to macrolides 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 the study participants in the current study. Although *C. trachomatis* is commonly associated with sexually transmitted diseases (Malhotra et al., 2013), it has also been shown that the human gastrointestinal tract might be a site of persistent infection by this pathogen (Borel et al., 2018; Yeruva et al., 2013). Moreover, in the post-eradication state, we observed an increase in the AMR gene families, macrolide esterase and macrolide phosphotransferase, both of which contribute to macrolide antibiotic inactivation. Alterations in the number of AMR genes after *H. pylori* eradication therapy have been reported in a few studies (Olekhnovich et al., 2019; Wang et al., 2021). Nonetheless, little is known about the functional mechanisms of gut microbiome dynamics following antibiotic treatment.

Despite all this wealth of knowledge this study had several limitations. First, some of the targeted ESBL-coding gene clusters were absent in all patient samples and the reason behind their absence remained ambiguous. Therefore, the developed primers that were targeting ESBL-coding gene clusters should be further validated using such methods as RT-qPCR or digital droplet PCR. Next, while 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 ESBL and AMR coding genes, especially within 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 evaluate resistance to the prescribed antibiotics, amoxicillin and clarithromycin, and did not involve the genomic characterization of patients' *H. pylori.* 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 NGS based large-scale ESBL coding gene screening panel 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 ESBL encoding gene determination in the gut microbiome of *H. pylori*-infected patients, but could also be potentially applied to different samples, populations, and various infection cases that encounter 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 ESBL screening panel is suitable for screening changes in prevalence of ESBL coding genes and in-depth research of the resistome is required to better understand the reservoir of AMR genes in relation to antibacterial therapy, which in future could aid clinicians when choosing antibacterial therapy.

Declarations

Conflict of Interest

None declared.

Author Contributions

General design of the study: DF, ML, GS; funding acquisition: DF; data acquisition: DG, DP, GS, MU, KM, RV; data analysis and statistics: DG, IS, JP, ID; interpretation of the data: DG, DF, IS, MU; drafting of the manuscript: DG, DF, IS; critical revision of the manuscript: DG, DF.

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Data Availability Statement

Raw sequencing data were deposited in the European Nucleotide Archive under study accession No. PRJEB48983.

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Tables

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%)

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

Figures

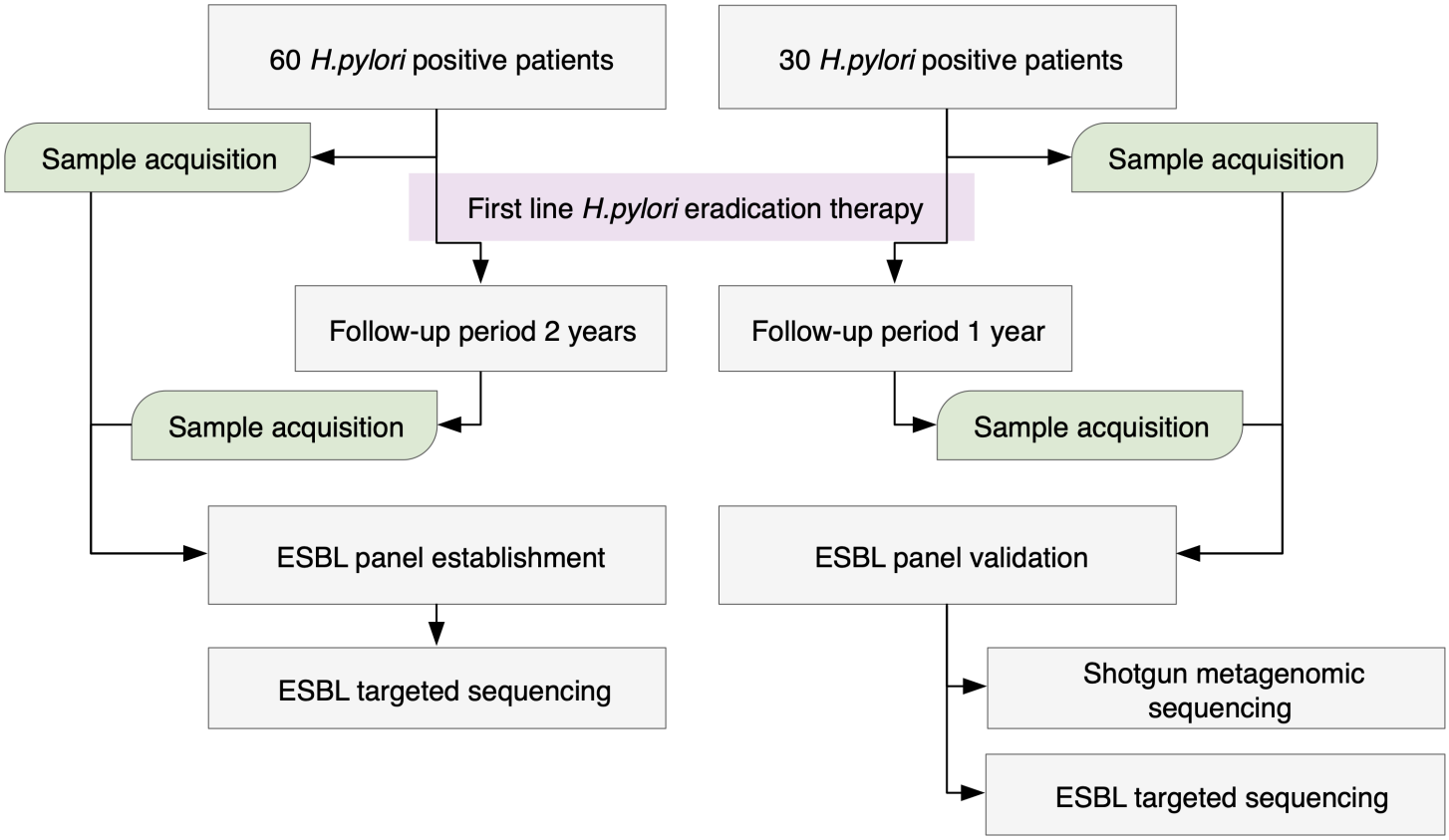

Figure 1

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.

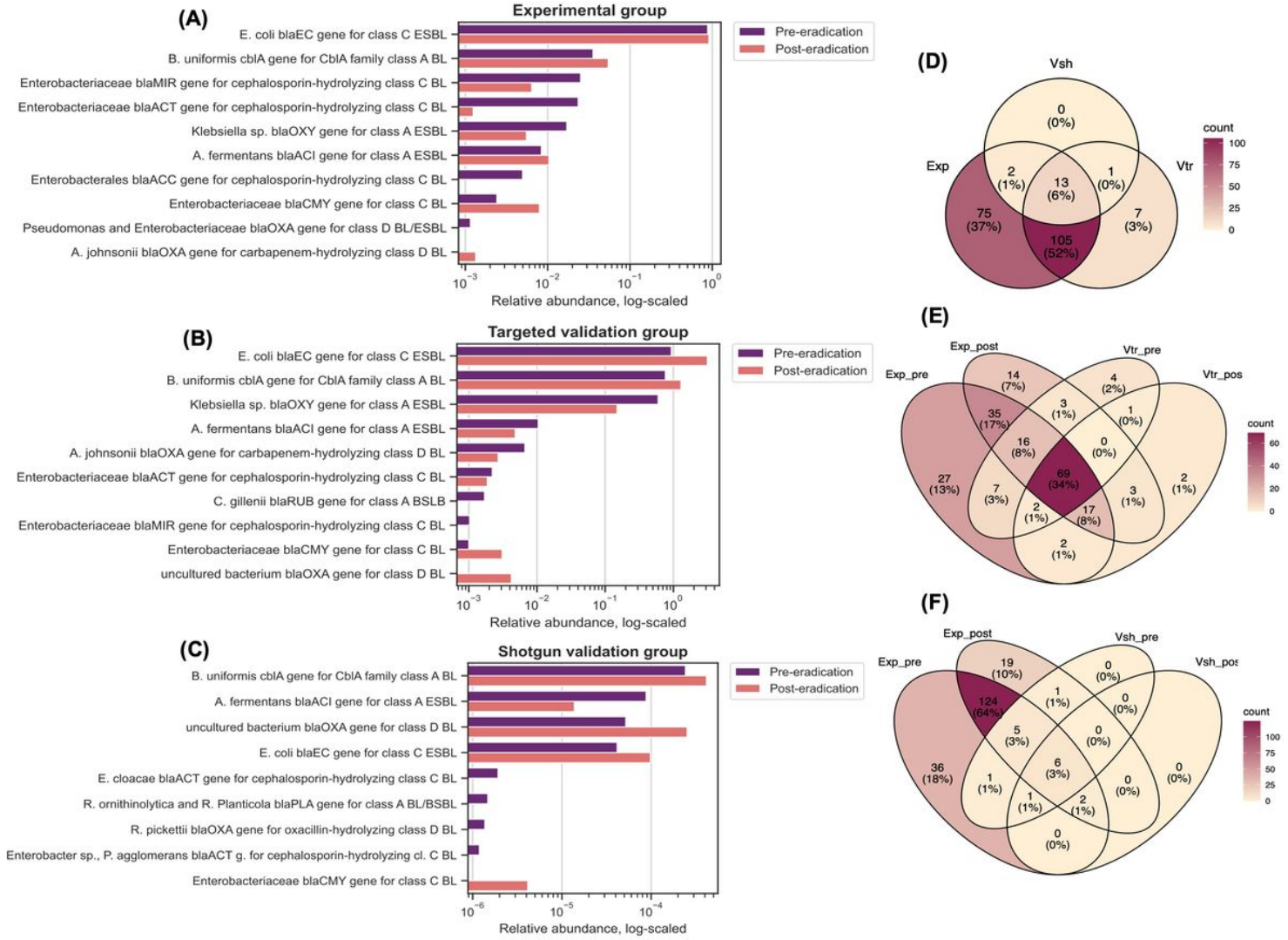

Figure 2

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.

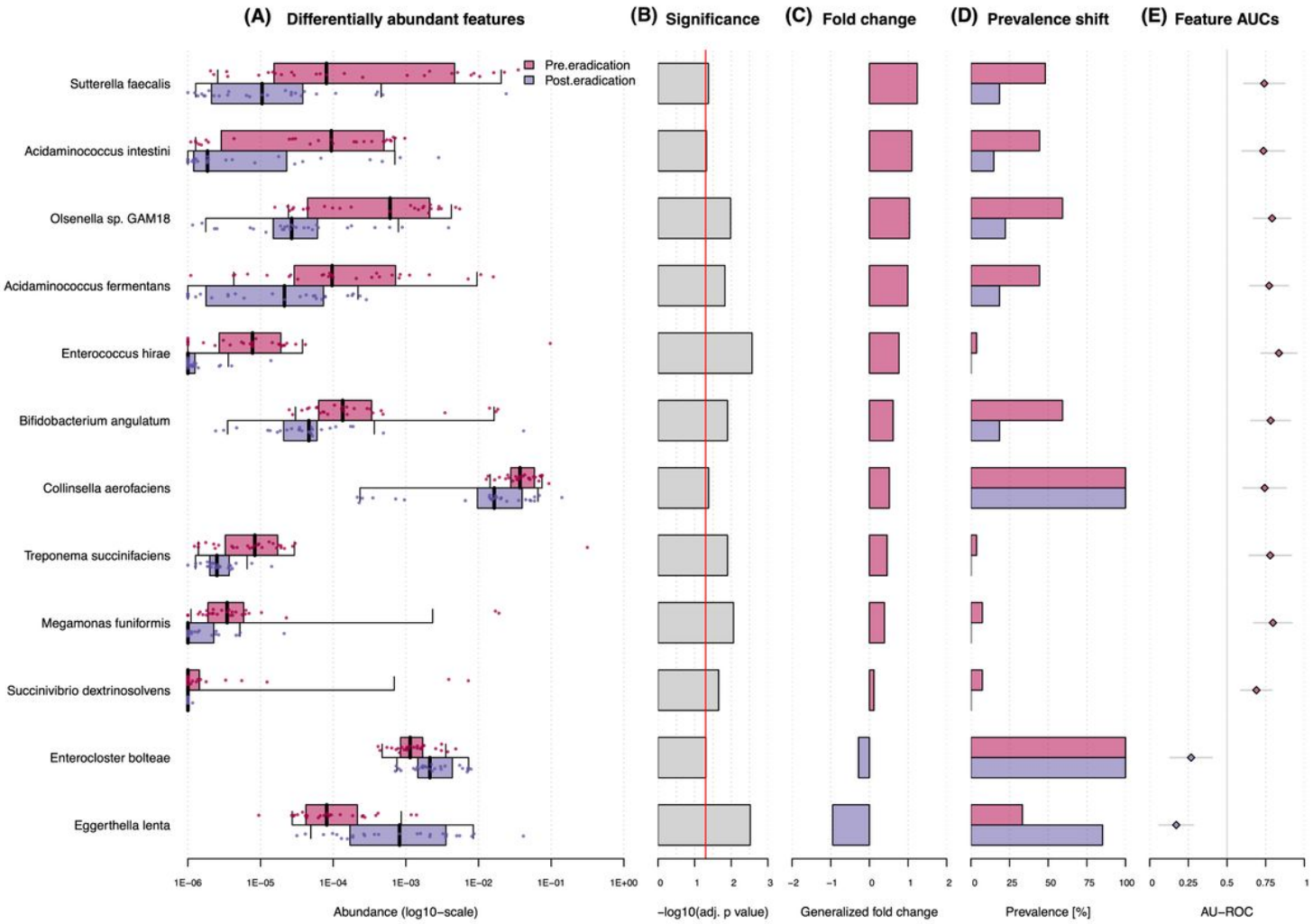

Figure 3

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.

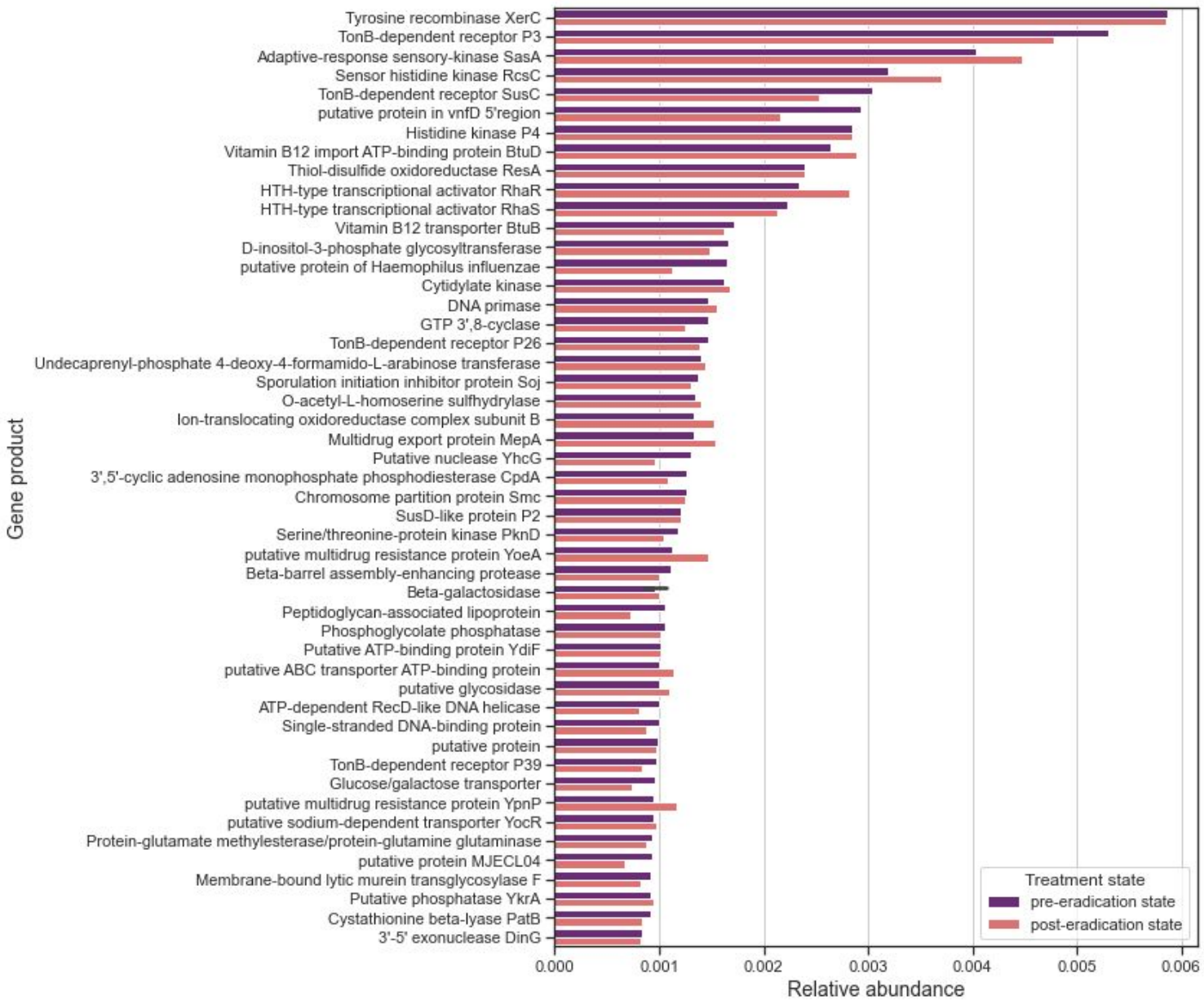

Figure 4

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

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementary1.xlsx
- Supplementary2.xlsx
- Supplementary3.docx
- Supplementary4.xlsx