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GASTRIC MICROENVIRONMENT: ANALYSIS OF MICROBIOTA COMPOSITION IN GASTRIC CANCEROUS AND NON-CANCEROUS MUCOSA AND EVALUATION OF MICROBIOTA AND MUCOSAL FEATURES IN TWINS

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CONTENTS

ABBREVIATIONS

AG	–	atrophic gastritis
BabA	–	blood-antigen binding protein A
CagA	–	cytotoxin-associated gene A
CFU/mL	–	colony-forming units per millilitre
DNA	–	deoxyribonucleic acid
DZ	–	dizygotic
GC	–	gastric cancer
GWAS	–	genome-wide association study
H2Ras	–	histamine H2 receptor antagonists
HopZ	–	Helicobacter outer membrane porins
IARC/WHO	–	International Agency for Research on Cancer / World Health Organization
IM	–	intestinal metaplasia
MAG	–	multifocal atrophic gastritis
MALT	–	mucosa associated lymphoid tissue
MZ	–	monozygotic
NAG	–	nonatrophic gastritis
NGS	–	next-generation sequencing
OLGA	–	Operative link on gastritis assessment
OLGIM	–	Operative link on gastric intestinal metaplasia assessment
PCoA		principal coordinate analysis
PERMANOVA	–	permutational multivariate analysis of variance
PPI	–	proton pump inhibitors
RNA	–	ribonucleic acid
rRNA	–	ribosomal ribonucleic acid
SabA	–	sialic-acid binding adhesin
VacA	–	vacuolating cytotoxin A

INTRODUCTION

Gastric cancer (GC) is one of the most common and lethal neoplasm, ranking as the fith most common and as the fourth most lethal neoplasm worldwide [1]. According to the pathohistological classification of Lauren's GC is classified into intestinal, diffuse and mixed (made up of both intestinal and diffuse) type [2]. Intestinal-type adenocarcinoma is the most common, followed by diffuse and mixed-type GC in decreasing order [3]. Both of Lauren's histological type is associated to *Helicobacter pylori* (*H. pylori*) [4]. In 1994, the International Agency for Research on Cancer, World Health Organization (IARC/WHO) categorised *H. pylori* as a class I carcinogen for gastric cancer (GC) [5]. Since then the gastric microbiota and its carcinogenic effects became a widespread topic and relevant field of the research. The role of gastric microbiota is the most explored in carcinogenesis of intestinal-type GC, meanwhile Lauren's diffuse-type GC has a stronger influence of genetic factors and is less associated with *H. pylori* and environmental factors [4], thus the importance of gastric microbiota in its cancerogenesis is less described.

Up to now, a widespread infection of *H. pylori* (affects >50 % of the world's population) is a predominant risk factor for GC [6], which triggers the Correa's precancerous cascade progressively leading to intestinal-type GC. Despite this until now it is not known why only 1–2 % of individuals infected with *H. pylori* develop GC [7] and its eradication does not necessarily prevent gastric malignancy [8, 9]. In recent years studies have been highlighted alterations and differences of gastric microbiota across the progression of Correa precancerous cascade (nonatrophic gastritis (NAG), athrophic gastritis (AG), instestinal metaplasia (IM)), dysplasia) and GC [10]. The key point is the modification of the dominant gastric microbiome in each stages of the Correa's precancerous cascade characterised by a progressive decreases of *H. pylori* accompanied by a parallel colonization (increases or decreases) of different and mixed collective microbial community [10]. These findings support the hypothesis that beside host [11] and environmental factors (smoking, salted food, etc.) [12, 13], differential susceptibility to GC is also associated with other non-*Helicobacter* bacteria. Besides this studies exploring metabolic effects of gastric microbiota in gastric carcinogenesis revealed differences in metabolome profiles between GC and adjacent non-malignant tisues, which may be associated to carcinogenic effects of gastric microbiome promoting GC carcinogenesis [14].

However the taxonomic pattern of gastric dysbiosis and differentiation of its causal or consequential effect on the gastric carcinogenesis is still a matter of debate. Therefore to depict a more thorough picture of the gastric microbiota and GC carcinogenesis it is important to identify transient and persistent gastric microbiome in health and GC populations with combined impact of the potential environment and host-genetics factors for microbial profile and tumorogenesis.

Under a genome-wide association study (GWAS) researches about 10 % of human microbial diversity could be impact by inheritable factors [15]. However, this relatonship still remains poorly exploreed. Thus, further studies are critical to determine the interplay of host genetic variation and microbiome. One of the best model to evaluate the effect of genetics for microbiome in association with other factors, particularly to environmental factors is twin studies. Several studies performed over the last years revealed impact of heritability for profile of gut microbiome [16, 17]. But to date studies analyzing gastric microbiome using twin model are scarce and still no genetic background on gastric microbiota profile was found [18].

Hereditary is also important for intestinal-type GC, which likewise diffuse-type adenocarcinoma is related with a strong family history of GC [19]. In contrast to the last one, it develops in the context of environmental exposures [20, 21] through a long standing progression of intermediate precancerous lesions of gastric mucosa. For this reason it is important to find out if there is a heritable patern of premaligant lesions, such as AG, which could be useful for assessing GC risk and survaillence of siblings. However, to date studies analyzing correlation of gastritis phenotype using twin model have not been performed yet.

The aim of the study

To analyse the composition of microbiota in gastric cancerous and non-cancerous mucosa and to evaluate gastric microbial similarity and phenotype of gastritis in twins.

The objectives of the study

1. To explore transient and persistent bacteria in gastric cancer and dyspepsia patients.
2. To compare the gastric microbiota of gastric cancer and dyspepsia patients.
3. To evaluate the influence of *H. pylori* on gastric microbiota composition.

4. To evaluate effect of zigosity on the microbial composition in twins.
5. To compare pathomorphological alterations of gastric mucosa in *H. pylori* positive and negative monozygotic and dizygotic twins, using the OLGA/OLGIM gastritis staging systems.

Novelty of the study

In the last decades the carcinogenic effects of gastric microbiota has became a relevant field of microbiota studies. Though the numerous previous studies of gastric microbiota with advanced genomic technics have revealed multiple species of non-*Helicobacter* bacteria as a part of gastric microbiota, no conclusion has been made of whether these represent a transient or persistent microbiota in the gastric mucosa. For this reason the first part of our study was to check hypothesis if *H. pylori* is the only core bacteria of the gastric, which is able to remain in the washed biopsies and affect composition of gastric microbiota. We have explored the resident and transient bacterial communities of gastric microbiota by comparing the changes in microbiota composition of washed and unwashed gastric mucosa samples. To our knowledge it is the first comprehensive research, which was used 16S ribosomal ribonucleic acid (rRNA) gene sequencing in order to distinguish between the transient and persistent bacteria of the gastric. In this part of study we have also compared the bacterial communities of GC and control patients. The results will be relevant to the issue about whether the gastric carcinogenesis is mediated through a microbial alterations caused by *H. pylori*.

Recently, interaction between host genetics and microbiome has became a relevant topic [22]. Although loci related to microbial characteristics, like bacterial taxonomies and pathways were identified by several studies, which have explored the interaction between gut microbiome composition and host genetics, using GWAS [23, 24], most of these results were population specific and difficult to replicate. Further studies are needed to determine if the host factors may impact the diversity of microbiome [25]. Therefore the second part of our study was aimed to analyze the impact of zigosity on microbial similarity in stomach mucosa with specific attention to *H. pylori* in monozygotic (MZ) and dizygotic (DZ) dyspeptic twins.

It is known that the progression of *H. pylori* associated gastritis towards the premalignant gastric lesions, such as AG is also modulated by host-related and environmental factors, however this relationship still remains poorly explored. Hence studies exploring the impact of environment and heredibility for morphologic features of chronic *H. pylori* gastritis are highly needed. Consequently the third part of our study was aimed to explore and compare the phenotype of chronic gastritis in two cohorts of dyspeptic MZ and DZ Lithuanian twins. To our knowledge, this is the first studies that evaluates gastric mucosa alterations in twins.

1. REVIEW OF LITERATURE

1.1. The Human gastric microbiota

1.1.1. *H. pylori* colonization and infection of the stomach

Helicobacter is a group of Gram- negative, curved or spiral-shaped bacteria, of which *H. pylori* is the most commonly known species isolated in 1982 by Barry Marshall and Robin Warren. According to phylogeographic studies *H. pylori* has been shown as a natural humans gastric colonizer co-existing in their populations 50,000–70,000 years [26] and nowadays affecting ≥50 % of the world's population [6]. A strict tropism to normal, metaplastic (in distal esophagus and proximal duodenum) [27, 28] or heterotopic (in Meckel's diverticulum) [29] gastric epithelium reflects its extensive co-evoliution with humans. However as all infected subjects develop a chronic gastritis [30], it is defined as host specific pathogens rather than commensal [31]. Despite that majority of infected individuals are asymptomatic, a long term colonisation is associated with various subsequent gastroduodenal diseases, such as gastroduodenal ulcers, GC or mucosa associated lymphoid tissue (MALT) lymphoma [32]. Besides this *H. pylori* is the only bacterium categorised as a class I carcinogen [5], therefore gastric colonisation by *H. pylori* is relevant topic, important for understanding gastric carcinogenesis.

It usually colonizes the human stomach during early childhood via fecal-oral, oral–oral or gastric–oral pathways in households and generally can persists for life [33]. Upon infection, the colonization features of *H. pylori* are expressed through specific factors and mechanisms adapted to withstand natural limiting factors of stomach (acidic pH, peristalsis, immune system, microbial competition for space) and persistenly colonise gastric microenvironment. Urease, that generates amonia from urea and neutralizes acidic gastric juice around own and neighboring bacteria [34, 35] and motility based by chemotaxis towards a higher pH [36, 37] and spiral shape with six polar flagella burrowing a thick mucin layer [38] are essential virulence factors for colonisation. Within the gastric mucosa, the majority of *H. pylori* are found in the mucus layer close to the gastric epithelium [39]. It adheres to mucin and surface with different adhesins (blood-antigen binding protein A (BabA) and sialic-acid binding adhesin (SabA), *Helicobacter* outer membrane porins (HopZ), AlpA and AlpB) [40–44] and resists to peristalsis. Pathogenicity of *H. pylori* is associated to cytotoxin-associated gene A (CagA) and vacuolating cytotoxin (VacA). These *H. pylori* factors induce [45] and modulate [46] inflammation, increase nutrient availability from gastric tissue [47] and also

affect various host cellular process with oncogenic potential, such as proliferation [48], differentiation [49] and apoptosis [50].

Genetic diversity among *H. pylori* strains is influenced by extensive mutagenesis within chromosome and a frequent interstrain recombination during natural infection with multiple *H. pylori* strains [51–53]. Therefore stomach is usually colonised by heterogenous strains of *H. pylori* with distinct combination of virulence factors. This permits *H. pylori* to adapt to host stomach changes during prolonged colonization and after transmission to new host and are associated to a different risk of gastroduodenal desease [54, 55].

1.1.2. Composition of the gastric microbiota

First studies of gastric microbiota based on culture and histological methods of gastric samples have proved, that non-*Helicobacter* bacteria can contaminate the gastric juice and its mucosa [56, 57]. With the advance of culture based and new genomic methods over the last decades, further studies proved that gastric microbiota apart of *H. pylori* includes multiple various species of non-*Helicobacter* bacteria with the microbial load around 10^2 to 10^4 colony-forming units (CFU)/mL [58]. However, definition of transient and persistent gastric microbiota as well as microbial composition of the stomach in health and desease remain a core issues of gastric microbiota research.

According to several studies >65 % of the bacterial phylotypes in the gastric originate from oral cavity [59, 60] and share similarities to the duodenum microbiota [61, 62], therefore a portion of bacteria, such as *Veillonella*, *Lactobacillus*, and *Clostridium* detected in the gastric juice can be considered as transient colonizers swallowed from oral cavity or flowed by duodenogastric reflux [59, 63]. On the other hand compositional differences between gastric juice and mucosa [61] shows a unique stomach microbiota with possibly acid-resistant bacterial strains [58, 64]. According to several studies of health population *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Fusobacteria* were defined as the most representatives phyla of non-*Helicobacter* bacteria in healthy populations from different geographical regions [58, 65, 66] with the most prevalent genera of *Streptococcus*, *Prevotella*, *Veillonella*, and *Rothia* [64]. *Firmicutes*, *Actinobacteria* and *Bacteroidetes* were dominant phyla in gastric juice, while *Proteobacteria* and *Firmicutes* were dominated in gastric mucosa (in decreasing order) [59, 65, 67].

However characterization of gastric microbiota is still complicated in relation with different factors (age, sex, ethnicity, genetic variations, childbirth mode, *H. pylori*, diet, lifestyle, geography, exposure of antibiotics,

proton pump inhibitors (PPI), histamine H2 receptor antagonists (H2RAs), etc.) between individuals contributing to inter-subjects variations in biodiversity of gastric microbiota [68–72].

1.1.3. *H. pylori* and the non-*Helicobacter* microbial community

Although chronic *H. pylori* infection induced modifications in gastric microenvironment, such as acid acclimation due to reduced gastric acidity, changes in nutrient availibility, increased inflammation and development of gastric atrophy is likely to predispose the shift in the gastric microbiota [73–75]. The effect of *H. pylori* colonization and its extent on the bacterial community has not been finally understood yet.

H. pylori is a core bacteria in the gastric microbiota (accounted for 90 % of the bacteria) of *H. pylori* positive individuals without AG or GC. Besides this they have a reduced diversity and abudance of other microbial taxa compared to *H. pylori*-negative patients [76–80]. This can be also supported by other data of a large variation in the bacterial community depending on the status of *H. pylori* and its reversibility after *H. pylori* eradication [81–84]. According to the study by Maldonado-Contreras et al. *H. pylori* colonization was related to relative abudances of *Proteobacteria*, *Spirochetes* and *Acidobacteria* and decrease abudances of *Actinobacteria*, *Bacteroidetes* and *Firmicutes* [84]. Another study by Llorca et al. demonstrated *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* as the most abundant phyla in a pediatric population with a lower alpha diversity in *H. pylori* positive childrens [81]. Besides this Li et al. reported increased diversity of gastric microbial post *H. pylori* eradication [85].

In contrast to the observation of negative *H. pylori* impact on bacterial diversity, other research have showed inverse findings [65, 76, 86, 87]. Bik et al. showed no significant differences in the composition of the human gastric microbiota according to *H. pylori* status [65]. This was confirmed by findings from other studies of the murine [86] and human stomach [88]. Moreover several studies have demonstrated that *H. pylori* can be negatively affected by non-*Helicobacter* microbial species. In a few studies *Lactobacillus* species (*Lactobacillus johnsonii*, *Lactobacillus murinus*, *Lactobacillus reuteri*) and *Streptococcus mitis* were found to inhibit the growth of *H. pylori* [89–91]. In addition to this variations in the interactions between non-*Helicobacter* bacteria and *H. pylori* can be influenced by the progression of AG [92, 93] and geographical differences [84, 88] in the biodiversity of the human stomach microbiota and consequently associated to different dominant bacterial phyla [94].

The discrepancies of these results are influenced by multiple factors such as different methods used in studies, the time of *H. pylori* and stage of premalignant lesions of gastric mucosa, therefore findings from different studies cannot be objectively compared and relationship between the *H. pylori* and other bacteria populations in the gastric niche remains controversial.

1.1.4. Gastric microbiota in twins

Every individual has a specific gastric microbiota with a great inter-subject variation [72, 95]. Stearns et al. identified the different most abundant phylum in each fourth healthy subjects of the study [66]. It is known that individual exposure to environmental factors contributes to intersubjects differences in gastric microbiota [72, 96–98], however little is known about the role of host genetic factors in the gastric microbiota variation.

A study of Dong et al. compared gastric microbiota between four healthy MZ twin pairs and 8 unrelated individuals by 16S rRNA gene amplicon pyrosequencing [18]. Analyses found a distinct composition of microbiota in each subjects, consisted of the five predominant phyla such as *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Fusobacteria* with no significant similarity in the α and β diversities of gastric microbiota among twins, but it showed differences between *H. pylori* positive and negative individuals. It concluded that genetic determinants had no effect on gastric microbiota profile as well as similar results reported in different anatomical niches [99–101]. However the small sample size of the study made it difficult to detect potential similiarity in microbiota diversity between twins.

Besides this fecal specimens from MZ and DZ twins and their mothers analysed by Turnbaugh et al showed similarity in microbiome pattern associated with either leanness or obesity among family members [16]. The twins cohort with 1,126 pairs in United Kingdom revealed an association between fecal microbiome taxa and genes related to diet, metabolism and olfaction [17]. Therefore interplay between host genetics and gastric microbiome remains to be further clarified.

1.1.5. Alterations of gastric microbiota in gastric carcinogenesis

Many studies focusing on link between microbial diversity and gastric carcinogenesis intestinal-type adenocarcinoma have found significant compositional differences of the gastric microbiota among the different stages of premalignant lesions (AG, IM and dysplasia) and GC [95, 102–106]. These evidences support hypothesis that dysbiotic microbiota changes across the precancerous Correa sequelae can be associated to the development of GC

[107]. Following the systematic reviews, findings in the composition and diversity of the native gastric microbiota during stomach carcinogenesis are diverse [83, 108, 109].

Some studies have concluded, that microbial diversity is significantly reduced in AG, IM and GC compared to patients with NAG [102, 103, 105]. Like one of the first studies by Aviles-Jimenez et al. showing a gradual reversal in gastric microbiota structure from NAG to IM and GC, with significantly higher microbial diversity in NAG compare to GC group [105] as well as the progressively reduced bacterial diversity across NAG, IM and GC reported by Wang Z. et al. [110]. Beside this Liu et al. showed decreased microbial richness from normal gastric tissue to peritumoral and tumoral tissues implying variation of microbiota based on stomach microhabitat within the same GC patient [111].

Meanwhile, other studies reported inversely or no correlation among bacterial diversity and progression from NAG to GC through the Correa cascade. For instance Wang et al. reported significantly increased bacterial load with more diversified structure of microbiota in GC compared to chronic gastritis group [95]. Increased microbial richness and diversity in cancerous tissues as compared to controls were also found by Chen et al. [112] and Castaño-Rodríguez et al. [106]. Whereas, Dicksved et al. identified no significant differences in the microbial compositions between GC and dyspepsia patients [113].

Despite methodological discrepancies such as subject groups, ethnicity sample sizes or analytic methods and mixed findings across studies, some consistent principal among research of microbial dysbiosis regarding gastric gastric carcinogenesis have been found. A gradual loss of active *H. pylori* infection during the progression of the Correa sequelae and GC posits that stomach tumorogenesis may be *H. pylori* independent [76, 102, 104, 114, 115] and frequently enriched by commensal and opportunistic oral or intestinal flora including genera of *Streptococcus*, *Lactobacillus* and *Lactococcus*, *Fusobacterium*, *Haemophilus*, *Veillonella*, *Leptotrichia*, *Dialister* [104–106] or *Phyllobacterium*, *Achromobacter*, *Citrobacter*, *Lactobacillus*, *Clostridium*, and *Rhodococcus*, respectively [102] As evidences emphasize the significancy of collective microbial community in gastric malignancy better than a unique bacterial species [102, 103], *H. pylori* remains the most crucial and specific gastric carcinogenesis associated pathogen [116].

Opposed to intestinal-type GC the absence of intermediate precancerous states across carcinogenesis process of Lauren's diffuse-type GC complicates the differentiation of dysbiotic gastric microbiota changes, therefore gastric microbiota composition associated to diffuse-type adenocarcinoma is less understood and explore [117]. For all that some studies revealed a potential

association of non-*Helicobacter* bacterial phyla with significantly worse overall survival in patients with this Lauren's histological type of GC [118, 119]. Besides this the importance of gastric microbiota in diffuse-type carcinogenesis can be based on epidemiological aspect, such as an increasing incidence of diffuse-type adenocarcinoma in high- and low-risk population for GC despite the decreased *H. pylori* rates [4] and support by hypothetical statement, that eradication of *H. pylori* may promote overgrowth of non-*Helicobacter* flora important for diffuse- type carcinogenesis [4].

1.2. Premalignant gastric alterations

1.2.1. The gastric precancerous cascade

Most of GC cases are intestinal-type adenocarcinoma, which develops trough a multistep process, including a stepwise sequence of phenotypic modifications of the native gastric tissue from healthy gastric mucosa towards NAG, AG, IM, dysplasia (low/high-grade noninvasive neoplasia) and GC [120], which is defined as precancerous Correa's cascade [121]. A sequential-causative effect of each of these steps was based by epidemiological studies [122–124]. The initial step in the Correa's cascade is chronic gastritis commonly caused by *H. pylori* mainly starting in the antrum [125]. In this step *H. pylori* – associated inflammation affects a normal gastric mucosa with well-preserved normal gastric glands and is called NAG. At this stage NAG can be cured by eradicating *H. pylori* otherwise it remains and may progresses to AG with the loss and/or metaplastic replacement of native gastric mucosal glands [126, 127]. Within spectrum of Correa's cascade lesions, AG is considered the elective "field of cancerization" prone to GC development [120]. *H. pylori* is by far the main determinant agent of gastric atrophy, and, all over the world, the prevalence of the *H. pylori* infection is consistently linked with both, AG and GC [120, 128, 129]. In some peculiar epidemiological contexts, however, a high prevalence of bacterial infection is associated with a low prevalence of gastric precancerous/cancer lesions, or equivalent rates of bacterial infection are associated with significantly different risk of gastric malignancy [130, 131]. These unexpected findings would support the hypothesis that etiological factors, other than *H. pylori*, may be involved in the modulation of the oncogenetic "cascade". Among these factors, both host-related (genetic variations, noncoding RNAs, methylation, etc) and other environmental factors or even gastric dysbiosis in line with epidemiological determinants (ethnic, culture, etc.) have been considered [132–138]. At this point a possible contribution of gastric dysbiosis to further progression of the Correa's cascade has been also considered [103], however

the role of dysbiosis along the preneoplastic cascade remains uncertain. Naturally *H. pylori*-associated AG progresses with time in severity and extension and become multifocal atrophic gastritis (MAG) [125].

Chronologically continuing chronic inflammation progress to advanced stage of atrophy with metaplastic replacement of native gastric mucosal glands, defined as IM [125]. Histologically it is a heterogeneous lesion, classified into two main subtypes: complete and incomplete types, which resembles the small intestine or colonic epithelium, respectively [139]. IM tends to occur at the *incisura angularis* and further extend to the antrum and corpus [125, 126, 140]. Up to this point accumulation of cells genetic lesions within atrophic/metaplastic mucosa become the background for dysplasia and invasive gastric adenocarcinoma as the final stages of cascade [120, 141].

Although the Correa's cascade demonstrates a linear progression of preneoplastic lesions, it may also envolve in several ways: either it remains without any changes over many years or it develops with regression and/or progression of lesions along cascade [142]. Therefore it remains unclear which point of cascade is irreversible. The findings of systematic reviews used AG and IM comparison suggest that IM can be defined as irreversible preneoplastic lesion beyond which a histopathological steps of cascade may progress regardless of *H. pylori* eradication [143, 144]. On the contrary a regression of IM has been also observed in several cohort studies [145, 146]. Besides this only a small proportion of patients with IM progress to dysplasia and GC [142]. For this reason, further studies are necessary to understand the mechanisms responsible for the development of this premalignant cascade.

1.2.2. Gastric cancer risk in patients with premalignant gastric lesions

Even though AG, IM and dysplasia are considered as preneoplastic gastric lesions of intestinal-type GC, they are often disregared in routine clinical practice [147]. Consequently, most of the GC cases are still diagnosed at late stages with a high mortality rate [148]. Therefore identification and surveillance of individuals with a high risk of GC are important for early diagnosis of GC.

The Operative link on gastritis assessment (OLGA) and the Operative link on gastric intestinal metaplasia assessment (OLGIM) staging systems have been suggested to identify patients with AG or IM and stratify their risk for GC [149–151]. Both OLGA and OLGIM gastritis staging systems combine atrophy or IM scores of the the updated Sydney system [152] assessed histologically on antral and corpus biopsies [150, 151]. Each OLGA/OLGIM stages (0–IV) of gastritis respectively express increasing

severity and extent of atrophy or IM [150, 151] and according to follow-up studies progressively correlate with a significantly higher risk of GC [153, 154]. As a result, to improve secondary prevention and early diagnosis of GC, international guidelines on this topic recommend endoscopic follow up to patient in a high- risk OLGA/OLGIM stages (III/IV), especially with a family history of GC [155–157].

However as the majority of individuals with precancerous lesions are asymptomatic, epidemiologic data of these premalignant alterations are mainly unknown, especially in low risk regions [147, 158–160]. Therefore international guidelines for GC screening, such as Japan or Korea nationwide screening programs [161], based on high prevalence of GC or British Society of Gastroenterology guidelines, which offers screening endoscopy for the high risk subjects [157] are scarce. Consequently, evidence to conclude a risk profile of premalignant lesions and international screening recommendations for GC are still insufficient.

2. METHODS

2.1. Ethics

The study was approved by the Lithuanian Bioethics Committee (Protocol No. 8/2011) and Kaunas Regional Ethics Committee of Biomedical Surveys (Protocol No. BE-2-10). All participants have signed an informed consent form to take part in the study.

2.2. A flowchart of the study

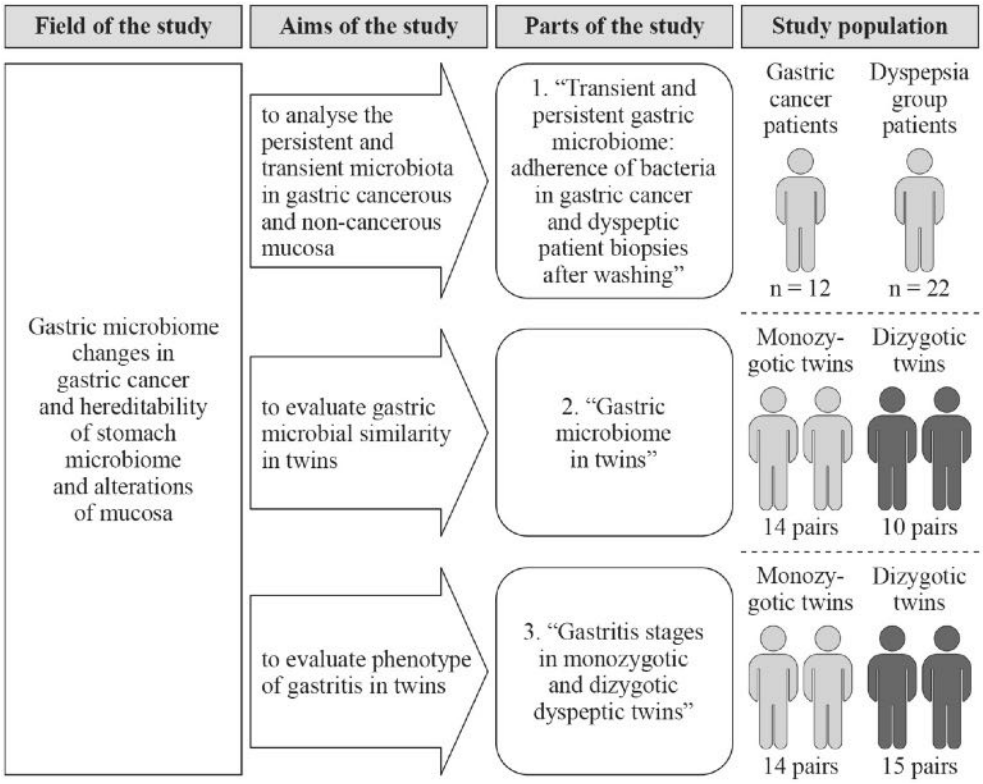

Fig. 2.2.1. *A flowchart of the study*

A flowchart of the study in Fig. 2.2.1 graphically demonstrates the context of our research. Microbiome changes in GC and heritability of gastric microbiome and pathomorphological alterations of mucosa may be potentially important for the origin of GC, but are still poorly explored fields. To bring a more comprehensive perspective on this fields, our study was consisted of three parts. The first part of our study was aimed to analyse

persistent and transient microbiota in gastric cancerous and non-cancerous mucosa, by comparing GC and dyspepsia patients. In the second and the third part of the study we evaluated the effect of zigosity on the microbial composition and phenotype of gastritis in twins, respectively.

2.3. Design of the study: "Transient and persistent gastric microbiome: adherence of bacteria in gastric cancer and dyspeptic patient biopsies after washing"

2.3.1. Study population

The study consisted of twelve patients with GC (case group) and twenty-two patients with dyspepsia (control group) referred to the Department of Gastroenterology of Hospital of Lithuanian University of Health Sciences Kauno klinikos for upper gastrointestinal endoscopy between 2017–2019. The inclusion criteria for patients included the following: >18 years old, no previous eradication of *H. pylori*, no prior exposure to proton pump inhibitors (PPI) or antibiotics for the last 3 months period. Additional inclusion criterion for GC group was histologically confirmed gastric adenocarcinoma without any previous therapeutic and surgical approaches. The exclusion criteria for participants included the following: < 18 years old, previous eradication of *H. pylori*, use of PPI or antibiotics within the last 3 months prior to inclusion and previous treatment approaches of GC.

2.3.2. Protocol of gastric biopsy sampling and handling

All biopsies were obtained and handled for futher research at the Department of Gastroenterology of Hospital of Lithuanian University of Health Sciences Kauno klinikos and Institute for Digestive Research of Lithuanian University of Health Sciences.

Protocol of both groups included three antral biopsies obtained 4 cm from the pylorus and three additional biopsies obtained from the cancer area in the corpus for the gastric cancer group. Each of these samples were collected by sterile biopsy forceps.

The first biopsy was immediately fixed in 10 % neutral buffered formalin solution and used for histopathologic examination of *H. pylori* at the Department of Pathological Anatomy of Hospital of Lithuanian University of Health Sciences Kauno klinikos. Histological slides were stained with haematoxylin/eosin and Giemsa stains to detect the presence of *H. pylori*.

The second biopsy was directly placed to the commercial transport medium (Portagerm pylori, bioMérieux, Marcy L'Etoile, France) and stored at –80 °C.

The third biopsy was immediately washed twice in separate tubes filled with sterile 4 °C Phosphate buffered saline (PBS, Sigma-Aldrich Chemie GmbH, USA) for 15 s to disengage the transient microflora. After the second wash, the biopsy was transferred in the same medium as the second biopsy and stored at –80 °C. The second and third biopsies were transported to the Copenhagen University Hospital (Rigshospitalet) for culture study.

2.3.3. Culture of gastric biopsies and identification of bacterial species

Biopsies were cultivated on 7% defibrinated horse blood agar plates (SSI Diagnostica A/S, Hillerød, Denmark), placed in serum bouillon with 10 % glycerol (SSI Diagnostica, Hillerød, Denmark), and frozen at −80 °C. The agar plates were incubated 6 days at 37 ◦C in microaerobic conditions (10 % CO_2, 5 % O_2). The observed growth was indicated, and single bacterial colonies of each morphology were isolated on new 5 % horse blood agar plates (SSI Diagnostica A/S, Hillerød, Denmark). The disks with the isolated cultures were incubated at 37 °C in microaerobic conditions until visible growth was noticed (1–3 days). Each isolate was transferred to a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFF) target plate, then treated with 1 µL HCAA matrix (Bruker Daltonics, Billerica, MA, USA) and analyzed with MALDI-TOF Mass Spectrometry (Bruker Daltonics, Billerica, MA, USA). To identify the species the protein profile was compared to the database Compass (Bruker Daltonics, Billerica, MA, USA).

2.3.4. Microbiota analysis

The microbiota analysis, including deoxyribonucleic acid (DNA) extraction, library preparation and sequencing, was performed at Statens Serum Institute.

2.3.4.1. DNA extraction and 16S rRNA gene amplicon sequencing

DNA was extracted from the biopsies using a QIAamp DNA mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. For each batch of DNA extraction, a negative control without sample material was included for downstream analysis. To amplify the extracted DNA a two-step polymerase chain reaction (PCR) was used by applying a modified version of the published universal prokaryotic primers 341F (ACTCCTAYGGGRBG CASCAG) and 806R (ACTCCTAYGGGRBGCASCAG). Both primers target the hyper-variable region V3–V4 regions of the 16S rRNA gene.

Afterwards amplicons were sequenced on the Illumina MiSeq desktop sequencer (Illumina Inc., San Diego, CA, USA), using the V2 Reagent Kit.

2.3.4.2. Library preparation

Purified genomic DNA from each sample was initially amplified in a 25 µL reaction, using the REDExtract-N-Amp PCR ReadyMix (Sigma-Aldrich, St Louis, MO, USA) with 0.4 µM of each 16S rRNA gene primer and 2 µL template. In this study the 16S PCR conditions were the following: an initial denaturation at 95 °C for 2 min, 20 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 30 s and final elongation at 72 °C for 7 min. This PCR run is considered to as PCR1. The product from PCR1 was prepared for sequencing by a second PCR (regarded to as PCR2), repeating the same PCR protocol of PCR1. PCR2 attached an adaptor A, an index i5 and a forward sequencing primer site (FSP) in the 5' end of the amplicons and an adaptor B, an index i7 and a reverse sequencing primer site (RSP) to the 3' end of the amplicons. DNA was quantified using the Quant-ITTM dsDNA High Sensitive Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and then PCR2 products were pooled in equimolar amounts between samples. Undesirable DNA amplicons from the pooled amplicon library (PAL) were removed by using Agencourt AMPure XP bead (Beckman Coulter, Brea, CA, USA) purification in a two-step process. First, DNA fragments below 300 nucleotides length were removed by a PAL AMPure beads 10:24 ratio, according to the manufacturer's instructions, and eluted in 40 µL TE buffer (AM1). Secondly, large DNA fragments above 1 kbp were removed by AM1 to AMPure beads 10:16 ratio as previously described. The resulting AMPure beads purified PAL (bPAL) was diluted to a final concentration of 11.5 pMDNA in a 0.001 NNaOH and used for sequencing on the Illumina MiSeq desktop sequencer (Illumina Inc., San Diego, CA, USA). We used the 500-cycle MiSeq Reagent Kit V2 in a 2 × 250 nt setup (Illumina Inc., San Diego, CA, USA) to sequence the library.

2.3.5. Bioinformatics

We used BION-META (*http://box.com/bion*) to analyse the sequence data, which were processed according to the previously described automated order [162]. Taxonomy tables were made with the identified phylum, class, order, family, genus, species and number of reads. Our data from the sequencing were submitted to European Nucleotide Archive with the accession number PRJEB38558.

2.3.6. Statistical analysis

The ten most abundant genera across all samples are demonstrated in staggered bar plots in which samples are ordered according to a hierarchical clustering based on Bray Curtis dissimilarities and ward-linkage. Analysis of the difference in the distribution of bacterial groups between biopsies was performed in the graphing and statistics program GraphPad Prism, version 5.01 (San Diego, CA, USA) by applying unpaired t-test.

The microbiota composition was analysed with program R, version 3.5.0 (R Foundation for Statistical Computing, Vienna, Austria) using the packages phyloseq v. 1.24.3 and vegan v. 2.5-2. Figures were created using the packages ggplot2 v. 3.2.0 and plotly v. 4.8.0. Alpha-diversity of samples as well as relative abundance of individual genera were compared pairwise between groups with Mann–Whitney rank sum tests and adjusted for multiple testing using Bonferroni correction. Principal coordinate analysis (PCoA) of samples was performed based BIONs species-level classification on Bray Curtis dissimilarity. The analysis of similarities (ANOSIM test) was used to compare within-group similarities and between-group similarities. 1000 random permutations were used to estimate *P* value.

2.4. Design of the study: "Gastric microbiome in twins"

2.4.1. Study population

"Gastric microbiome in twins" is a part of larger study of "Gut microbial similarity in twins is driven by shared environment and aging", which consisted of 108 twin pairs and was aimed to analyse the role of host genetics and environment factors on bacterial similarity in specimens of saliva, stomach, and fecal from twins. Twins were selected from the Twin Center at Lithuanian University of Health Sciences. They were interviewed by phone calls, and those that accepted the invitation were included in the study during the years 2016–2018. Twin pairs that reported clinical symptoms of dyspepsia were suggested to undergo upper gastrointestinal endoscopy. The inclusion criteria for participants included the following: >18 years old, no previous eradication of *H. pylori* and no prior exposure to PPI or antibiotics at least for the last 4-week period. According to inclusion criteria 24 out of 108 twin pairs were selected to take part in this section of study. The demographics and clinical data of the study participants are provided in Table 3.2.1.1.

2.4.2. Protocol of gastric biopsy sampling

Protocol of gastric biopsies included two biopsies from the antrum and two biopsies from the corpus obtained by biopsy forceps during endoscopy. Biopsies were placed at two different tubes labelled according to the site of the sample and immediately frozen in liquid nitrogen and stored at –80 °C.

2.4.3. Zygosity of twins (MZ *versus* DZ)

Zygosity of twins (MZ *versus* DZ) was confirmed by automated analysis of DNA microsatellite markers by targeting 15 different loci: D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TROX, D18S51, D5S818, and FGA (AmpFLSTR® Identifiler® Plus PCR Amplification Kit (Thermo Fisher Scientific, USA)).

2.4.4. DNA and RNA extraction

A Allprep DNA/RNA mini kit (Qiagen, Germany) was used to ectract bacterial DNA and RNA from gastric biopsy samples according to the manufacturer's instructions. On-column DNA digestion during RNA purification was performed using RNase-Free DNase Set (Qiagen). RNA was reverse transcribed to cDNA using Superscript IV First-Strand Synthesis System (Thermo Fisher Scientific).

2.4.5. Library preparation

Libraries were generated by amplification of the V1-V2 region of the 16S rRNA, taking as template either the DNA (16S rRNA gene) or the cDNA (16S rRNA) after 20 cycles PCR reaction. The 27F and 338R primers were used to target the V1–V2 region of the 16S rRNA. The libraries were sequenced on a MiSeq (2×250 bp, Illumina, Hayward, California, USA) [163, 164].

2.4.6. Sequencing analysis

We performed FastQ files analysis by using the dada package [165], version 1.10.1, in R. Samples with less than 4 000 reads were removed from downstream analysis. All samples were resampled to equal sequencing depth of 4838 reads using the phyloseq package [166]. The naïve Bayesian classification [167] with a pseudo-bootstrap threshold of 80 % was used to annotate phylotypes to taxonomic affiliation. Bacterial communities were analyzed from phylum to genus and at phylotype taxonomy ranks.

The sample-similarity matrix by Bray-Curtis algorithm (1000 bootstrap) at phylotype level and plotted using Past, Mega software or iTol Interactive Tree of Life website [168] were used to built dendrogram and PCoA.

Analysis of samples similarities was based on their Bray-Curtis-Similarity and the ROUT method (Q = 1 %) in Prism 7 (GraphPad Software, La Jolla, CA) was used to identify the outliers of samples. To evaluate the significant differences between groups defined a priori we applied Mann-Whitney test. The correlations were performed using Spearman test using Prism 7 (GraphPad Software, La Jolla, CA). Primer-e (Primer 7, Version 7.0.17, Add on: Permanova+) [169] was used to calculate permutational multivariate analysis of variance (PERMANOVA). *P* value < 0.05 was considered as statistically significant difference. A paired twins were compared only if the samples of both twins from the pair were successfully sequenced.

2.5. Design of the study: "Gastritis stages in monozygotic and dizygotic dyspeptic twins"

2.5.1. Study population

Two cohorts of twins were selected from the Twin Center at Lithuanian University of Health Sciences, during the period 2016-2018. The twins were interviewed by phone calls, and those that reported clinical symptoms of dyspepsia and accepted the invitation to be included in the study were referred for upper gastrointestinal endoscopy. Zygosity of twins (mono- *versus* dizygosity) was confirmed by automated analysis of DNA microsatellite markers. The study included 29 twin pairs (58 subjects): 14 pairs of MZ and 15 pairs (10 same-sex pairs and 5 mixed-sex pairs) of DZ twins. Exclusion criteria for participants included the following: < 18 years old, previous *H. pylori* eradication therapy, use of PPI or antibiotics for the last 4-week period prior to inclusion.

2.5.2. Endoscopy and protocol of gastric biopsy sampling

All the endoscopic procedures were performed at the Department of Gastroenterology of Hospital of Lithuanian University of Health Sciences Kauno klinikos. In all patients, the biopsy protocol included 5 gastric biopsy samples, according to the updated Sydney system to meet requirements for staging of gastritis: 2 from the distal antrum mucosa (1 from the greater curvature and 1 from the lesser curvature), 1 from mucosa of the incisura angularis at the lesser curvature and 2 from the proximal corpus mucosa (1 from the anterior wall and 1 from the posterior wall).

2.5.3. Preparation of gastric mucosa biopsies for histopathological examination

Biopsy specimens were fixed in 10 % neutral buffered formalin solution and placed at three different tubes labelled according to the site of the sample (antrum, *incisura angularis*, corpus) and embedded in paraffin, and stained with hematoxylin and eosin and Giemsa stain for *H. pylori* at the Department of Pathological Anatomy of Hospital of Lithuanian University of Health Science Kauno klinikos.

2.5.4. Histopathological examination of gastric mucosal biopsies

The gastric biopsies were evaluated for *H. pylori* status, gastritis and premalignant lesions (atrophy and intestinal metaplasia (IM)) according to the framework of the updated Sydney system [152]. In all cases, premalignant lesions were staged by aplying the OLGA and OLGIM staging systems, according to the defined criteria.

2.5.4.1. Grading of *H. pylori* chronic gastritis: the updated Sydney system

Gastric biopsy specimens from antrum (including incisura angularis) and corpus were evaluated separately for five histological parameters: density of *H. pylori*, chronic inflammation (lymphoid- monocytic infiltrates), inflammatory activity (neutrophils infiltrates), atrophy and IM. Each of these variables were graded by the visual analog scales (VAS) into normal or absent, mild, moderate and severe (0–3 respectively) categories. The values of each biopsy were averaged separately for antrum (including incisura angularis) and corpus. According to the topographic distribution of the intensity of inflammatory changes gastritis was classified into antral- / corpus-predominant or pangastritis [152].

2.5.4.2. Staging of *H. pylori* chronic gastritis: the OLGA and OLGIM systems

Staging of *H. pylori* chronic gastritis was based on OLGA and OLGIM staging systems, which classify gastritis into five stages (0–IV) by using atrophy and IM scores respectively as important risk factors significantly correlating with the risk of intestinal-type GC. According to the OLGA and OLGIM staging frame atrophy and IM scores were assessing at the single biopsy and gastric mucosal compartment (antrum with *incisura angularis* and corpus) levels.

2.5.4.2.1. Assessing the atrophy and IM scores at the single biopsy level

For each specimens mucosal atrophy was scored as the percentage of atrophic glands (including non- metaplastic and metaplastic phenotypes) on four-tiered scale by using VAS of the updated Sydney system: no atrophy (0 %) = score 0; mild atrophy (1–30 %) = score 1; moderate atrophy (31–60 %) = score 2; severe atrophy (>60 %) = score 3). Analogically, in each biopsy the degree of IM was scored as the percentage of metaplastic glands on a four- tiered scale (0- 3) by adopting VAS with the same cut-offs as for atrophy.

2.5.4.2.2. Assessing the atrophy and IM scores at the gastric mucosal compartment level

For each gastric mucosal compartment, the overall atrophy and IM scores were calculated as the average of 3 antrum/incisura angularis biopsies scores and the average of 2 corpus biopsies scores and ranked on a four- tiered scale (0–3) with the same cut-offs as for single biopsy level. A combination of the final antrum and corpus score for atrophy and IM were used to obtained the OLGA and OLGIM gastritis stages, respectively.

2.5.5. Statistical analysis

Statistical analysis was performed using IBM SPSS software (v 22.0). Age is presented as mean and median values, while age differences between the groups were evaluated comparing mean values using a T-test. The Pearson Chi-squared test to compare sample proportion was applied. The exact Pearson Chi-squared (χ^2) test was used to assess concordance of OLGA and OLGIM stages between MZ and DZ twins. $P < 0.05$ was considered significant.

3. RESULTS

3.1. Results of the study: "Transient and persistent gastric microbiome: adherence of bacteria in gastric cancer and dyspeptic patient biopsies after washing"

3.1.1. Characteristics of patient groups

The demographics and clinical data of patient groups are listed in Table 3.1.1.1. The GC group consisted of 12 patients with GC, whereas the control group consisted of 22 patients with dyspepsia. In the study, 68 % of patients (n = 23) were female and 32 % (n = 11) were male with the age range of 22–91 years (median 53 years). Among patients with GC the *H. pylori* status was as the following: 9 persons were *H. pylori*-negative and 3 persons were *H. pylori*-positive. Among patients with dyspepsia, 11 individuals were *H. pylori*-positive and 11 individuals were *H. pylori*-negative. The diffuse, intestinal and mixed types of gastric adenocarcinoma were diagnosed in six, four and two patients, respectively, whereas the second and third G stage of gastric adenocarcinoma were confirmed in five and seven patients, respectively. According to the topography, three individuals had proximal and nine patients had distal GC.

Table 3.1.1.1. *The demographics and clinical data of patient groups*

Diagnosis	**Mean age (years)**	**Gender**	***H. pylori* Status (Histology)**	**Lauren Classification**	**G Stage**	**Proximal/ Distal Part**
Gastric adenocarcinoma (n = 12)	62.2	Female (n = 5)	Negative (n = 9)	Diffuse (n = 6)	2 (n =5)	Proximal (n = 3)
		Male (n = 7)	Positive (n = 3)	Intestinal (n = 4)	3 (n =7)	Distal (n = 9)
				Mixed (n = 2)		
Dyspepsia (n = 22)	48.1	Female (n = 18)	Negative (n = 11)	NA	NA	NA
		Male (n = 4)	Positive (n = 11)			

NA: Not applicable.

3.1.2. Comparison of cultured bacteria composition in unwashed and washed biopsies of dyspepsia and gastric cancer patients

Results of bacterial colonies cultured from the biopsy pairs are summarised in Table 3.1.2.1. A total of 27 biopsy pairs showed reduced or no growth in the washed biopsy compared to the growth observed in the unwashed biopsy. Only 5 biopsy pairs showed unchanged growth, while 3 showed increased growth. The overall number of cultured non-*Helicobacter* species decreased in the washed biopsies compared to the unwashed biopsies, potentially indicating that most of bacteria do not adhere to the stomach mucosa.

Table 3.1.2.1. *Change in growth of bacterial species cultured from the biopsy pairs*

Growth Result	Dyspepsia Patients	Gastric Cancer Patients
No bacterial growth in either biopsy	8 biopsy pairs	3 biopsy pairs
Growth in unwashed but not in washed biopsy	4 biopsy pairs	5 biopsy pairs
Reduced growth in the washed biopsy	9 biopsy pairs	9 biopsy pairs
Unchanged growth	1 biopsy pairs	4 biopsy pairs
Increased growth in the washed biopsy	0 biopsy pairs	3 biopsy pairs

The total number of colonies cultured from unwashed and washed biopsies are shown in Fig. 3.1.2.1, whereas the percentage distribution of total cultured bacterial species in each patient groups is presented in Fig. 3.1.2.2 and Table 3.1.2.2.

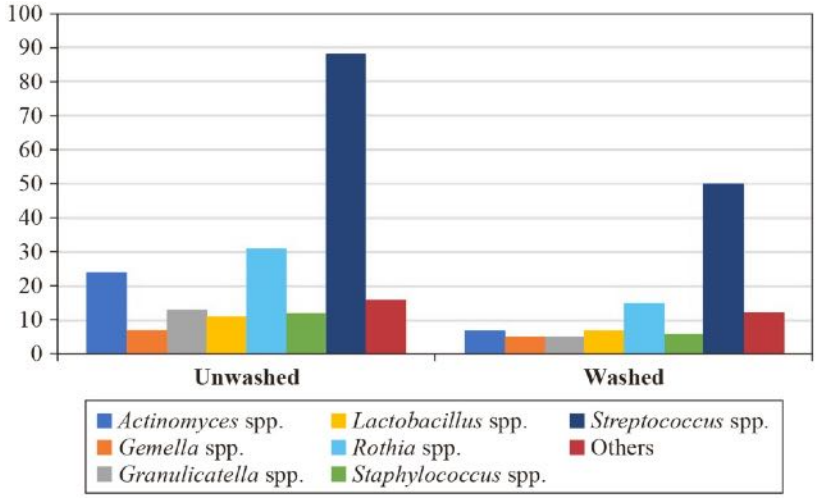

Fig. 3.1.2.1. *The number of bacterial species isolated from culture of gastric cancer and dyspepsia patient biopsies*

"Others" include *Bacillus* spp., *Corynebacterium* spp., *Enterobacter* spp., *Enterococcus* spp., *Haemophilus* spp, *Micrococcus* spp., *Neisseria* spp., *Stenotrophomonas* spp.

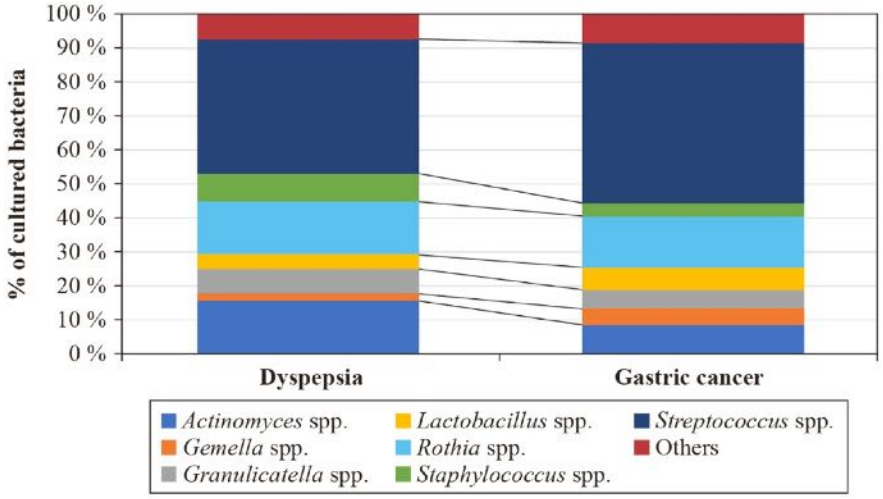

Fig. 3.1.2.2. *The relative distribution of non-Helicobacter bacteria cultured from the unwashed biopsies. The percentages can be found in Table 3.1.2.2*

"Others" include *Bacillus* spp., *Corynebacterium* spp., *Enterobacter* spp., *Enterococcus* spp., *Haemophilus* spp, *Micrococcus* spp., *Neisseria* spp., *Stenotrophomonas* spp.

Table 3.1.2.2. *Distribution of total cultured bacterial groups*

	Dyspepsia		Gastric cancer	
	Unwashed	**Washed**	**Unwashed**	**Washed**
Streptococcus spp.	40 %	43 %	47 %	49 %
Rothia spp.	16 %	14 %	15 %	14 %
Actinomyces spp.	16 %	9 %	9 %	6 %
Staphylococcus spp.	8 %	6 %	4 %	6 %
Granulicatella spp.	7 %	6 %	6 %	4 %
Lactobacillus spp.	4 %	3 %	7 %	8 %
Gemella spp.	2 %	9 %	5 %	3 %
Enterococcus spp.	1 %	3 %	6 %	8 %
Micrococcus spp.	2 %	3 %	2 %	0 %
Corynebacterium spp.	2 %	3 %	–	–
Stenotrophomonas spp.	1 %	0 %	–	–
Neisseria spp.	1 %	0 %	–	–
Enterobacter spp.	0 %	3 %	–	–
Bacillus spp.	–	–	1 %	0 %
Haemophilus spp.	–	–	0 %	3 %

The cultured bacteria from the unwashed and washed biopsies in dyspepsia and GC patients were dominated by *Streptococcus* spp., followed by *Rothia* spp. and *Actinomyces* spp. The total number of the listed bacterial colonies was lower in the washed biopsies compared to the unwashed biopsies (Fig. 3.1.2.1), whereas the relative abundance of *Streptococcus* spp. was higher in the washed biopsies (Table 3.1.2.2).

The distribution of cultured bacteria in biopsies from GC and dyspepsia patients showed an increase in the relative abundance of *Streptococcus* and a decrease in *Actinomyces* spp. (Table 3.1.2.2, Fig. 3.1.2.2).

3.1.3. Microbiome analysis

3.1.3.1. Comparison of bacterial composition in unwashed and washed biopsies

Bacterial DNA was detected in all biopsies, even in those were no growth was observed. The average distribution of the most prevalent bacterial groups in unwashed and washed biopsies according to patient groups is represented in Fig. 3.1.3.1.1 and Fig. 3.1.3.1.2.

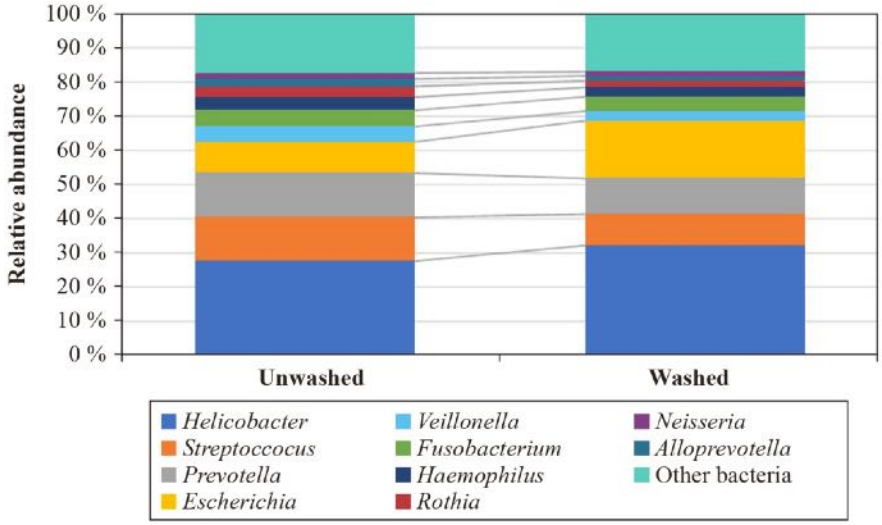

Fig. 3.1.3.1.1. *Comparison of the bacterial reads from microbiome analysis of unwashed and washed biopsies from dyspepsia patients (other bacteria are listed in text below)*

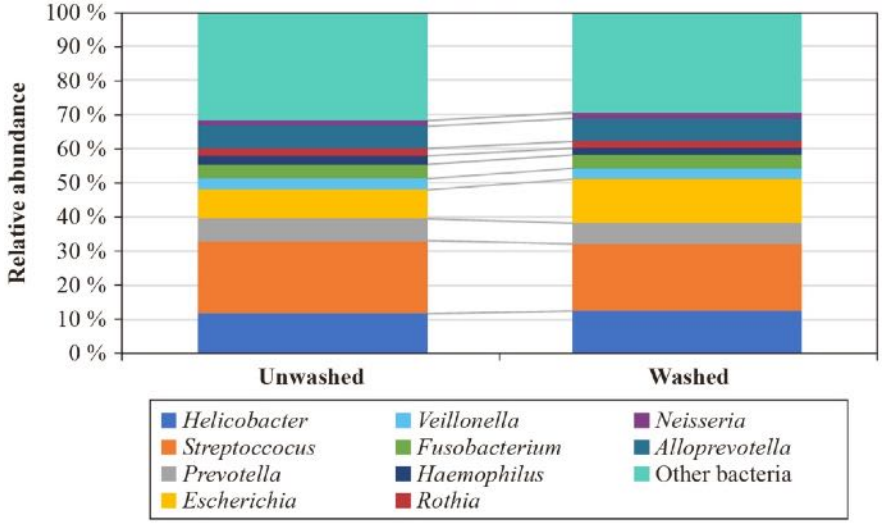

Fig. 3.1.3.1.2. *Comparison of the bacterial reads from microbiome analysis of unwashed and washed biopsies from gastric cancer patients (other bacteria are listed in text below)*

The ten most prevalent groups in the microbiome analysis were *Helicobacter* spp., *Streptococcus* spp., *Prevotella* spp., *Escherichia* spp., *Veillonella* spp., *Fusobacterium* spp., *Haemophilus* spp., *Rothia* spp., *Neisseria* spp., and *Alloprevotella* spp. The average relative abundance of *H. pylori* increased in

some of the washed biopsies compared to the unwashed, but this was not always observed in the individual samples (Table 3.1.3.1.1), therefore the increase in *H. pylori* was not significantly different.

Table 3.1.3.1.1. *Mean and standard error of the 10 most common bacterial groups as percentage of total bacterial reads in the microbiome analysis*

	Dyspepsia Biopsy Pairs (n = 22)		**Gastric Cancer Biopsy Pairs (n = 24)**	
	Unwashed (% ± SEM)	**Washed (% ± SEM)**	**Unwashed (% ± SEM)**	**Washed (% ± SEM)**
Helicobacter spp.	28.3 ± 7.9	31.2 ± 8.4	11.8 ± 4.4	12.7 ± 5.0
Streptococcus spp.	12.6 ± 1.8	9.6 ± 1.8	21.3 ± 4.3	20.1 ± 4.3
Prevotella spp.	13.9 ± 2.7	11.0 ± 2.5	6.4 ± 1.1	6.1 ± 1.3
Escherichia spp.	7.0 ± 2.9	10.4 ± 3.8	9.5 ± 3.6	11.9 ± 3.6
Veillonella spp.	5.3 ± 0.9	3.4 ± 0.9	3.5 ± 1.0	3.7 ± 0.7
Fusobacterium spp.	4.8 ± 1.0	4.3 ± 1.5	4.2 ± 1.4	3.7 ± 1.2
Haemophilus spp.	4.6 ± 1.3	3.2 ± 0.9	2.4 ± 0.5	2.0 ± 0.5
Rothia spp.	3.5 ± 0.8	1.9 ± 0.6	2.2 ± 0.4	2.0 ± 0.7
Neisseria spp.	2.2 ± 0.7	1.5 ± 0.6	6.5 ± 2.7	6.8 ± 2.5
Alloprevotella spp.	1.5 ± 0.5	1.4 ± 0.4	1.7 ± 0.7	1.8 ± 0.7
Other bacteria	17.4 ± 2.7	17.0 ± 3.1	30.57 ± 5.8	29.3 ± 5.2

Other bacteria are listed in text below.

The “other bacteria” belong to the bacterial groups that were found in several of the biopsies and are among the genera: *Abiotrophia*, *Aggregatibacter*, *Atopobium*, *Campylobacter*, *Capnocytophaga*, *Catonella*, *Corynebacterium*, *Dialister*, *Eubacterium*, *Filifacter*, *Flavobacterium*, *Gemella*, *Granulicatella*, *Lachnoanaerobaculum*, *Lactobacillus*, *Leptotrichia*, *Megasphaera*, *Oribacterium*, *Parvimonas*, *Peptostreptococcus*, *Porphyromonas*, *Propionibacterium*, *Selenomonas*, *Solobacterium*, *Staphylococcus*, *Stenotrophomonas*, *Stomatobaculum* and *Treponema* (percentages are listed in Table 3.1.3.1.2).

Table 3.1.3.1.2. *Mean and standard error of the most common bacterial groups belonging to "other bacteria" percentage of total bacterial reads in the microbiome analysis*

	Dyspepsia biopsy pairs (n = 22)		**Gastric cancer biopsy pairs (n = 24)**	
	Unwashed (% ± SEM)	**Washed (% ± SEM)**	**Unwashed (% ± SEM)**	**Washed (% ± SEM)**
Abiotrophia spp.	0.013 ± 0.007	0.007 ± 0.009	0.104 ± 0.055	0.128 ± 0.047
Aggregatibacter spp.	0.139 ± 0.043	0.134 ± 0.044	0.103 ± 0.038	0.187 ± 0.086
Atopobium spp.	0.359 ± 0.113	0.31 ± 0.128	0.328 ± 0.064	0.335 ± 0.086
Campylobacter spp.	0.507 ± 0.141	0.452 ± 0.104	0.423 ± 0.121	0.314 ± 0.116
Capnocytophaga spp.	0.455 ± 0.19	0.308 ± 0.123	0.329 ± 0.087	0.274 ± 0.079
Catonella spp.	0.311 ± 0.098	0.212 ± 0.056	0.093 ± 0.027	0.119 ± 0.061
Corynebacterium spp.	0.214 ± 0.112	0.257 ± 0.175	0.047 ± 0.017	0.08 ± 0.033
Dialister spp.	0.304 ± 0.089	0.199 ± 0.083	0.31 ± 0.132	0.242 ± 0.101
Eubacterium spp.	0.123 ± 0.03	0.181 ± 0.059	0.186 ± 0.07	0.277 ± 0.147
Filifactor spp.	0.172 ± 0.076	0.262 ± 0.139	0.043 ± 0.012	0.08 ± 0.044
Flavobcaterium spp.	1.235 ± 0.975	1.27 ± 0.927	0.164 ± 0.063	0.575 ± 0.375
Gemella spp.	0.827 ± 0.228	0.82 ± 0.313	0.861 ± 0.211	1.446 ± 0.433
Granulicatella spp.	1.046 ± 0.219	1.093 ± 0.206	1.333 ± 0.405	1.603 ± 0.431
Lachnoanaerobaculum spp.	0.296 ± 0.066	0.271 ± 0.106	0.457 ± 0.156	0.435 ± 0.137
Lactobacillus spp.	0.013 ± 0.007	0.012 ± 0.006	1.925 ± 1.245	10.5 ± 8.878
Leptotrichia spp.	0.459 ± 0.196	0.967 ± 0.516	0.728 ± 0.362	0.42 ± 0.177
Megasphaera spp.	0.554 ± 0.188	0.448 ± 0.143	0.219 ± 0.062	0.196 ± 0.059
Oribacterium spp.	0.432 ± 0.09	0.336 ± 0.11	0.329 ± 0.113	0.632 ± 0.327
Parvimonas spp.	0.229 ± 0.076	0.31 ± 0.188	1.478 ± 0.833	2.508 ± 1.555
Peptostreptococcus spp.	0.135 ± 0.045	0.13 ± 0.066	2.517 ± 1.448	3.319 ± 1.892
Porphyromonas spp.	1.765 ± 0.52	1.607 ± 0.653	0.666 ± 0.198	1.241 ± 0.566
Propionibacterium spp.	0.058 ± 0.031	0.082 ± 0.026	0.141 ± 0.056	0.256 ± 0.107
Selenomonas spp.	0.167 ± 0.059	0.231 ± 0.082	0.087 ± 0.022	0.088 ± 0.031
Solobacterium spp.	0.281 ± 0.062	0.282 ± 0.103	0.674 ± 0.29	0.603 ± 0.217
Staphylococcus spp.	0.403 ± 0.168	0.627 ± 0.153	0.292 ± 0.077	0.728 ± 0.351
Stenotrophomonas spp.	0.679 ± 0.54	1.128 ± 0.769	0.156 ± 0.052	0.495 ± 0.325
Stomatobaculum spp.	0.328 ± 0.12	0.365 ± 0.115	0.561 ± 0.332	0.775 ± 0.483
Treponema spp.	0.211 ± 0.076	0.391 ± 0.218	0.067 ± 0.031	0.056 ± 0.019

SEM: standard error of the mean.

The relative abundance of other non- *Helicobacter* bacteria was similar between unwashed and washed samples and neither of the listed bacterial

groups showed a significant change in relative abundance in both dyspepsia or GC groups (Fig. 3.1.3.1.1, 3.1.3.1.2). The bacterial distribution between the patient groups did not reach statistical significance. The bacterial diversity within the samples showed no statistical differences between the unwashed and washed biopsies (P = 0.22581) (Fig. 3.1.3.1.3), and similar bacterial species were clustered in both groups (P = 0.801199) (Fig. 3.1.3.1.4).

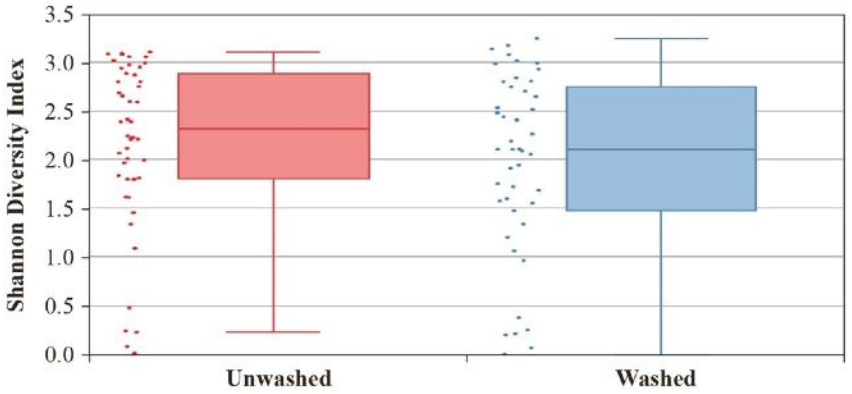

Fig. 3.1.3.1.3. *Comparison of the alpha-diversity shown by a Shannon Index (P = 0.22581) within the samples*

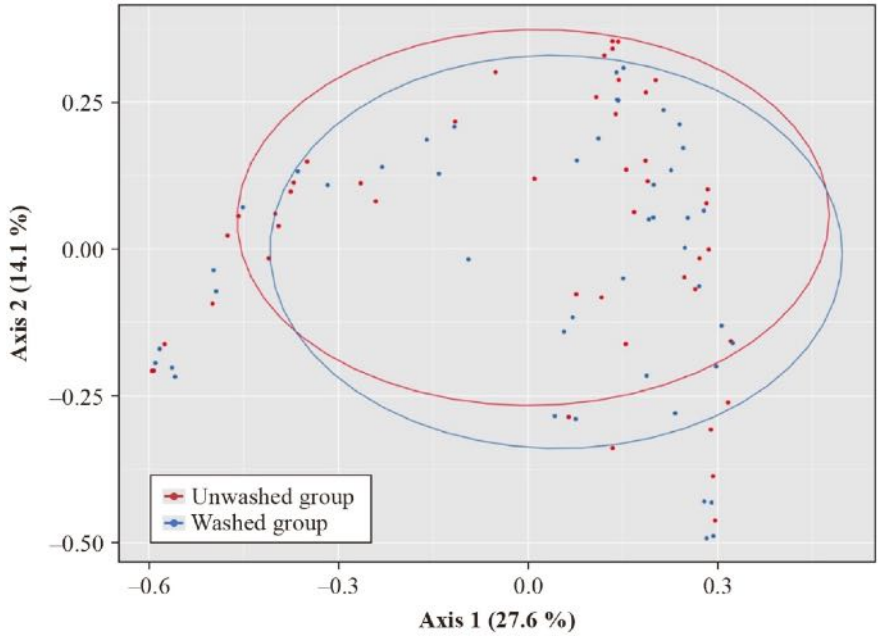

Fig. 3.1.3.1.4. *Comparison of the beta-diversity between the two groups shown by PCoA plot (P = 0.801199) in unwashed biopsies (red) and washed biopsies (blue)*

3.1.3.2. Comparison of bacterial composition in dyspepsia and gastric cancer patients

The average relative abundance of *H. pylori* was not significantly different in untreated biopsies from dyspepsia patients and GC patients (Fig. 3.1.3.2.1).

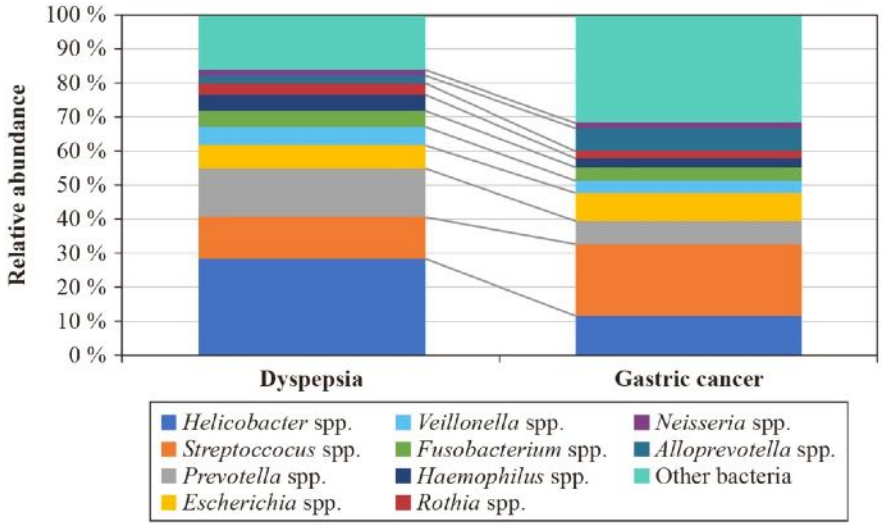

Fig. 3.1.3.2.1. *The average relative abundance of the ten most common bacterial genera of untreated biopsies from dyspepsia patients and gastric cancer patients*

For the majority of bacterial groups, no statistical difference was found in the distribution of bacteria between dyspepsia and GC patient biopsies. Nevertheless, a significant increase in the presence of *Prevotella* spp. was found in dyspepsia patients (P = 0.0109) and of "other bacteria" in GC patients (P = 0.0349). This statistical difference in "other bacteria" may be due to the dominance of *Enterococcus* spp. in one biopsy pair, whereas more than 95 % of the bacterial reads were recognized as this. But without this biopsy pair the difference in "other bacteria" between the patient groups was not statistically significant. The bacterial diversity in biopsies from dyspepsia and GC groups did not reach statistical significance within the groups (P = 0.556831) (Fig. 3.1.3.2.2) or between the distribution of species in the groups (Fig. 3.1.3.2.3).

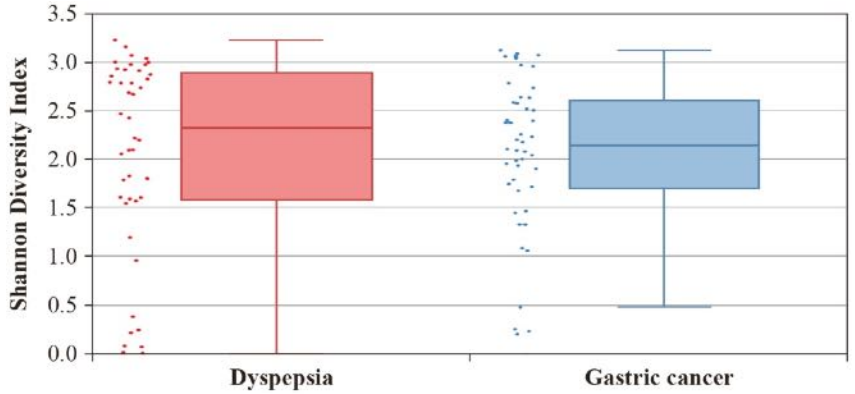

Fig. 3.1.3.2.2. *Comparison of the alpha-diversity shown by a Shannon Index (P = 0.556831) within the groups*

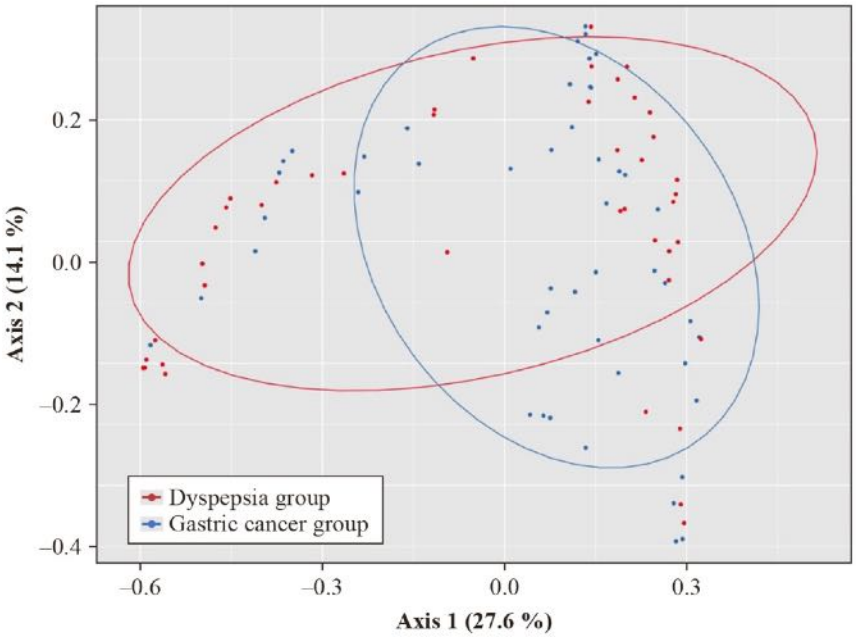

Fig. 3.1.3.2.3. *Comparison of the beta-diversity between the two groups shown by PCoA plot (P = 0.052947) in biopsies from dyspepsia patients (red) and gastric cancer patients (blue)*

A low percentage of the bacterial reads in biopsies from dyspepsia patients were identified as *Lactobacillus* spp. (< 0.1 % of total bacterial reads). Most biopsy pairs from GC patients (30 pairs) did not show presence of *Lactobacillus* spp.; 6 biopsy pairs showed a relative abundance of 0–0.5 % of total bacterial reads, and 7 biopsy pairs showed a relative abundance of 0.5–2 % *Lactobacillus* spp., while three biopsy pairs showed a high relative abundance

of *Lactobacillus* spp., which both increased (from 11 to 27 %) and decreased (from 23 % to 0.4 % and from 29 % to 5 %) between the unwashed and washed biopsies. However this difference in *Lactobacillus* spp. abundance was not significant between dyspepsia and gastric cancer patients.

3.1.3.3. Comparison of bacterial composition in antrum and cancer area biopsies of gastric cancer patients

The distribution of bacterial groups showed no significant differences between the antrum and cancer area biopsies (Fig. 3.1.3.3.1 and Table 3.1.3.3.1).

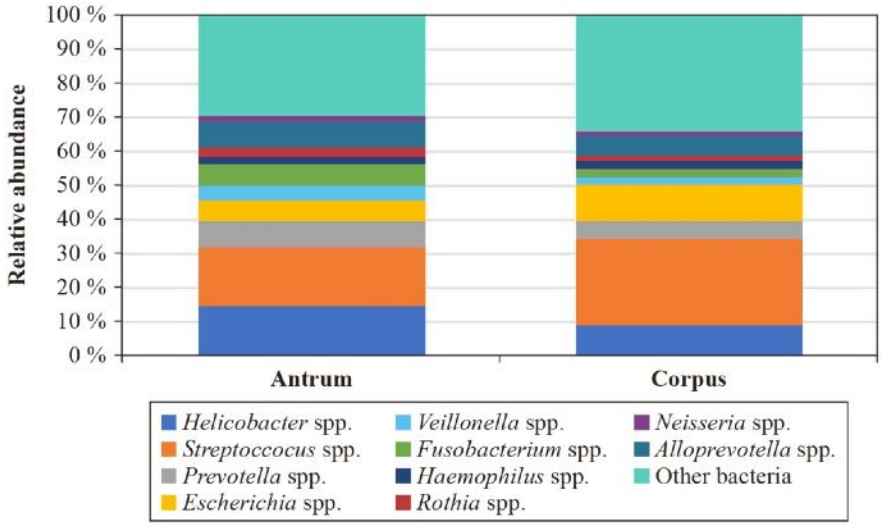

Fig. 3.1.3.3.1. *The relative abundance of bacteria in untreated biopsies from the antrum and corpus area of gastric cancer patients*

Table 3.1.3.3.1. *Mean and standard error as percentage of the 10 most common bacteria as percentage of total bacterial reads in the microbiome analysis of biopsies from gastric cancer patients (n = 12)*

	Antrum area (% ± SEM)	**Cancer area (% ± SEM)**
Helicobacter spp.	14.5 ± 7.3	9.1 ± 4.9
Streptococcus spp.	17.6 ± 5.1	25.1 ± 6.9
Prevotella spp.	7.4 ± 1.6	5.3 ± 1.5
Escherichia spp.	6.1 ± 2.7	12.9 ± 6.6
Veillonella spp.	4.3 ± 1.8	2.7 ± 2.0
Fusobacterium spp.	6.2 ± 2.6	2.1 ± 0.8
Haemophilus spp.	2.2 ± 0.7	2.6 ± 0.9

Table 3.1.3.3.1. *Continued*

	Antrum area (% ± SEM)	**Cancer area (% ± SEM)**
Rothia spp.	2.8 ± 0.8	1.6 ± 0.5
Neisseria spp.	7.3 ± 4.4	5.6 ± 3.2
Alloprevotella spp.	1.9 ± 1.0	1.5 ± 0.9
Other bacteria	29.6 ± 7.6	34.1 ± 8.8

SEM: standard error of the mean.

The diversity of bacteria in biopsies from antrum and cancer area in the corpus did not reach statistical differences in the bacterial diversity within the sample areas ($P = 0.960995$) (Fig. 3.1.3.3.2) or between the two sample areas ($P = 0.111888$) (Fig. 3.1.3.3.3).

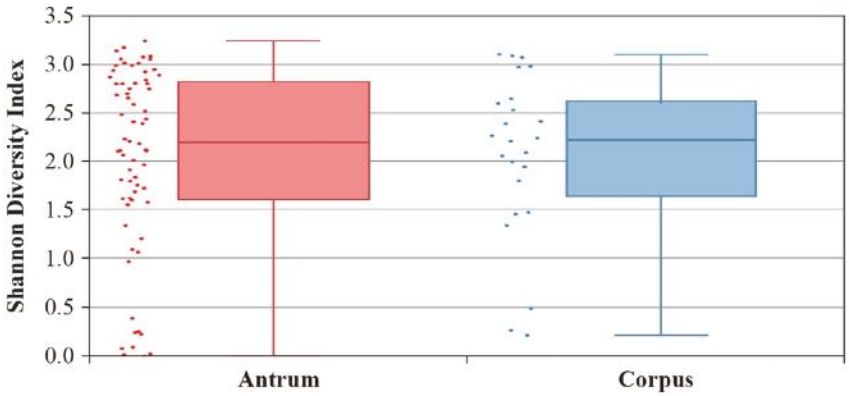

Fig. 3.1.3.3.2. *Comparison of the alpha-diversity shown by a Shannon Index (P = 0.960995) within the sample areas*

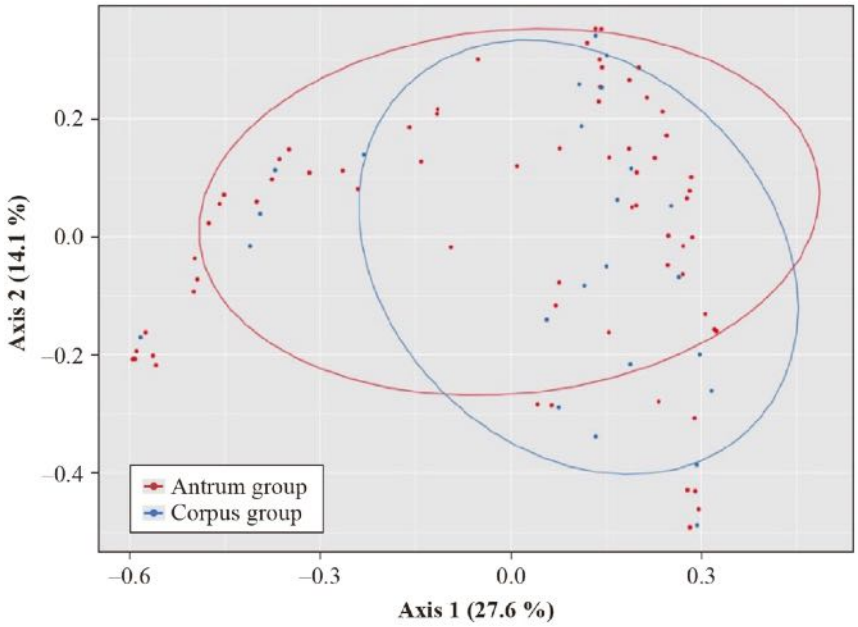

Fig. 3.1.3.3.3. *Comparison of the beta-diversity between shown by PCoA plot (P = 0.111888) in biopsies from antrum area (red) and corpus area (blue) in the stomach*

3.1.4. Analysis of bacterial composition in association with *H. pylori*

H. pylori was detected in 14 of 34 patients (41 %) by histology and only in 4 biopsies from 2 patients by culture, despite incubation for additional days. Microbiome analysis found DNA from *H. pylori* in 16 of 34 patients (47 %) as the only species of *Helicobacter* in the biopsies.

Three different distribution types of *H. pylori* and non-*Helicobacter* were identified: 4 biopsy pairs showed almost complete dominance of *H. pylori* (> 90 % of total bacterial reads in at least one of the biopsies) (Fig. 3.1.4.1), 16 biopsy pairs showed a mixed relative abundance of *H. pylori* and other bacteria (Fig. 3.1.4.2) and 26 biopsy pairs showed none or less than 1 % of total bacterial reads identified as *H. pylori* (Fig. 3.1.4.3).

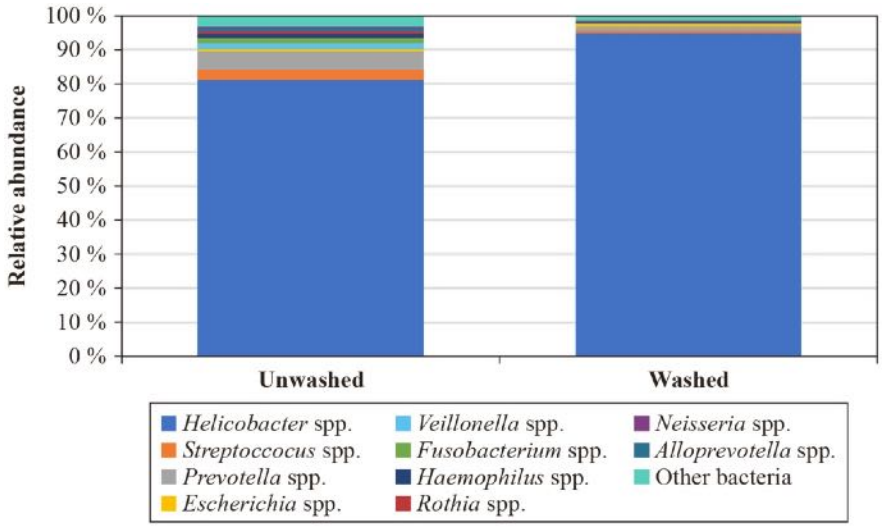

Fig. 3.1.4.1. *The distribution of H. pylori and non-Helicobacter species by dominance of H. pylori*

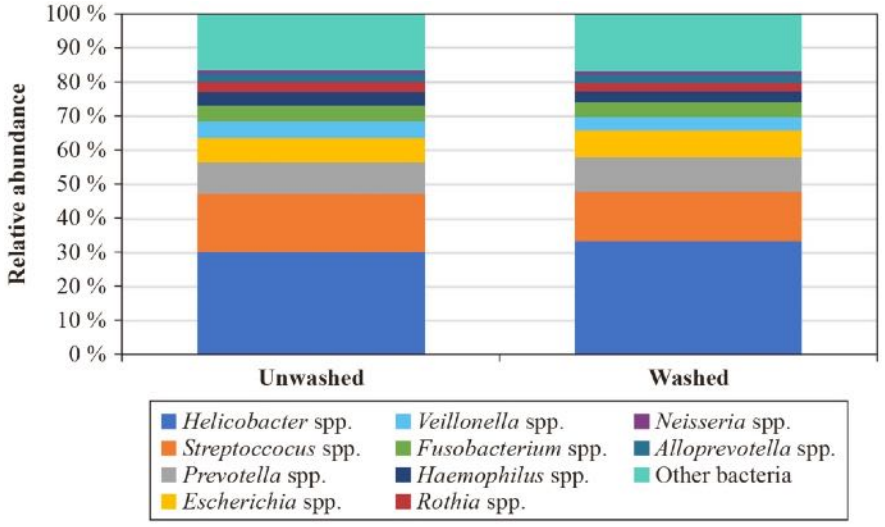

Fig. 3.1.4.2. *The distribution of H. pylori and non-Helicobacter species by mixed presence of non-Helicobacter and H. pylori*

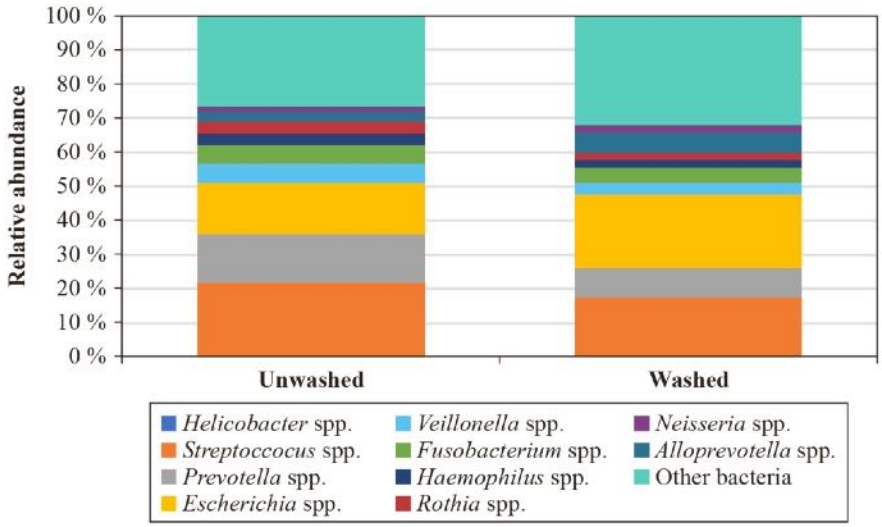

Fig. 3.1.4.3. *The distribution of H. pylori and non-Helicobacter species by absence of H. pylori*

The bacterial diversity in the gastric samples defined as positive or negative for *H. pylori* by histology has reached statistical significant differences. The *H. pylori*-negative biopsies showed a significantly higher diversity compare to *H. pylori*-positive biopsies ($P = 0.004353$) (Fig. 3.1.4.4).

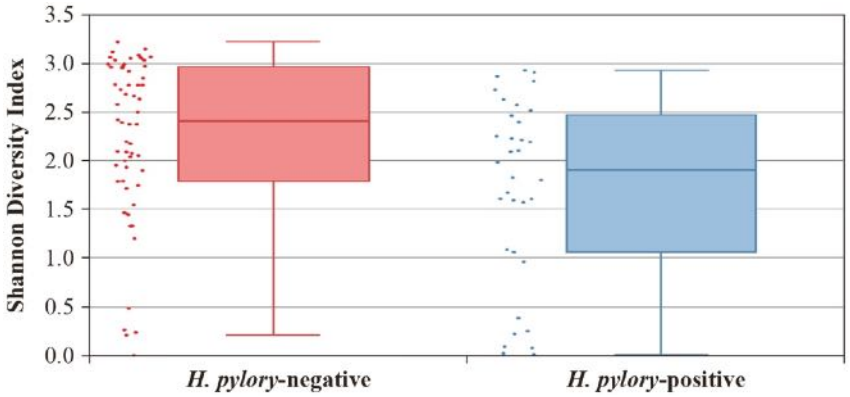

Fig. 3.1.4.4. *The alpha-diversity shown by a Shannon Index (P = 0.004353) within the group*

A significant difference in the bacterial diversity was observed between biopsies determined positive or negative for *H. pylori* ($P = 0.0009999$), showing that the presence of *H. pylori* may change the bacterial community to allow for a unique composition (Fig. 3.1.4.5).

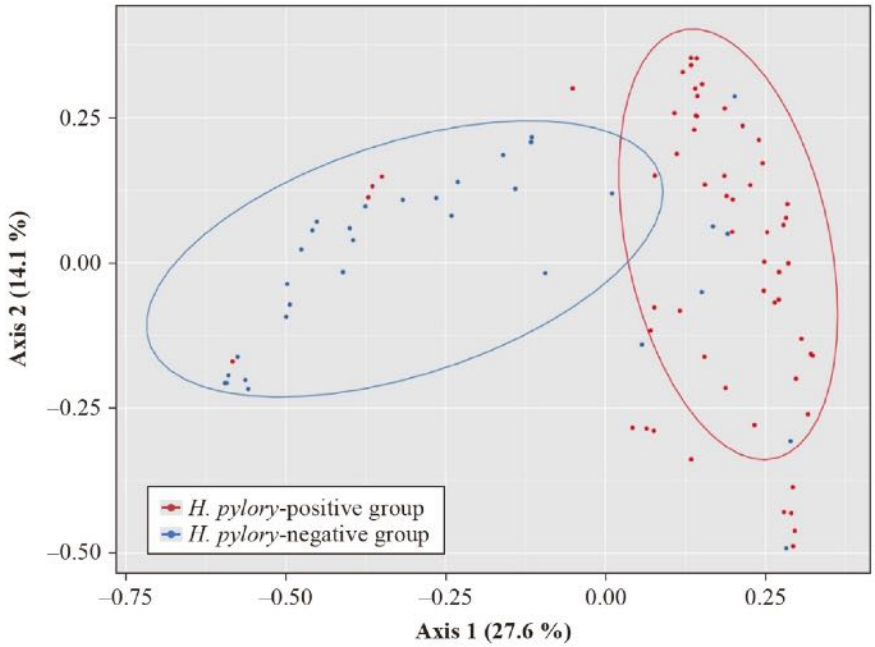

Fig. 3.1.4.5. *The beta-diversity between the two groups shown by PCoA plot (P = 0.000999.4)*

3.2. Results of the study: "Gastric microbiome in twins"

3.2.1. Characteristics of twin groups

The demographics and clinical data of twins groups are listed in Table 3.2.1.1.

Table 3.2.1.1. *The demographics and clinical characteristics of patient groups*

Twin pairs	Monozygotic n (14)			Dizygotic n (10)		
Gender of twins within a pair	MM n (3)	FF n (11)	MF –	MM n (1)	FF n (5)	MF n (4)
Mean (median) age	40.3 (39.5)			43.2 (43)		

MM: both twins of male gender; FF: both twins of female gender; MF: twin pair comprised of a male and a female twin.

3.2.2. Gastric microbiome profile

Whereas *H. pylori* totally colonized the stomach (Fig. 3.2.2.1), the structure of the microbiome in the gastric biopsies was defined by *H. pylori* status. Hence, *H. pylori* twins were grouped separately from negative twins.

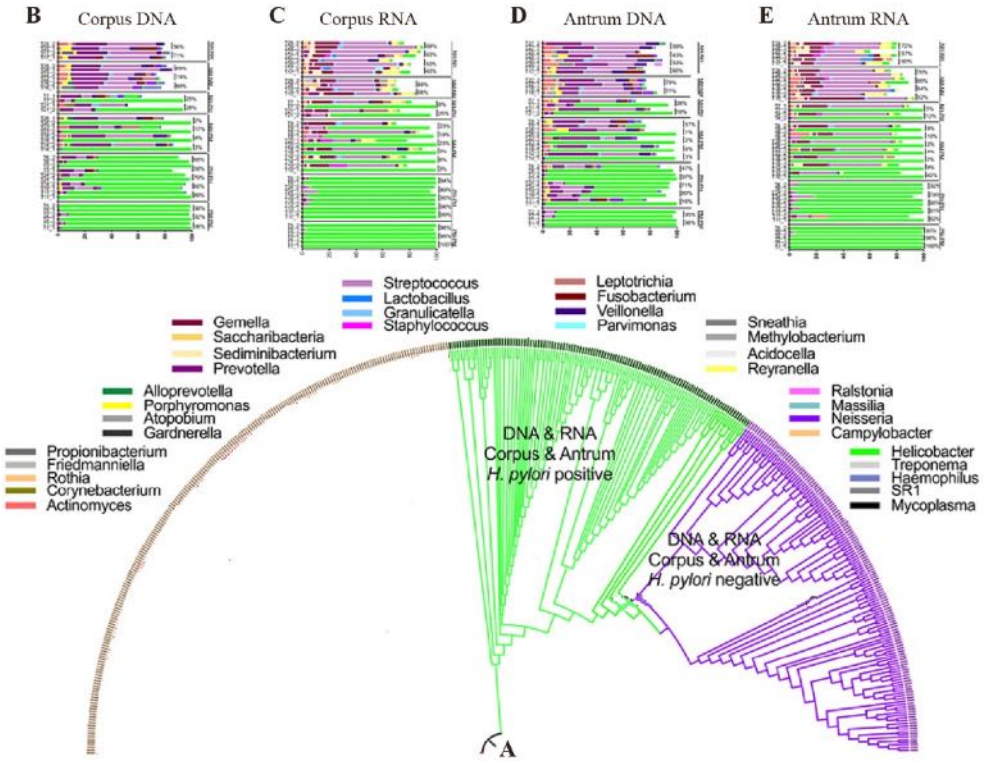

Fig. 3.2.2.1. *Gastric microbiome in twins*

Global bacteria composition analysis of stomach demonstrates that gastric (corpus and antrum biopsies) in *H. pylori* negative twins is mainly colonized by *Streptococcaceae* (Fig. 3.2.2.1).

3.2.3. *H. pylori* status

The overall prevalence of *H. pylori* among twins was 55 %. (Fig. 3.2.2.1 B to E). 16 out of 24 twin pairs were concordant for the *H. pylori* and 8 out of 24 twin pairs were discordant. *H. pylori* status was similar in MZ and DZ twins. According to PCoA *H. pylori* was the main factor for differentiating twins based on their microbial composition in the gastric (Fig. 3.2.3.1).

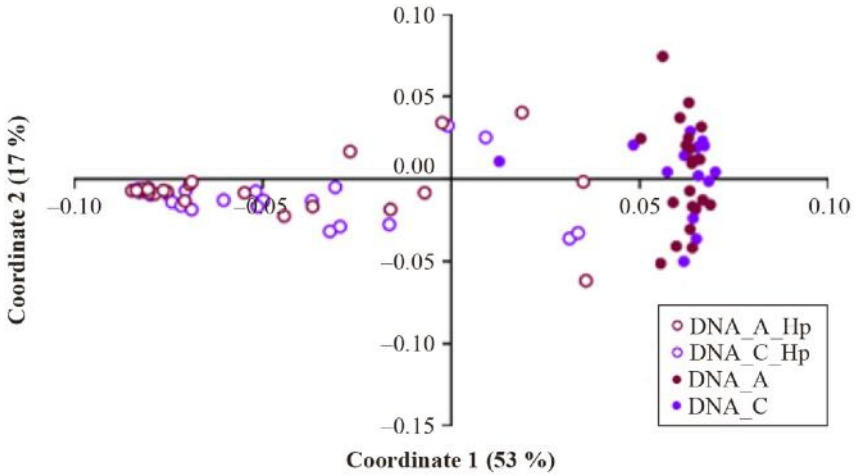

Fig. 3.2.3.1. *Principal coordinates analysis (PCoA) of the microbial communities in gastric*

DNA_C: corpus DNA, DNA_A: antrum DNA, Hp: *H. pylori*.

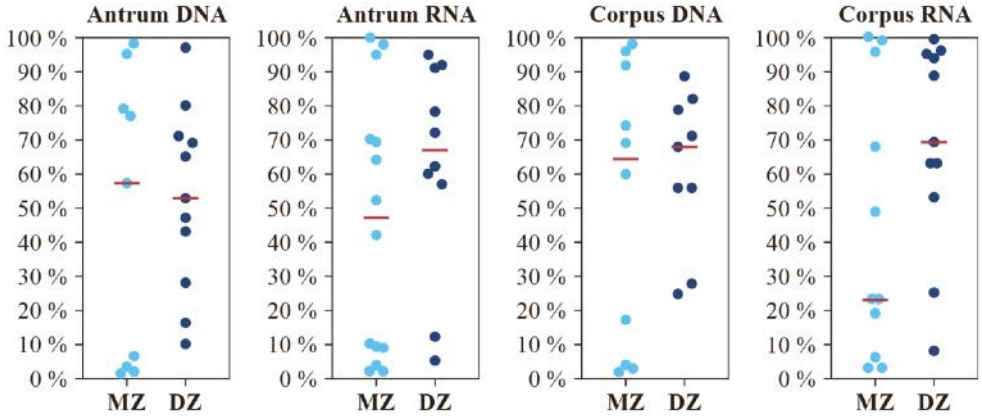

Fig. 3.2.3.2. *Percentage of Bray-Curtis similarities of the bacterial communities in twin pairs in gastric between monozygotic and dizygotic twins*

MZ: monozygotic, DZ: dizygotic.

Besides this the percentage of Bray-Curtis similarities was not statistical different between MZ and DZ twins (Fig. 3.2.3.2), indicating that zygosity posibly does not affect the gastric microbial composition.

3.3. Results of the study: "Gastritis stages in monozygotic and dizygotic dyspeptic twins"

3.3.1. Demographic characteristics and *H. pylori* status of subjects

Major demographic data of twins are presented in Table 3.3.1.1. The study included 29 twin pairs (58 subjects): 14 pairs of MZ and 15 pairs (10 same-sex pairs and 5 mixed-sex pairs) of DZ twins. The mean and median age (years) of MZ and DZ twins was 40.3 (39.5) and 38.6 (36), respectively ($P = 0.623$).

Table 3.3.1.1. *Demographics and H. pylori status distinguishing monozygotic versus dizygotic twins*

Twin pairs	**Monozygotic n (14)**			**Dizygotic n (15)**			***P* value**
Gender of twins within a pair	MM n (3)	FF n (11)	MF –	MM n (1)	FF n (9)	MF n (5)	
Mean (median) age	40.3 (39.5)			38.6 (36)			0.623
Concordant *H. pylori*-positive status	5			5			0.893
Concordant *H. pylori*-negative status	4			5			
Discordant *H. pylori*-negative status	5			5			

MM: both twins of male gender; FF: both twins of female gender; MF: twin pair comprised of a male and a female twin.

The overall prevalence of *H. pylori* infection was 51.7 %. Concordance rates for *H. pylori* infection prevalence among monozygotic and dizygotic twins was 35.7 % and 33.3 %, respectively ($P = 0.891$). Among monozygotic twins, the *H. pylori* status (Hp status) was as the following: five pairs were Hp-positive, four pairs were Hp-negative, and five pairs were Hp-discordant. Among dizygotic twins, five pairs were Hp-positive and five pairs were Hp-negative, and in five pairs, the Hp-status was discordant. Concordant Hp-status (both Hp-positive and Hp-negative) in monozygotic and dizygotic twins was determined in 9/14 (64.3 %) and in 10/15 (66.7 %) twin pairs, respectively ($P = 0.893$) (Table 3.3.1.1).

3.3.2. Histological alterations of gastric mucosa in twins

Concordance of topographical extension of atrophic lesions among monozygotic and dizygotic twins is shown in Table 3.3.2.1.

Table 3.3.2.1. *Concordance of topographical extension of atrophy and IM in monozygotic and dizygotic twins*

Twin pairs	Monozygotic		Dizygotic		*P* value
	n (14)	%	n (15)	%	
Concordance according to antrum athrophy	11	78.6	11	73.3	0.742
Concordance according to corpus athrophy	13	92.9	13	86.7	0.584
Concordance according to antrum IM	12	85.7	11	73.3	0.411
Concordance according to corpus IM	12	85.7	13	86.7	0.941

IM: intestinal metaplasia.

The distribution of monozygotic and dizygotic twins according to OLGA and OLGIM gastritis stages is demonstrated in Table 3.3.2.2. The prevalence of low-risk OLGA and OLGIM stages among mono- and dizygotic twins was 100 % and 93.3 %, respectively, whereas the prevalence of high-risk stages was 0 % and 6.7 %, respectively. Concordance according to OLGA and OLGIM stages between monozygotic and dizygotic twins did not reach statistical significance ($P = 0.097$ and $P = 0.175$, respectively).

Table 3.3.2.2. *Gastritis stage (OLGA and OLGIM systems) in monozygotic and dizygotic twins*

Stage	OLGA-staging				*P* value	OLGIM-staging				*P* value
	Monozygotic twins		Dizygotic twins			Monozygotic twins		Dizygotic twins		
	n (28)	%	n (30)	%		n (28)	%	n (30)	%	
0	18	64.3	24	80		19	67.9	24	80	
I	9	32.1	3	10		8	28.6	3	10	
II	1	3.6	1	3.3	0.097	1	3.6	1	3.3	0.175
III	–	–	2	6.7		–	–	2	6.7	
IV	–	–	–	–		–	–	–	–	

OLGA: Operative Link on Gastritis Assessment; OLGIM: Operative Link on Gastritis/ Intestinal-Metaplasia Assessment.

Tables 3.3.2.3 and 3.3.2.4 show the prevalence of similar or different gastritis stages (grouped in low *versus* high risk) in monozygotic and dizygotic twins distinguished by their Hp-status (Hp-positive pair, Hp-negative-pair, and Hp-discordant pair). Low-risk stages (stages 0-I-II) largely prevailed among both mono- and dizygotic twins (only two cases featured a high-risk stage (stage III, by OLGA and OLGIM). By applying both OLGA and OLGIM staging, no differences emerged in the distribution of twins by stage after performing a comparison of low- *versus* high-risk stages.

Table 3.3.2.3. *Gastritis OLGA-stage according to the H. pylori status: twins are distinguished according to zygosity*

Group of stages	**Twins pair**					
	***H. pylori*-positive (10 pairs)**		***H. pylori*-negative (9 pairs)**		***H. pylori*-discordant (10 pairs)**	
	MZ	**DZ**	**MZ**	**DZ**	**MZ**	**DZ**
Similar low-risk stage (0-I-II)	5	4	4	5	5	4
Similar high-risk stage (III-IV)	–	–	–	–	–	–
Discordant stage (0-I-II *versus* III-IV)	–	1	–	–	–	1

MZ: Monozygotic; DZ: Dizygotic.

Table 3.3.2.4. *Gastritis OLGIM-stage according to the H. pylori status distinguished by zygosity*

Group of stages	**Twins pair**					
	***H. pylori*-positive (10 pairs)**		***H. pylori*-negative (9 pairs)**		***H. pylori*-discordant (10 pairs)**	
	MZ	**DZ**	**MZ**	**DZ**	**MZ**	**DZ**
Similar low-risk stage (0-I-II)	5	4	4	5	5	4
Similar high-risk stage (III-IV)	–	–	–	–	–	–
Discordant stage (0-I-II *versus* III-IV)	–	1	–	–	–	1

MZ: Monozygotic; DZ: Dizygotic.

4. DISCUSSION

4.1. "Transient and persistent gastric microbiome: adherence of bacteria in gastric cancer and dyspeptic patient biopsies after washing "

4.1.1. Comparison of washed and unwashed biopsies

A decreased bacterial growth from the washed biopsies compared to the unwashed biopsies observed in our study (Fig. 3.1.2.1, Table 3.1.2.1) might indicate that the bacteria removed by washing were not adhering to the biopsy and should be considered as a transient part of the gastric microbiota. We observed that *Streptococcus* spp. was predominant non-*Helicobacter* bacterial group in the biopsies. This may either be explained by a higher starting concentration in the unwashed biopsy or an increased ability to adhere to the tissue, compared to the other bacteria in the biopsies.

The non-*Helicobacter* species identified in our study have been described by previous studies as oral, upper airways or intestinal commensals, including species of the dominant groups *Streptococcus*, *Rothia* and *Actinomyces* [73, 83, 84, 113, 170]. Intestinal-originated bacteria like *Enterococcus* spp. as well as *Escherichia* spp. were also identified with both culture and microbiome analysis and may be attributed to the overgrowth from the intestines. The low detection of *Staphylococcus* spp. in microbiome analysis compared to culture may be explained by selection of growth of *Staphylococcus*, as species of this bacterial group are facultative aerobic with a fast growth rate.

Based on our hypothesis, it was expected, that only *H. pylori* would remain in the washed biopsies. However, the number of non-*Helicobacter* bacteria remained in washed biopsies. Besides this, the average relative abundance and diversity of these bacteria within the biopsies did not significantly change after several washing steps (Fig. 3.1.3.1.1 and 3.1.3.1.2). This imply that some of non-*Helicobacter* bacterial groups must be able to adhere to the biopsies and may remain in the gastric mucosa, therefore should be further explored.

4.1.2. Comparison of gastric cancer and dyspepsia patients

Although carcinogenesis of GC has been associated with a progressive shift in gastric microbiota towards oral and intestinal bacteria [58], there is still no specific bacterial genera in the only of the patient groups [58, 171]. Our expectation was that a different bacterial composition would be observed in the GC compared to dyspepsia patients. However, in our study the bacterial

diversity between biopsies from control group and GC patients showed no significant differences (Fig. 3.1.3.3.1).

We observed a higher abundance of *Lactobacillus* spp. in GC group biopsies compared to control group biopsies in both the culture and microbiome analysis, however this difference was not statistical significant. Therefore the statement that species of *Lactobacillus* are involved in the carcinogenesis of GC might not be generally correct. Species of *Lactobacillus* were cultured from four biopsies, whereas no reads were identified in the microbiome analysis. In comparison 16 biopsies were negative for culture of *Lactobacillus* spp., but positive in the microbiome analysis. An explanation for the discrepancy in culture and microbiome analysis can be that 16S rRNA-based analysis is more sensitive, and able to to detect the presence of bacteria in small quantities. On the other hand 16S rRNA gene amplicon sequencing is not genus-specific, hence, the 16S rRNA genes from several bacteria are amplified simultaneously. For instance, if *Lactobacillus* spp. are present in small quantities, the presence of a high amount of other DNA may drown it out, and it will not be detected. Combined with a selection in the culture for microaerobic-growing bacteria, the abundance of *Lactobacillus* spp. may appear higher in this experiment setting. Therefore this difference between methods should also be considered in future culture- and sequencing-based studies.

A study by Blaser and Atherton discussed whether *H. pylori* is a main factor of GC development, or if its presence is enough to change the gastric microenvironment and allow colonization of other bacteria, which in turn may promote the development of GC [172]. It was agree that long-term colonization causes inflammation and damage to the host tissues but that the mechanism leading to development of GC is not well understood [172]. In order to fully understand the pathological changes of other non-*Helicobacter* species in the stomach, infection assays should be performed on human cell cultures or model organisms with gastrointestinal tracts more similar to humans.

4.1.3. Bacterial composition in association with *H. pylori*

H. pylori was only cultured from two biopsies after extended culture, despite that its presence in other patients was confirmed by histology and microbiome analysis. Moreover, microbiome analysis identified *H. pylori* in two biopsies, while the histology was negative for *H. pylori*. This comparison of methods demonstrates that culture, histology and 16S rRNA gene sequencing are not always in accordance, and investigation of bacterial communities using several methods is preferred. Similarly, it was demonstrated

by previous study, where *H. pylori* was detected with sensitive DNA-based methods in subjects who previously were found to be negative for *H. pylori* with conventional methods [58]. Beside this, identification of *H. pylori* through the culture is ussually challenging or even impossible because of its slow growth and fastidious nature or ability to take a coccoid form during prolonged incubation [73]. The culture results are still considered to be valid, as other species were cultivated, and thus, the medium and growth conditions must have been sufficient for bacterial growth. However, in order to culture all the present bacteria in the biopsies, several other medium types and atmospheres might have been considered.

We expected that the relative distribution of *H. pylori* should be increased after wash, along with the removal of contaminating bacteria. However this was not observed in all cases (Fig. 3.1.3.1.1 and 3.1.3.1.2). The findings from microbiome sequencing demonstrated three types of *H. pylori* and non-*H. pylori* bacteria distrubution in the gastric environment, suggesting that the stomach is more complex than previously thought (Fig. 3.1.4.1–3.1.4.3). The geographical origin of the patients can be also important for natural variations in the microbiota. Our findings contribute to the theory that relatioship between *H. pylori* and non-*Helicobacter* bacteria is a dynamic process, and previous discrepancies between studies may be caused by natural difference between patients. The ten most prevalent groups with microbiome analysis agrees with the groups detected in other reported next-generation sequencing (NGS)-studies [58].

The biopsies showing positive or negative for *H. pylori* by histology analysis showed significant differences in the bacterial diversity within ($P = 0.004353$) and between ($P = 0.000999.4$) the groups (Fig. 3.1.4.4 and 3.1.4.5), presupposing that *H. pylori* may promote changes in the environment to allow for survival of other bacteria as suggested by Blaser and Atherton [172]. Similar findings were also obtained by Maldonado-Contreras et al., which discovered a difference in the bacterial diversity based on *H. pylori*-status [84]. Patients negative for *H. pylori* presented a higher relative abundance of *Actinobacteria* and *Firmicutes*, whereas *H. pylori*-patients represented a higher abundance of *Proteobacteria* and *Acidobacteria*.

The most common bacterial groups identified in the culture and microbiome analysis shows few similarities, and only two genera in the 10 most abundant groups from the microbiome analysis were cultured. Even though, both *Gemella* and *Granulicatella* were detected in several biopsies but not in high enough reads to be included in the top 10. The difference between the methods may be determined by selection of certain bacteria in the microaerobic incubation. Four of the 10 most common groups have been described as anaerobes or strict anaerobes (*Prevotella, Veillonella, Fusobacterium,*

Neisseria), preventing them from growing in the microaerobic environment used in this study. *H. pylori* was found in 47 % of the biopsies by microbiome analysis but only cultured from two biopsy pairs. The biopsies were stored in the transport medium at −80 °C, which might have decreased the viability of *H. pylori*. It may be suggested that other bacteria identified by microbiome analysis were also present in the biopsies but were inhibited by the storage/ freezing, and therefore unable to be cultured.

4.2. "Gastric microbial in twins"

It has been hypothesized that host genetics may determine the composition of the gut microbiome [17], even though the genetic inheritance of microbial composition has been recently questioned [173]. The role of host genetics in shaping of microbiome has been proposed earlier [174], however, more recent evidence highlight the predominant role of environmental factors, (such as age, sugar consumption, toothbrushing habits) *versus* the host factors in defining the bacterial composition in twins [175–177]. The concept of stomach microbiome is still evolving with an ongoing discussion about what constitutes a gastric microbiome [83, 178].

While the largest studies related to twins have been dealing with easily accessible specimens like saliva and feces, only limited data were available for systematic characterization of mucosal microbiome in other body niches such as stomach. In the only available study at present, the authors evaluated four pairs of twins with very heterogeneous results [18]. In our study, we evaluated microbiome in the largest cohort (20 twin pairs) that were representative for various combinations of MZ and DZ including concordant and discordant for *H. pylori* status. Even though larger cohorts are needed to estimate the minor effects in concordant groups, we clearly demonstrate the predominant role of *H. pylori* status in defining composition of the gastric microbiome in twins irrespective of zygosity status. Our data are in agreement with previous studies where *H. pylori* is the major gastric microbiome shaping species [61, 179]. In earlier high-quality studies, familial environment and *H. pylori* status were considered as the predominant risk factors for peptic ulcer development which is supported by our results [180].

This work provides important findings on the stomach microbiome in twins, even so, various further aspects need more accurate evaluation. Even though this work is so far the largest to assess the stomach microbiota in twins, we are unable to address the microbial similarity in depth for the substantial effect of *H. pylori* on stomach microbiota. Further studies in larger cohorts of MZ, DZ and predominantly non-twin siblings would likely help to

estimate the dynamics in microbial patterns. This cohort included only a single time point, whereas multiple time points would be needed to assess. Furthermore, this work is based on 16s rRNA, therefore, whole genome sequencing including metatranscriptomic would likely provide a much more comprehensive view on the similarity and function of the stomach microbiome.

4.3. "Gastritis stages in monozygotic and dizygotic dyspeptic twins"

Our study has investigated the phenotype of *H. pylori*-associated gastritis in two cohorts of monozygotic (14 pairs) and dizygotic (15 pairs) twins. The results of our study revealed high concordance rates of gastric mucosal alterations both in monozygotic and dizygotic twins. To our best knowledge, to date, there have been no previous reports, which have assessed the importance of shared genetic influences on susceptibility and phenotype of chronic *H. pylori* gastritis and premalignant gastric alterations on a well defined twin cohort.

Epidemiological contexts, case-controls, and twins studies have addressed the issue of the possible interaction between different host-related and environmental risk factors in the promotion of gastric precancerous lesions and gastric mucosal atrophy. These factors have been extensively studied among the patients with precancerous gastric lesions and included virulence factors of *H. pylori* strains, environmental factors (smoking, salted food, etc.), or genetic predisposition of the host [145, 181]. To date, however, the exact factors that trigger malignant transformation from normal gastric mucosa towards gastric cancer are not completely understood.

A previous study conducted in Sweden involving monozygotic (36 pairs) and dizygotic (88 pairs) twins reared apart revealed that the concordance rate for *H. pylori* infection was significantly higher among monozygotic twins (82 %) than among dizygotic (66 %) twins [180]. Slightly higher concordance rates for *H. pylori* infection prevalence among monozygotic (35.7 %) *versus* dizygotic (33.3 %) have also been observed in the present study. The difference, however, did not reach statistical significance, probably due to a small number oftwin pairs. Based on case-control studies in different geographical regions, which evaluate the prevalence of *H. pylori* infection and the development of gastric atrophy and IM, first-degree relatives of GC patients are associated with an increased risk for GC. A Marcos-Pinto et al. [181] study showed that first-degree relatives of early-onset gastric carcinoma patients have significantly higher prevalence of *H. pylori*, AG, and advanced stages

of OLGA. *H. pylori* was present in 82 % of cases and in 59 % of controls ($P = 0.001$); AG was diagnosed in 70 % of cases and in 32% ofcontrol individuals, respectively ($P < 0.001$), while OLGA stages III and IV were present in 10% and 9% in both groups, respectively ($P < 0.001$). The results of Rokkas et al. [182] meta-analysis showed similar results: their pooled OR of *H. pylori* infection, AG, and IM between individuals with family history of GC were 1.92-fold ($P \leq 0.001$), 2.2-fold ($P = 0.005$), and 1.98-fold ($P \leq 0.001$) higher, respectively, in comparison with controls. These results can be influenced by the exposition of shared environmental factors (alcohol, smoking, salted and smoked food, and hygiene) between family members, circulation of intrafamilial *H. pylori* strains [183–185], and genetic susceptibility [186–188].

Our study showed that there was no difference in concordance rates for chronic *H. pylori* gastritis among monozygotic twins (64.3 %, 9/14 pairs) as compared to dizygotic twins 66.7 % (10/15 pairs) ($P = 0.893$). This could partly relate to the fact that twins share the same environment in early childhood and genetic predisposition for *H. pylori* acquisition might be less important. Concordance of topographical extension of atrophy and IM was higher among monozygotic twins but did not reach statistical significance. There were no statistical differences in concordance rates according to OLGA and OLGIM stages between monozygotic and dizygotic twins ($P = 0.097$ and 0.175, respectively). It is very important to point out that 11 of 14 monozygotic twin pairs and 10 out of 15 dizygotic twins had absolute concordance for OLGA stages. Similarly, 10 out of 14 monozygotic twin pairs and 10 out of 15 dizygotic twins had absolute concordance for OLGIM stages. These findings suggest that if one twin is identified with high- risk premalignant gastric lesions, the other twin should also be assessed for these alterations.

A relatively small number of the study population represent the main limitation of the present study. The numbers of individual twins within our study group were not very large, and certain effects might have been biased. The design of the study also did not allow us to evaluate as to what extent gastric mucosal alterations in twins are related to the common shared genetic factors per se or shared environmental factors during childhood and later on in lifetime. There might also have been a certain selection bias, because our study included only twins with dyspeptic symptoms.

CONCLUSIONS

1. The number of cultured non-*Helicobacter* bacteria decreased in the washed biopsies, suggesting that they might be a transient contamination from oral cavity. However, in the microbiome analysis, no significant differences in the bacterial diversity were observed between unwashed and washed biopsies.

2. The bacterial diversity in the biopsies from dyspepsia and gastric cancer patients showed no significant differences.

3. The bacterial diversity in biopsies that were *H. pylori*-positive and *H. pylori*-negative was significantly different, implying that *H. pylori* is the major modulator of gastric microbiome.

4. The zygosity does not affect the stomach bacterial composition. However, the presence of *H. pylori* seems to be the determinant for differentiating twins based on their bacterial composition in the stomach irrespective of zygosity.

5. Pathomorphological alterations of gastric mucosa in monozygotic and dizygotic twins showed high rates of concordance. However the prema-lignant lesions rated by OLGA and OLGIM gastritis stages were not modulated by the zygosity of the twins.

SUMMARY IN LITHUANIAN

1. Įvadas

Skrandžio adenokarcinoma yra viena labiausiai paplitusių onkologinių ligų pasaulyje, pagal sergamumą ir mirtingumą užimantį penktąją ir ketvirtąją vietą iš visų piktybinių navikų [1]. Ši skrandžio vėžio forma pagal *Lauren* klasifikaciją histologiškai skirstoma į tris pagrindinius tipus (išvardyti pagal dažnumą klinikinėje praktikoje mažėjančia seka): žarninį, difuzinį ir mišrų [2, 3]. Šių morfologinių adenokarcinomos tipų išsivystymui įtakos turi *H. pylori* [4], kuri 1994 m. Tarptautinio vėžio tyrimo centro prie PSO buvo pripažinta I klasės kancerogenu [5], o su skrandžio vėžiu taip pat buvo pradėtos sieti ir kitos, skrandyje egzistuojančios, potencialiai patogeninės bakterijos. Didžioji dalis skrandžio mikrobiotos studijų pasaulyje atliekamos, siekiant išsiaiškinti galimą kancerogeninį mikrobiotos poveikį žarninio tipo adenokarcinomai. Difuzinio tipo skrandžio vėžys lyginant su žarninio tipo adenokarcinoma dažniau siejamas su žmogaus genetiniais veiksniais nei su *H. pylori* ar aplinkos veiksniais [4], todėl skrandžio mikrobiotos reikšmė jo atžvilgiu yra ištirta mažiau.

Pasaulyje plačiai paplitusi *H. pylori* infekcija (užsikrėtę apie 50 proc. pasaulio populiacijos) iki šiol yra pagrindinis skrandžio vėžio rizikos veiksnys [6], aktyvinantis daugiapakopę Correa P. pasiūlytą ikivėžinių pokyčių seką, kuriai progresuojant išsivysto žarninio tipo skrandžio adenokarcinoma. Vis dėlto lieka neaišku, kodėl tik 1–2 proc. asmenų, užsikrėtusių *H. pylori*, išsivysto skrandžio vėžys [7], o jos eradikacija nebūtinai sumažina skrandžio vėžio išsivystymo riziką [8, 9]. Paskutiniais metais atlikti tyrimai parodė, kad tarp atskirų ikivėžinių pokyčių stadijų (lėtinio gastrito, atrofinio gastrito, žarninės metaplazijos, displazijos) ir skrandžio vėžio egzistuoja skrandžio mikrobiotos sudėties pokyčių ir skirtumų [10]. Pagrindinis šių pokyčių bruožas yra progresuojantis *H. pylori* sumažėjimas, susijęs su įvairių skrandyje kolonijas formuojančių mikroorganizmų pagausėjimu arba sumažėjimu [10]. Šie radiniai pagrindžia hipotezę, kad polinkis susirgti skrandžio vėžiu yra susijęs ne tik su genetiniais [11] ir aplinkos veiksniais (rūkymu, nesaikingu druskos vartojimu ir kt.) [12, 13], bet gali būti nulemtas ir skrandyje aptinkamų bakterijų. Be to, įmanomą šių bakterijų įtaką skrandžio vėžiui formuotis rodo skrandžio mikrobiotos metabolitų skirtumai tarp navikinio audinio ir jį supančios aplinkinės gleivinės, nustatyti studijomis, tiriančiomis skrandžio mikrobiotos medžiagų apykaitą [14].

Vis dėlto tikslus taksonominis skrandžio disbiozės modelis bei priežastinio ryšio grandinė tarp disbiozinių skrandžio pokyčių ir jo karcinogenezės

šiuo metu nėra nustatyti. Siekiant atrasti tikslesnį skrandžio mikrobiotos ir karcinogenezės modelį, svarbu nustatyti sveikų ir skrandžio vėžiu sergančių asmenų laikiną ir nuolatinę skrandžio mikrobiotą, kartu ištirti kompleksinį aplinkos ir žmogaus genetinių veiksnių poveikį mikrofloros sudėčiai ir tumorogenezei.

Plataus masto genomo asociacijos tyrimų duomenimis, apie 10 proc. žmogaus mikrobiotos įvairovės priklauso nuo jo genetinių veiksnių [15], tačiau šis ryšys dar nėra visiškai ištirtas. Todėl studijos, atliekamos siekiant išsiaiškinti sąveiką tarp žmogaus genetinių variacijų ir mikrofloros sudėties, yra labai svarbios. Dvynių studijos yra vienas iš geriausių tyrimo metodų, vertinant genetikos įtaką mikrobiomui bendroje aplinkoje. Paskutiniais metais atliktos dvynių studijos įrodė paveldimumo įtaką žarnyno mikrobiomo sudėčiai [16, 17]. Tačiau studijų, analizuojančių skrandžio mikrobiomą panaudojant dvynių modelį, nėra daug, todėl paveldimumo įtaka jo mikrobiomo sudėčiai iki šiol nėra įrodyta [18].

Paveldimumas taip pat yra svarbus žarninio tipo skrandžio adenokarcinomai, kuriai kaip ir difuziniam jos tipui, yra būdinga šeiminė skrandžio vėžio anamnezė [19]. Priešingai nei difuzinė šio vėžio forma, žarninis skrandžio vėžys, veikiamas aplinkos veiksnių [20, 21], vystosi pereidamas daugiapakopę ikivėžinių skrandžio gleivinės pokyčių seką. Dėl šios priežasties pavykus išsiaiškinti, ar ikivėžinių pokyčių, tokių kaip atrofinis gastritas, fenotipas yra paveldimas, būtų galima kurti naujus personalizuotus skrandžio vėžio rizikos vertinimo ir stebėsenos metodus pirmos eilės giminaičiams. Tačiau studijų, analizuojančių gastrito fenotipo koreliaciją panaudojant dvynių modelį šiuo metu nėra atlikta.

2. Tikslas ir uždaviniai

Tikslas

Nustatyti su skrandžio vėžiu siejamus mikrobiotos pakitimus ir palyginti dvynių skrandžio mikrobiotos bei lėtinio gastrito fenotipų skirtumus.

Uždaviniai

1. Ištirti laikinos ir nuolatinės skrandžio mikrobiotos pakitimus pacientams, sergantiems skrandžio vėžiu, bei pacientams, turintiems dispepsijos simptomų.
2. Palyginti pacientų, sergančių skrandžio vėžiu, bei pacientų, turinčių dispepsijos simptomų, skrandžio mikrobiotos sudėtį.
3. Įvertinti *H. pylori* įtaką skrandžio mikrobiotos sudėčiai.
4. Įvertinti zigotiškumo įtaką dvynių skrandžio mikrobiotai.

5. Palyginti monozigotinių ir dizigotinių dvynių skrandžio gleivinės patomorfologinius pokyčius naudojant OLGA/OLGIM gastrito histologinį klasifikavimą.

Darbo naujumas

Karcinogeninis skrandžio mikrobiotos poveikis pastaraisiais dešimtmečiais tapo aktualia mikrobiotos studijų sritimi. Nors, pasitelkus naujas genomines bakterijų tyrimų technologijas, ankstesnės studijos įrodė, kad, be *H. pylori*, dalį skrandžio mikrobiotos sudaro daugybė kitų bakterijų rūšių, tačiau apibendrinamosios išvados, kurios iš bakterijų rūšių yra laikinos, o kurios sudaro nuolatinį skrandžio mikrobiomą, nėra suformuluotos. Todėl pirmoji mūsų tyrimo dalis buvo skirta patikrinti hipotezę, ar *H. pylori* yra vienintelė bakterija, skrandyje egzistuojanti kaip nuolatinė skrandžio mikrobiotos dalis, galinti išlikti dominuojančia bakterija biopsijose po taikyto plovimo. Mes įvertinome nuolatinės ir laikinos skrandžio mikrobiotos sudėtį, palygindami bakterijų sudėties pokyčius plautose ir neplautose skrandžio biopsijose. Tai yra pirmasis pasaulyje tyrimas, kuriame, naudojant bakterijų 16S rRNR variabilaus regiono pagausinimą PGR metodu, buvo bandoma nuodugniai išskirti laikiną ir nuolatinę skrandžio mikrobiotą sudarančias bakterijas ir palyginti skrandžio vėžiu sergančių bei dispepsijos simptomų turinčių pacientų skrandžio mikrobiotą. Tyrimo rezultatai yra svarbūs siekiant nustatyti, ar *H. pylori* nulemti mikrobiotos pokyčiai gali skatinti skrandžio karcinogenezę.

Pastaruoju metu didelio susidomėjimo taip pat sulaukė žmogaus genetikos ir mikrobiotos sąveika [22]. Pasitelkus viso genomo asociacijos tyrimus, ryšys tarp genetinių variacijų ir žarnyno mikrobiomo buvo analizuojamas keletoje studijų [23][24]. Nors daugelis identifikuotų genų lokusų buvo susieti su mikroorganizmų taksonomine įvairove ir sudėties skirtumais, tačiau daugelis šių radinių buvo nustatyti tiriant tikslines populiacijas ir yra sunkiai atkartojami. Taigi, svarstant, ar žmogaus skrandžio mikrobiomo įvairovę gali nulemti paveldimi veiksniai, reikalingos tolimesnės studijos siekiant nustatyti žmogaus genetikos ir mikrobiomo sąveiką [25]. Todėl antroji mūsų tyrimo dalis buvo skirta nustatyti zigotiškumo įtaką dvynių skrandžio mikrobiotos sudėčiai.

Žinoma, kad skrandžio gleivinės ikivėžiniai pakitimai, pavyzdžiui, atrofinis gastritas, vystosi progresuojant lėtiniam *H. pylori* gastritui, kuris yra veikiamas žmogaus genetinių ir aplinkos veiksnių, tačiau šis ryšys dar nėra galutinai ištirtas. Taigi, studijos, tiriančios aplinkos ir paveldimumo įtaką morfologinėms lėtinio *H. pylori* gastrito savybėms, yra labai svarbios. Todėl trečiosios mūsų tyrimo dalies tikslas buvo įvertinti ir palyginti monozigotinių

ir dizigotinių dvynių lėtinio gastrito fenotipų skirtumus. Mūsų žiniomis tai yra pirmasis tyrimas, ištyręs dvynių skrandžio gleivinės pokyčius.

3. Metodai

Tyrimas atliktas gavus Lietuvos Bioetikos komiteto leidimą (Protokolo Nr. 8/2011) ir Kauno regioninio Biomedicininių tyrimų etikos komiteto leidimą (Protokolo Nr. BE-2-10). Visi asmenys pasirašė sutikimo dalyvauti tyrime formas.

Tiriamieji

Į pirmąją tyrimo dalį buvo įtraukti Lietuvos sveikatos mokslų universiteto ligoninės Kauno klinikų (toliau – Kauno klinikų) Gastroenterologijos klinikos pacientai, sergantys naujai diagnozuotu skrandžio vėžiu, ir pacientai, turintys dispepsijos simptomų. Jie sudarė kontrolinę tyrimo grupę. Į tyrimą buvo įtraukti tik vyresni nei 18 metų amžiaus pacientai, kuriems anksčiau nebuvo taikyta *H. pylori* eradikacija bei 3 mėnesius iki endoskopinio tyrimo nebuvo skiriami protonų siurblio inhibitoriai (PSI) ir antibiotikai. Anksčiau netaikytas skrandžio vėžio gydymas buvo papildomas kriterijus skrandžio vėžio grupės pacientams. Iš viso į tyrimą įtraukėme 12 pacientų, sergančių skrandžio vėžiu, ir 22 kontrolinės grupės pacientus.

Antrosios ir trečiosios tyrimo dalies tiriamųjų grupes sudarė Lietuvos sveikatos mokslų universiteto (LSMU) Biologinių sistemų ir genetinių tyrimų instituto Dvynių centro dvynių poros. Tyrime dalyvavo tik vyresni nei 18 metų amžiaus dvyniai, kuriems anksčiau nebuvo taikyta *H. pylori* eradikacija bei 1 mėnesį iki endoskopinio tyrimo nebuvo skirtas gydymas PSI ir antibiotikais. Antrosios tyrimo dalies imtį sudarė 20 dvynių porų, turinčių dispepsijos simptomų ir dalyvaujančių studijoje, kuri buvo sudaryta iš 108 dvynių porų ir skirta ištirti dvynių seilių, skrandžio ir storojo žarnyno mikrobiotos sudėčiai.

Trečiosios tyrimo dalies imtį sudarė 29 dvynių poros: dvidešimt dvynių porų iš antrosios tyrimo dalies ir devynios naujai į šią tyrimo dalį įtrauktos dvynių poros.

Tyrimo eiga ir metodika

Pirmojoje tyrimo dalyje tiriamiesiems ezofagogastroduodenoskopijos (EFGDS) metu atskiromis biopsinėmis žnyplėmis atliktos antralinės skrandžio dalies gleivinės biopsijos bei paimtos skrandžio vėžio grupės pacientų papildomos biopsijos iš vėžio apimtos zonos gleivinės. Pirmoji biopsija iš kiekvienos srities buvo perkeliama į mėgintuvėlį su specialia terpe ir šaldoma

–80 °C temperatūroje, antroji prieš perkeliant į specialią terpę ir užšaldant buvo du kartus plaunama PBS buferyje, o trečioji naudojama histologinei *H. pylori* identifikacijai. Bakterijos skrandžio gleivinės mėginiuose buvo identifikuojamos naudojant biopsijų pasėlius ir MALDI-TOFF (angl. *matrix-assisted laser desorption/ionization time-of-flight mass spectrometry*) masės spektometrijos tyrimą bei atliekant bioinformatinę 16S rRNR bibliotekų duomenų analizę (BION-META (*http://box.com/bion*) paketas). Statistinė duomenų analizė atlikta naudojant „GraphPad Prism“ (5.01 versija) programą ir R paketą.

Antrojoje tyrimo dalyje dalyviams EFGDS metu atliktos prievarčio bei skrandžio kūno gleivinės biopsijos, kurios buvo iš karto perkeliamos į mėgintuvėlius be terpės ir šaldomos –80 °C temperatūroje. Bakterijų DNR ir RNR iš biopsinės medžiagos buvo išskirtos naudojant „Allprep DNA/RNA mini kit“ (Qiagen, Vokietija) rinkinį, o 16S rRNR bibliotekos buvo paruoštos naudojant 16S rRNR variabilaus regiono pagausinimą PGR metodu. Statistinė duomenų analizė atlikta naudojant R paketą.

Atliekant trečiąją tyrimo dalį dvyniams EFGDS metu paimtos skrandžio gleivinės biopsijos pagal atnaujintos Sidnėjaus klasifikacijos protokolą. Šios biopsijos pagal ėminio topografinę vietą buvo perkeltos į atskirus mėgintuvėlius, užpildytus 10 proc. formalino tirpalu. Vėliau šie mėginiai buvo liejami į parafininius blokelius ir ruošiami histologiniams preparatams. *H. pylori* identifikacijai histologiniai preparatai buvo dažomi hematoksilino-eozino (HE) ir Gimzos metodais. Histologiniai audinių preparatai buvo vertinami pagal atnaujintą Sidnėjaus klasifikaciją bei OLGA ir OLGIM gastrito stadijavimo sistemas. Statistinė duomenų analizė atlikta naudojant *IBM SPSS Statistics for Windows* (22.0 versija) programą.

4. Rezultatai

Pirmosios tyrimo dalies pacientų demografiniai ir klinikiniai duomenys pateikti 4.1 lentelėje.

4.1 lentelė. *Demografiniai ir klinikiniai pacientų duomenys*

Diagnozė	**Vidutinis amžius (metai)**	**Lytis**	***H. pylori* statusas (Histologija)**	**Lauren klasifikacija**	**G stadija**	**Skrandžio vėžio sritis – Proksimalinė/ Distalinė**
Skrandžio adenokarcinoma (n = 12)	62,2	Moterys (n = 5)	Neigiamas (n = 9)	Difuzinis (n = 6)	2 (n =5)	Proksimalinė (n = 3)
		Vyrai (n = 7)	Teigiamas (n = 3)	Žarninis (n = 4)	3 (n =7)	Distalinė (n = 9)
				Mišrus (n = 2)		
Dispepsija (n = 22)	48,1	Moterys (n = 18)	Neigiamas (n = 11)	–	–	–
		Vyrai (n = 4)	Teigiamas (n = 11)	–	–	–

Iš 4.2 lentelėje pateiktų duomenų matyti, kad 27 biopsijų porose po plovimo pasėliai buvo neigiami arba juose nustatytas sumažėjęs ne-*Helicobacter* bakterijų kolonijų skaičius, 5 biopsijų porų pasėliuose ne-*Helicobacter* bakterijų kolonijų skaičiaus pokyčių nenustatyta, o 3 porose plautų biopsijų pasėliuose kolonijų skaičius buvo didesnis už kolonijų skaičių neplautų biopsijų pasėliuose. Tai rodo, kad dalis bakterijų, kaip ir buvo tikėtasi, buvo išplautos iš skrandžio gleivinės kaip laikina mikroflora.

4.2 lentelė. *Neplautų ir plautų skrandžio gleivinės pasėlių rezultatų palyginimas*

Neplautų ir plautų biopsijų pasėlių rezultatai	**Dispepsijos grupė**	**Skrandžio vėžio grupė**
Abiejose biopsijų grupėse bakterijų augimo nestebėta	8 biopsijų poros	3 biopsijų poros
Bakterijų augimas stebėtas tik neplautų biopsijų pasėliuose	4 biopsijų poros	5 biopsijų poros
Sumažėjęs bakterijų augimas plautų biopsijų pasėliuose	9 biopsijų poros	9 biopsijų poros
Abiejose biopsijų grupėse bakterijų augimo pokyčių nestebėta	1 biopsijų pora	4 biopsijų poros
Pagausėjęs bakterijų augimas plautų biopsijų pasėliuose	0 biopsijų porų	3 biopsijų poros

Atlikus mikrobiotos analizę, statistiškai reikšmingų bakterijų rūšių įvairovės (α įvairovės) (p = 0,22581) bei jų rūšinio pasiskirstymo (β įvairovės) (p = 0,801199) skirtumų neplautuose ir plautuose skrandžio gleivinės mėgi-

niuose nenustatyta. Statistiškai reikšmingų bakterijų α (p = 0,556831) ir β (p = 0,052947) įvairovės skirtumų taip pat nenustatyta ir tarp pacientų grupių. Tačiau statistiškai reikšmingai gausesnė (p = 0,004353) ir skirtinga (p = 0,0009999) rūšinė bakterijų įvairovė nustatyta mėginiuose be *H. pylori* palyginti su mėginiais, kuriuose *H. pylori* buvo nustatyta.

Antrojoje tyrimo dalyje atlikę dvynių skrandžio mikrobiotos analizę, nustatėme, kad skrandžio gleivinės mėginiuose esanti *H. pylori* yra dominuojanti bakterijų rūšis. *Streptococcaceae* buvo vyraujanti ne-*Helicobacter* bakterijų šeima. 8 iš 20 dvynių porų *H. pylori* atžvilgiu buvo diskordantiškos. Analizė parodė, kad *H. pylori* yra pagrindinis veiksnys, įtakojantis dvynių skrandžio mikrobiotos sudėtį. Be to, lyginant bakterijų sudėties panašumą porų mėginiuose pagal Brei-Kurtis (angl. *Bray-Curtis*) kriterijų, statistiškai reikšmingų skirtumų tarp dvynių grupių nenustatyta.

Trečiosios tyrimo dalies dvynių porų demografiniai ir klinikiniai duomenys pateikti 4.3 lentelėje.

4.3 lentelė. *Demografiniai ir klinikiniai dvynių porų duomenys*

Dvynių poros	**Monozigotinės n (14)**			**Dizigotinės n (15)**			**p vertė**
Dvynių lytis poroje	VV n (3)	MM n (11)	VM –	VV n (1)	MM n (9)	VM n (5)	
Amžiaus vidurkis (mediana)	40,3 (39,5)			38,6 (36)			0,623

VV: abu dvyniai vyriškosios lyties; MM: abu dvyniai moteriškosios lyties; VM: dvynių pora sudaryta iš vyriškosios ir moteriškosios lyties individų.

H. pylori konkordantiškumas tarp monozigotinių ir dizigotinų dvynių porų statistiškai reikšmingai nesiskyrė (p = 0,893). Įvertinus OLGA ir OLGIM gastrito stadijų konkordantiškumą tarp monozigotinių ir dizigotinių dvynių, statistiškai reikšmingų skirtumų taip pat nebuvo nustatyta (p = 0,097, p = 0,175).

5. Išvados

1. Po plovimo mėginiuose sumažėjęs bakterijų kolonijų skaičius įrodo, kad dalis skrandyje aptinkamų bakterijų rūšių yra laikinos, o bakterijų rūšių įvairovė bei jų rūšinis pasiskirstymas neplautuose ir plautuose skrandžio gleivinės mėginiuose statistiškai reikšmingai nesiskyrė.
2. Statistiškai reikšmingų skrandžio mikrobiotos pokyčių sergant skrandžio vėžiu nenustatyta.

3. Statistiškai reikšmingi bakterijų įvairovės pokyčiai mėginiuose, esant *H. pylori,* rodo, kad *H. pylori* yra pagrindinis veiksnys, įtakojantis skrandžio mikrobiotos sudėtį.
4. Zigotiškumo įtakos skrandžio mikrobiotos sudėčiai nenustatėme. *H. pylori* buvo pagrindinis veiksnys, įtakojantis dvynių skrandžio mikrobiotos sudėtį, neatsižvelgiant į jų zigotiškumą.
5. Patomorfologinių skrandžio gleivinės pokyčių konkordantiškumo dažnis tarp monozigotinių ir dizigotinių dvynių buvo aukštas, tačiau ikivėžiniai pokyčiai, stadijuojami pagal OLGA ir OLGIM gastrito klasifikavimo sistemas, nebuvo nulemti dvynių zigotiškumo.

REFERENCES

1. Sung H, Ferlay J, Siegel RL, et al. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 2021; 71: 209–249.
2. Berlth F, Bollschweiler E, Drebber U, et al. Pathohistological classification systems in gastric cancer: Diagnostic relevance and prognostic value. *World J Gastroenterol* 2014; 20: 5679–5684.
3. López-Carrillo L, Vega-Ramos B, Costa-Dias R, et al. Histological types of gastric cancer in Mexico. *Int J Epidemiol* 1997; 26: 1166–1171.
4. Assumpção PP, Barra WF, Ishak G, et al. The diffuse-type gastric cancer epidemiology enigma. *BMC Gastroenterol* 2020; 20: 1–7.
5. Schistosomes, liver flukes and Helicobacter pylori. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7-14 June 1994. IARC Monogr Eval Carcinog Risks Hum. 1994;61:1–241.
6. Hooi JKY, Lai WY, Ng WK, et al. Global Prevalence of Helicobacter pylori Infection: Systematic Review and Meta-Analysis. *Gastroenterology* 2017; 153: 420–429.
7. Björkholm B, Falk P, Engstrand L, et al. Helicobacter pylori: Resurrection of the cancer link. *J Intern Med* 2003; 253: 102–119.
8. Hu YL, Pang W, Huang Y, et al. The Gastric Microbiome Is Perturbed in Advanced Gastric Adenocarcinoma Identified Through Shotgun Metagenomics. *Front Cell Infect Microbiol* 2018; 8: 1–11.
9. Gao JJ, Zhang Y, Gerhard M, et al. Association between gut microbiota and Helicobacter pylori-Related gastric lesions in a high-risk population of gastric cancer. *Front Cell Infect Microbiol* 2018; 8: 1–12.
10. Wen J, Lau HCH, Peppelenbosch M, et al. Gastric microbiota beyond h. Pylori: An emerging critical character in gastric carcinogenesis. *Biomedicines* 2021; 9: 1–18.
11. Persson C, Canedo P, MacHado JC, et al. Polymorphisms in inflammatory response genes and their association with gastric cancer: A HuGE systematic review and meta-analyses. *Am J Epidemiol* 2011; 173: 259–270.
12. Gonázlez CA, López-Carrillo L. Helicobacter pylori, nutrition and smoking interactions: Their impact in gastric carcinogenesis. *Scand J Gastroenterol* 2010; 45: 6–14.
13. Raei N, Behrouz B, Zahri S, et al. Helicobacter pylori infection and dietary factors act. *Asian Pacific J Cancer Prev* 2016; 17: 917–921.
14. Dai D, Yang Y, Yu J, et al. Interactions between gastric microbiota and metabolites in gastric cancer. *Cell Death Dis*; 12. Epub ahead of print 2021. DOI: 10.1038/s41419-021-04396-y.
15. Jha S, Rollins MG, Fuchs G, et al. Genome-wide association analysis identifies variation in vitamin D receptor and other host factors influencing the gut microbiota. *Nat Genet* 2017; 546: 651–655.
16. Turnbaugh PJ, Hamady M, Yatsunenko T, et al. A core gut microbiome between lean and obesity twins. *Nature* 2009; 457: 480–484.
17. Goodrich JK, Davenport ER, Beaumont M, et al. Genetic Determinants of the Gut Microbiome in UK Twins. *Cell Host Microbe* 2016; 19: 731–743.
18. Dong Q, Xin Y, Wang L, et al. Characterization of Gastric Microbiota in Twins. *Curr Microbiol* 2017; 74: 224–229.

19. Shin CM, Kim N, Lee HS, et al. Intrafamilial aggregation of gastric cancer: A comprehensive approach including environmental factors, Helicobacter pylori virulence, and genetic susceptibility. *Eur J Gastroenterol Hepatol* 2011; 23: 411–417.
20. Li Y, Eshak ES, Shirai K, et al. Alcohol consumption and risk of gastric cancer: The Japan collaborative cohort study. *J Epidemiol* 2021; 31: 30–36.
21. Jakszyn P, González CA. Nitrosamine and related food intake and gastric and oesophageal cancer risk: A systematic review of the epidemiological evidence. *World J Gastroenterol* 2006; 12: 4296–4303.
22. Kurilshikov A, Wijmenga C, Fu J, et al. Host Genetics and Gut Microbiome: Challenges and Perspectives. *Trends Immunol* 2017; 38: 633–647.
23. Turpin W, Espin-Garcia O, Xu W, et al. Association of host genome with intestinal microbial composition in a large healthy cohort. *Nat Genet* 2016; 48: 1413–1417.
24. Bonder MJ, Kurilshikov A, Tigchelaar EF, et al. The effect of host genetics on the gut microbiome. *Nat Genet* 2016; 48: 1407–1412.
25. Weissbrod O, Rothschild D, Barkan E, et al. Host genetics and microbiome associations through the lens of genome wide association studies. *Curr Opin Microbiol* 2018; 44: 9–19.
26. Manuscript A. Europe PMC Funders Group An African origin for the intimate association between humans and Helicobacter pylori. 2007; 445: 915–918.
27. Lin BPC, Harmata PA. Gastric and esophageal brush cytology. *Pathology* 1983; 15: 393–397.
28. Wyatt JI, Rathbone BJ, Dixon MF, et al. Campylobacter pyloridis and acid induced gastric metaplasia in the pathogenesis of duodenitis. *J Clin Pathol* 1987; 40: 841–848.
29. Suhardja TS, Kua H, Hrabovszky Z. Helicobacter pylori Colonisation in Heterotopic Gastric Mucosa in Meckel Diverticulum: Case Report and Review of the Literature. *Clin Med Insights Case Reports* 2019; 12: 10–12.
30. IJsselmuiden CB, Faden RR. Research and Informed Consent in Africa — Another Look. N Engl J Med 1992; 326: 830–834.
31. Cover TL, Blaser MJ. Helicobacter pylori in Health and Disease. Gastroenterology 2009; 136: 1863–1873.
32. Progress M. INCE the first culture of. 2002; 347: 1175–1186.
33. Brown LM. Helicobacter pylori: Epidemiology and routes of transmission. *Epidemiol Rev* 2000; 22: 283–297.
34. Krakowka S, Eaton KA, Rings DM, et al. Gastritis induced by helicobacter pylori in gnotobiotic piglets. *Rev Infect Dis* 1991; 13: S681–S685.
35. Miller EF, Maier RJ. Ammonium metabolism enzymes aid Helicobacter pylori acid resistance. *J Bacteriol* 2014; 196: 3074–3081.
36. Nakamura H, Yoshiyama H, Takeuchi H, et al. Urease plays an important role in the chemotactic motility of Helicobacter pylori in a viscous environment. *Infect Immun* 1998; 66: 4832–4837.
37. Williams SM, Chen YT, Andermann TM, et al. Helicobacter pylori chemotaxis modulates inflammation and bacterium-gastric epithelium interactions in infected mice. *Infect Immun* 2007; 75: 3747–3757.
38. Eaton KA, Morgan DR, Krakowka S. Motility as a factor in the colonisation of gnotobiotic piglets by Helicobacter pylori. *J Med Microbiol* 1992; 37: 123–127.
39. Hessey SJ, Spencer J, Wyatt JI, et al. Bacterial adhesion and disease activity in Helicobacter associated chronic gastritis. *Gut* 1990; 31: 134–138.

40. Lindén SK, Sheng YH, Every AL, et al. MUC1 limits Helicobacter pylori infection both by steric hindrance and by acting as a releasable decoy. *PLoS Pathog*; 5. Epub ahead of print 2009. DOI: 10.1371/journal.ppat.1000617.
41. Van de Bovenkamp JHB, Mahdavi J, Korteland-Van Male AM, et al. The MUC5AC Glycoprotein is the Primary Receptor for Helicobacter pylori in the Human Stomach. *Helicobacter* 2003; 8: 521–532.
42. Mahdavi J, Sondén B, Hurtig M, et al. Helicobacter pylori sabA adhesin in persistent infection and chronic inflammation. *Science (80-)* 2002; 297: 573–578.
43. Peck B, Ortkamp M, Diehl KD, et al. Conservation, localization and expression of HopZ, a protein involved in adhesion of Helicobacter pylori. *Nucleic Acids Res* 1999; 27: 3325–3333.
44. Senkovich OA, Yin J, Ekshyyan V, et al. Helicobacter pylori AlpA and AlpB Bind host laminin and influence gastric inflammation in gerbils. *Infect Immun* 2011; 79: 3106–3116.
45. Supajatura V, Ushio H, Wada A, et al. Cutting Edge: VacA, a Vacuolating Cytotoxin of Helicobacter pylori , Directly Activates Mast Cells for Migration and Production of Proinflammatory Cytokines . *J Immunol* 2002; 168: 2603–2607.
46. Torres VJ, VanCompernolle SE, Sundrud MS, et al. Helicobacter pylori Vacuolating Cytotoxin Inhibits Activation-Induced Proliferation of Human T and B Lymphocyte Subsets. *J Immunol* 2007; 179: 5433–5440.
47. Papini E, Satin B, Norais N, et al. Selective increase of the permeability of polarized epithelial cell monolayers by Helicobacter pylori vacuolating toxin. *J Clin Invest* 1998; 102: 813–820.
48. Umehara S, Higashi H, Ohnishi N, et al. Effects of Helicobacter pylori CagA protein on the growth and survival of B lymphocytes, the origin of MALT lymphoma. *Oncogene* 2003; 22: 8337–8342.
49. Murata-Kamiya N, Kurashima Y, Teishikata Y, et al. Helicobacter pylori CagA interacts with E-cadherin and deregulates the β-catenin signal that promotes intestinal transdifferentiation in gastric epithelial cells. *Oncogene* 2007; 26: 4617–4626.
50. Galmiche A, Rassow J, Doye A, et al. The N-terminal 34 kDa fragment of Helicobacter pylori vacuolating cytotoxin targets mitochondria and induces cytochrome c release. *EMBO J* 2000; 19: 6361–6370.
51. Grande R, Giulio M Di, Di Campli E, et al. A model of Helicobacter pylori persistence in a case of gastric cancer. *New Microbiol* 2010; 33: 343–349.
52. Kersulyte D, Chalkauskas H. Emergence of recombinant strains of Helicobacter pylori during human infection. 1999; 31: 31–43.
53. Salama N, Guillemin K, McDaniel TK, et al. A whole-genome microarray reveals genetic diversity among Helicobacter pylori strains. *Proc Natl Acad Sci U S A* 2000; 97: 14668–14673.
54. Kennemann L, Didelot X, Aebischer T, et al. Helicobacter pylori genome evolution during human infection. *Proc Natl Acad Sci U S A* 2011; 108: 5033–5038.
55. Gerhard M, Lehn N, Neumayer N, et al. Clinical relevance of the Helicobacter pylori gene for blood-group antigen-binding adhesin. *Proc Natl Acad Sci U S A* 1999; 96: 12778–12783.
56. Sanduleanu S, Jonkers D, De Bruine A, et al. Non-Helicobacter pylori bacterial flora during acid-suppressive therapy: Differential findings in gastric juice and gastric mucosa. *Aliment Pharmacol Ther* 2001; 15: 379–388.

57. Ryan KA, Jayaraman T, Daly P, et al. Isolation of lactobacilli with probiotic properties from the human stomach. *Lett Appl Microbiol* 2008; 47: 269–274.
58. Delgado S, Cabrera-Rubio R, Mira A, et al. Microbiological Survey of the Human Gastric Ecosystem Using Culturing and Pyrosequencing Methods. *Microb Ecol* 2013; 65: 763–772.
59. Nardone G, Compare D. The human gastric microbiota: Is it time to rethink the pathogenesis of stomach diseases? *United Eur Gastroenterol J* 2015; 3: 255–260.
60. Kazor CE, Mitchell PM, Lee AM, et al. Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients. *J Clin Microbiol* 2003; 41: 558–563.
61. Schulz C, Schütte K, Koch N, et al. The active bacterial assemblages of the upper Gi tract in individuals with and without Helicobacter infection. *Gut* 2016; 67: 216–225.
62. Bassis CM, Erb-Downward JR, Dickson RP, et al. Analysis of the upper respiratory tract microbiotas as the source of the lung and gastric microbiotas in healthy individuals. *MBio* 2015; 6: 1–10.
63. Zilberstein B, Quintanilha AG, Santos MAA, et al. Digestive tract microbiota in healthy volunteers. *Clinics* 2007; 62: 47–54.
64. Engstrand L, Lindberg M. Helicobacter pylori and the gastric microbiota. *Best Pract Res Clin Gastroenterol* 2013; 27: 39–45.
65. Bik EM, Eckburg PB, Gill SR, et al. Molecular analysis of the bacterial microbiota in the human stomach. *Proc Natl Acad Sci U S A* 2006; 103: 732–737.
66. Stearns JC, Lynch MDJ, Senadheera DB, et al. Bacterial biogeography of the human digestive tract. *Sci Rep* 2011; 1: 1–9.
67. Sung J, Kim N, Kim J, et al. Comparison of Gastric Microbiota Between Gastric Juice and Mucosa by Next Generation Sequencing Method. *J Cancer Prev* 2016; 21: 60–65.
68. A. BN, Jennifer C, Thomas B, et al. Antibiotics, birth mode, and diet shape microbiome maturation during early life. Sci Transl Med 2016; 8: 343ra82-343ra82.
69. Haro C, Rangel-Zúñiga OA, Alcalá-Díaz JF, et al. Intestinal microbiota is influenced by gender and body mass index. *PLoS One* 2016; 11: 1–16.
70. Tsuda A, Suda W, Morita H, et al. Influence of proton-pump inhibitors on the luminal microbiota in the gastrointestinal tract. *Clin Transl Gastroenterol* 2015; 6: e89-9.
71. Lloyd-Price J, Abu-Ali G, Huttenhower C. The healthy human microbiome. *Genome Med* 2016; 8: 1–11.
72. Yang I, Woltemate S, Piazuelo MB, et al. Different gastric microbiota compositions in two human populations with high and low gastric cancer risk in Colombia. *Sci Rep* 2016; 6: 1–10.
73. Lofgren JL, Whary MT, Ge Z, et al. Lack of commensal flora in helicobacter pylori-infected INS-GAS mice reduces gastritis and delays intraepithelial neoplasia. *Gastroenterology* 2011; 140: 210-220.e4.
74. Newton JL, Jordan N, Oliver L, et al. Helicobacter pylori in vivo causes structural changes in the adherent gastric mucus layer but barrier thickness is not compromised. *Gut* 1998; 43: 470–475.
75. Mustapha P, Paris I, Garcia M, et al. Chemokines and antimicrobial peptides have a cag-dependent early response to Helicobacter pylori infection in primary human gastric epithelial cells. *Infect Immun* 2014; 82: 2881–2889.

76. Yu G, Torres J, Hu N, et al. Molecular characterization of the human stomach microbiota in Gastric Cancer Patients. *Front Cell Infect Microbiol* 2017; 7: 1–11.
77. Bravo D, Hoare A, Soto C, et al. Helicobacter pylori in human health and disease: Mechanisms for local gastric and systemic effects. *World J Gastroenterol* 2018; 24: 3071–3089.
78. Parsons BN, Ijaz UZ, D'Amore R, et al. Comparison of the human gastric microbiota in hypochlorhydric states arising as a result of Helicobacter pylori-induced atrophic gastritis, autoimmune atrophic gastritis and proton pump inhibitor use. *PLoS Pathog* 2017; 13: 1–18.
79. Troncoso C, Pavez M, Cerda A, et al. Maldi-tof ms and 16s rna identification of culturable gastric microbiota: Variability associated with the presence of helicobacter pylori. *Microorganisms* 2020; 8: 1–15.
80. Andersson AF, Lindberg M, Jakobsson H, et al. Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLoS One*; 3. Epub ahead of print 2008. DOI: 10.1371/journal.pone.0002836.
81. Llorca L, Pérez-Pérez G, Urruzuno P, et al. Characterization of the gastric microbiota in a pediatric population according to Helicobacter pylori status. *Pediatr Infect Dis J* 2017; 36: 173–178.
82. Ianiro G, Molina-Infante J, Gasbarrini A. Gastric Microbiota. *Helicobacter* 2015; 20: 68–71.
83. Rajilic-Stojanovic M, Figueiredo C, Smet A, et al. Systematic review: gastric microbiota in health and disease. *Aliment Pharmacol Ther* 2020; 51: 582–602.
84. Maldonado-Contreras A, Goldfarb KC, Godoy-Vitorino F, et al. Structure of the human gastric bacterial community in relation to Helicobacter pylori status. *ISME J* 2011; 5: 574–579.
85. Li TH, Qin Y, Sham PC, et al. Alterations in Gastric Microbiota after H. Pylori Eradication and in Different Histological Stages of Gastric Carcinogenesis. *Sci Rep* 2017; 7: 1–8.
86. Mai PT, Kaparakis M, Galic M, et al. Chronic Helicobacter pylori infection does not significantly alter the microbiota of the murine stomach. *Appl Environ Microbiol* 2007; 73: 1010–1013.
87. Monstein HJ, Tiveljung A, Kraft CH, et al. Profiling of bacterial flora in gastric biopsies from patients with Helicobacter pylori-associated gastritis and histologically normal control individuals by temperature gradient gel electrophoresis and 16S rDNA sequence analysis. *J Med Microbiol* 2000; 49: 817–822.
88. Khosravi Y, Dieye Y, Poh BH, et al. Culturable bacterial microbiota of the stomach of helicobacter pylori positive and negative gastric disease patients. *Sci World J*; 2014. Epub ahead of print 2014. DOI: 10.1155/2014/610421.
89. Zaman C, Osaki T, Hanawa T, et al. Analysis of the microbial ecology between Helicobacter pylori and the gastric microbiota of Mongolian gerbils. *J Med Microbiol* 2013; 63: 129–137.
90. Delgado S, Leite AMO, Ruas-Madiedo P, et al. Probiotic and technological properties of Lactobacillus spp. Strains from the human stomach in the search for potential candidates against gastric microbial dysbiosis. *Front Microbiol* 2014; 5: 1–8.
91. Khosravi Y, Dieye Y, Loke MF, et al. Streptococcus mitis induces conversion of helicobacter pylori to coccoid cells during co-culture in vitro. *PLoS One* 2014; 9: 1–11.

92. Guo Y, Zhang Y, Gerhard M, et al. Effect of Helicobacter pylori on gastrointestinal microbiota: A population-based study in Linqu, a high-risk area of gastric cancer. *Gut* 2020; 69: 1598–1607.
93. Yan R, Guo Y, Gong Q, et al. Microbiological evidences for gastric cardiac microflora dysbiosis inducing the progression of inflammation. *J Gastroenterol Hepatol* 2020; 35: 1032–1041.
94. Pereira V, Abraham P, Nallapeta S, et al. Gastric bacterial Flora in patients Harbouring Helicobacter pylori with or without chronic dyspepsia: Analysis with matrix-assisted laser desorption ionization time-of-flight mass spectroscopy. *BMC Gastroenterol* 2018; 18: 1–8.
95. Wang L, Zhou J, Xin Y, et al. Bacterial overgrowth and diversification of microbiota in gastric cancer. *Eur J Gastroenterol Hepatol* 2016; 28: 261–266.
96. Lang D. Opportunities to assess factors contributing to the development of the intestinal microbiota in infants living in developing countries. *Microb Ecol Heal Dis* 2015; 26: 1–11.
97. Grazul H, Kanda LL, Gondek D. Impact of probiotic supplements on microbiome diversity following antibiotic treatment of mice. *Gut Microbes* 2016; 7: 101–114.
98. Kashtanova DA, Popenko AS, Tkacheva ON, et al. Association between the gut microbiota and diet: Fetal life, early childhood, and further life. *Nutrition* 2016; 32: 620–627.
99. Lee S, Sung J, Lee JE, et al. Comparison of the gut microbiotas of healthy adult twins living in South Korea and the United States. *Appl Environ Microbiol* 2011; 77: 7433–7437.
100. Lee JE, Lee S, Lee H, et al. Association of the Vaginal Microbiota with Human Papillomavirus Infection in a Korean Twin Cohort. *PLoS One*; 8. Epub ahead of print 2013. DOI: 10.1371/journal.pone.0063514.
101. Simões CD, Maukonen J, Kaprio J, et al. Habitual dietary intake is associated with stool microbiota composition inmonozygotic twins. *J Nutr* 2013; 143: 417–423.
102. Ferreira RM, Pereira-Marques J, Pinto-Ribeiro I, et al. Gastric microbial community profiling reveals a dysbiotic cancer-associated microbiota. *Gut* 2018; 67: 226–236.
103. Coker OO, Dai Z, Nie Y, et al. Mucosal microbiome dysbiosis in gastric carcinogenesis. *Gut* 2018; 67: 1024–1032.
104. Eun CS, Kim BK, Han DS, et al. Differences in gastric mucosal microbiota profiling in patients with chronic gastritis, intestinal metaplasia, and gastric cancer using pyrosequencing methods. *Helicobacter* 2014; 19: 407–416.
105. Aviles-Jimenez F, Vazquez-Jimenez F, Medrano-Guzman R, et al. Stomach microbiota composition varies between patients with non-atrophic gastritis and patients with intestinal type of gastric cancer. *Sci Rep* 2014; 4: 1–11.
106. Castaño-Rodríguez N, Goh KL, Fock KM, et al. Dysbiosis of the microbiome in gastric carcinogenesis. *Sci Rep* 2017; 7: 1–9.
107. Caguazango JC. Ecological models of gastric microbiota dysbiosis: Helicobacter pylori and gastric carcinogenesis. *Med Microecol* 2020; 3: 100010.
108. Stewart OA, Wu F, Chen Y. The role of gastric microbiota in gastric cancer. *Gut Microbes* 2020; 11: 1220–1230.
109. Yang J, Zhou X, Liu X, et al. Role of the Gastric Microbiome in Gastric Cancer: From Carcinogenesis to Treatment. *Front Microbiol*; 12. Epub ahead of print 2021. DOI: 10.3389/fmicb.2021.641322.

110. Wang Z, Gao X, Zeng R, et al. Changes of the Gastric Mucosal Microbiome Associated With Histological Stages of Gastric Carcinogenesis. *Front Microbiol* 2020; 11: 1–13.
111. Liu X, Shao L, Liu X, et al. Alterations of gastric mucosal microbiota across different stomach microhabitats in a cohort of 276 patients with gastric cancer. *EBioMedicine* 2019; 40: 336–348.
112. Chen XH, Wang A, Chu AN, et al. Mucosa-associated microbiota in gastric cancer tissues compared with non-cancer tissues. *Front Microbiol* 2019; 10: 1–12.
113. Dicksved J, Lindberg M, Rosenquist M, et al. Molecular characterization of the stomach microbiota in patients with gastric cancer and in controls. *J Med Microbiol* 2009; 58: 509–516.
114. Galiatsatos P, Wyse J, Szilagyi A. Accuracy of biopsies for Helicobacter pylori in the presence of intestinal metaplasia of the stomach. *Turkish J Gastroenterol* 2014; 25: 19–23.
115. Li J, Perez Perez GI. Is there a role for the non-helicobacter pylori bacteria in the risk of developing gastric cancer? *Int J Mol Sci*; 19. Epub ahead of print 2018. DOI: 10.3390/ijms19051353.
116. Engstrand L, Graham DY. Microbiome and Gastric Cancer. *Dig Dis Sci* 2020; 65: 865–873.
117. Barra WF, Sarquis DP, Khayat AS, et al. Gastric Cancer Microbiome. *Pathobiology* 2021; 88: 156–169.
118. Hsieh YY, Tung SY, Pan HY, et al. Increased Abundance of Clostridium and Fusobacterium in Gastric Microbiota of Patients with Gastric Cancer in Taiwan. *Sci Rep* 2018; 8: 1–11.
119. Boehm ET, Thon C, Kupcinskas J, et al. Fusobacterium nucleatum is associated with worse prognosis in Lauren's diffuse type gastric cancer patients. *Sci Rep* 2020; 10: 1–12.
120. Genta RM, Rugge M. Gastric precancerous lesions: heading for an international consensus. *Gut* 1999; 45 Suppl 1: I5-8.
121. Kapadia CR. Gastric atrophy, metaplasia, and dysplasia: A clinical perspective. *J Clin Gastroenterol* 2003; 36: 29–36.
122. Ohata H, Kitauchi S, Yoshimura N, et al. Progression of chronic atrophic gastritis associated with Helicobacter pylori infection increases risk of gastric cancer. *Int J Cancer* 2004; 109: 138–143.
123. Conteduca V, Sansonno D, Lauletta G, et al. H. pylori infection and gastric cancer: State of the art (Review). *Int J Oncol* 2013; 42: 5–18.
124. González CA, Agudo A. Carcinogenesis, prevention and early detection of gastric cancer: Where we are and where we should go. *Int J Cancer* 2012; 130: 745–753.
125. Correa P, Piazuelo MB. The gastric precancerous cascade. *J Dig Dis* 2012; 13: 2–9.
126. Rugge M, Pennelli G, Pilozzi E, et al. Gastritis: The histology report. *Dig Liver Dis*; 43. Epub ahead of print 2011. DOI: 10.1016/S1590-8658(11)60593-8.
127. Rugge M, Correa P, Dixon MF, et al. Gastric mucosal atrophy: Interobserver consistency using new criteria for classification and grading. *Aliment Pharmacol Ther* 2002; 16: 1249–1259.
128. Farinati F, Cardin R, Russo VM, et al. Helicobacter pylori CagA status, mucosal oxidative damage and gastritis phenotype: A potential pathway to cancer? *Helicobacter* 2003; 8: 227–234.

129. Ghasemi Basir HR, Ghobakhlou M, Akbari P, et al. Correlation between the intensity of helicobacter pylori colonization and severity of gastritis. *Gastroenterol Res Pract* 2017; 2017: 10–14.
130. Tsukanov VV, Butorin NN, Maady AS, et al. Helicobacter pylori Infection, Intestinal Metaplasia, and Gastric Cancer Risk in Eastern Siberia. *Helicobacter* 2011; 16: 107–112.
131. Tsukanov V V., Kasparov E V., Tonkikh JL, et al. Peptic Ulcer Disease and Helicobacter pylori Infection in Different Siberian Ethnicities. *Helicobacter* 2017; 22: 1–7.
132. Petersson F, Borch K, Franzén LE. Prevalence of subtypes of intestinal metaplasia in the general population and in patients with autoimmune chronic atrophic gastritis. *Scand J Gastroenterol* 2002; 37: 262–266.
133. Heinrichs SKM, Hess T, Becker J, et al. Evidence for PTGER4, PSCA, and MBOAT7 as risk genes for gastric cancer on the genome and transcriptome level. *Cancer Med* 2018; 7: 5057–5065.
134. Petkevicius V, Salteniene V, Juzenas S, et al. Polymorphisms of microRNA target genes IL12B, INSR, CCND1 and IL10 in gastric cancer. *World J Gastroenterol* 2017; 23: 3480–3487.
135. Link A, Kupcinskas J. MicroRNAs as non-invasive diagnostic biomarkers for gastric cancer: Current insights and future perspectives. *World J Gastroenterol* 2018; 24: 3313–3329.
136. Polk DB, Jr RMP. Helicobacter Pylori: Gástric Cancer. NIH Public Access. 2010; 10: 403–414.
137. Abdeljawad K, Wehbeh A, Qayed E. Low Prevalence of Clinically Significant Endoscopic Findings in Outpatients with Dyspepsia. *Gastroenterol Res Pract*; 2017. Epub ahead of print 2017. DOI: 10.1155/2017/3543681.
138. Rugge M, Sugano K, Scarpignato C, et al. Gastric cancer prevention targeted on risk assessment: Gastritis OLGA staging. *Helicobacter*; 24. Epub ahead of print 2019. DOI: 10.1111/hel.12571.
139. Niwa T, Tsukamoto T, Toyoda T, et al. Inflammatory processes triggered by Helicobacter pylori infection cause aberrant DNA methylation in gastric epithelial cells. *Cancer Res* 2010; 70: 1430–1440.
140. González CA, Sanz-Anquela JM, Gisbert JP, et al. Utility of subtyping intestinal metaplasia as marker of gastric cancer risk. A review of the evidence. *Int J Cancer* 2013; 133: 1023–1032.
141. Gutierrezgonzalez L, Graham TA, Rodriguezjusto M, et al. The clonal origins of dysplasia from intestinal metaplasia in the human stomach. *Gastroenterology* 2011; 140: 1251-1260.e6.
142. Koulis A, Buckle A, Boussioutas A. Premalignant lesions and gastric cancer: Current understanding. *World J Gastrointest Oncol* 2019; 11: 665–678.
143. Rokkas T, Pistiolas D, Sechopoulos P, et al. The long-term impact of Helicobacter pylori eradication on gastric histology: A systematic review and meta-analysis. *Helicobacter* 2007; 12: 32–38.
144. Chen HN, Wang Z, Li X, et al. Helicobacter pylori eradication cannot reduce the risk of gastric cancer in patients with intestinal metaplasia and dysplasia: evidence from a meta-analysis. *Gastric Cancer* 2016; 19: 166–175.

145. Den Hollander WJ, Holster IL, Den Hoed CM, et al. Surveillance of premalignant gastric lesions: A multicentre prospective cohort study from low incidence regions. *Gut* 2019; 68: 585–593.
146. González CA, Sanz-Anquela JM, Companioni O, et al. Incomplete type of intestinal metaplasia has the highest risk to progress to gastric cancer: Results of the Spanish follow-up multicenter study. *J Gastroenterol Hepatol* 2016; 31: 953–958.
147. de Vries AC, van Grieken NCT, Looman CWN, et al. Gastric Cancer Risk in Patients With Premalignant Gastric Lesions: A Nationwide Cohort Study in the Netherlands. *Gastroenterology* 2008; 134: 945–952.
148. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; 68: 394–424.
149. Rugge M, Genta RM. Staging and grading of chronic gastritis. *Hum Pathol* 2005; 36: 228–233.
150. Rugge M, Correa P, Di Mario F, et al. OLGA staging for gastritis: A tutorial. *Dig Liver Dis* 2008; 40: 650–658.
151. Capelle LG, de Vries AC, Haringsma J, et al. The staging of gastritis with the OLGA system by using intestinal metaplasia as an accurate alternative for atrophic gastritis. *Gastrointest Endosc* 2010; 71: 1150–1158.
152. Dixon MF, Genta RM, Yardley JH, et al. Classification and grading of Gastritis: The updated Sydney system. *Am J Surg Pathol* 1996; 20: 1161–1181.
153. Rugge M, De Boni M, Pennelli G, et al. Gastritis OLGA-staging and gastric cancer risk: A twelve-year clinico-pathological follow-up study. *Aliment Pharmacol Ther* 2010; 31: 1104–1111.
154. Lee JWJ, Zhu F, Srivastava S, et al. Severity of gastric intestinal metaplasia predicts the risk of gastric cancer: A prospective multicentre cohort study (GCEP). *Gut* 2021; 1–10.
155. Sugano K, Tack J, Kuipers EJ, et al. Kyoto global consensus report on Helicobacter pylori gastritis. *Gut* 2015; 64: 1353–1367.
156. Pita I, Libânio D, Nunes PP. Diagnosis and Management of Epithelial Precancerous Conditions and Lesions in the Stomach. *Curr Treat Options Gastroenterol* 2021; 19: 277–294.
157. Banks M, Graham D, Jansen M, et al. British Society of Gastroenterology guidelines on the diagnosis and management of patients at risk of gastric adenocarcinoma. *Gut* 2019; 68: 1545–1575.
158. Den Hoed CM, Van Eijck BC, Capelle LG, et al. The prevalence of premalignant gastric lesions in asymptomatic patients: Predicting the future incidence of gastric cancer. *Eur J Cancer* 2011; 47: 1211–1218.
159. De Vries AC, Meijer GA, Looman CWN, et al. Epidemiological trends of pre-malignant gastric lesions: A long-term nationwide study in the Netherlands. *Gut* 2007; 56: 1665–1670.
160. De Vries AC, Kuipers EJ. Epidemiology of premalignant gastric lesions: Implications for the development of screening and surveillance strategies. *Helicobacter* 2007; 12: 22–31.
161. Yuan Y. A survey and evaluation of population-based screening for gastric cancer. *Cancer Biol Med* 2013; 10: 72–80.

162. McDonald JE, Larsen N, Pennington A, et al. Characterising the canine oral microbiome by direct sequencing of reverse-transcribed rRNA molecules. *PLoS One* 2016; 11: 1–17.
163. Camarinha-Silva A, Jáuregui R, Chaves-Moreno D, et al. Comparing the anterior nare bacterial community of two discrete human populations using Illumina amplicon sequencing. *Environ Microbiol* 2014; 16: 2939–2952.
164. Chaves-Moreno D, Plumeier I, Kahl S, et al. The microbial community structure of the cotton rat nose. *Environ Microbiol Rep* 2015; 7: 929–935.
165. Ombrello AK. Dada2. *Encycl Med Immunol* 2020; 13: 1–7.
166. McMurdie PJ, Holmes S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLoS One*; 8. Epub ahead of print 2013. DOI: 10.1371/journal.pone.0061217.
167. Wang Q, Garrity GM, Tiedje JM, et al. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 2007; 73: 5261–5267.
168. Letunic I, Bork P. Interactive Tree of Life (iTOL) v4: Recent updates and new developments. *Nucleic Acids Res* 2019; 47: 256–259.
169. Anderson MJ, Gorley RN, Clarke KR. PERMANOVA+ Primer V7: User Manual. *Prim Ltd, Plymouth, UK* 2008; 93.
170. O'Hara AM, Shanahan F. The gut flora as a forgotten organ. *EMBO Rep* 2006; 7: 688–693.
171. Cover TL, Blaser MJ. Helicobacter pylori in Health and Disease. *Gastroenterology* 2009; 136: 1863–1873.
172. Blaser MJ, Atherton JC. Helicobacter pylori persistence: Biology and disease. *J Clin Invest* 2004; 113: 321–333.
173. Rothschild D, Weissbrod O, Barkan E, et al. Environment dominates over host genetics in shaping human gut microbiota. *Nature* 2018; 555: 210–215.
174. Gomez A, Espinoza JL, Harkins DM, et al. Host Genetic Control of the Oral Microbiome in Health and Disease. *Cell Host Microbe* 2017; 22: 269-278.e3.
175. Freire M, Moustafa A, Harkins DM, et al. Longitudinal Study of Oral Microbiome Variation in Twins. *Sci Rep* 2020; 10: 1–10.
176. Wu H, Zeng B, Li B, et al. Research on oral microbiota of monozygotic twins with discordant caries experience - In vitro and in vivo study. *Sci Rep* 2018; 8: 1–8.
177. Mukherjee C, Moyer CO, Steinkamp HM, et al. Acquisition of oral microbiota is driven by environment, not host genetics. *Microbiome* 2021; 9: 1–13.
178. Kupcinskas J, Hold GL. Other Helicobacters and the gastric microbiome. *Helicobacter* 2018; 23: 1–6.
179. Vasapolli R, Schütte K, Schulz C, et al. Analysis of Transcriptionally Active Bacteria Throughout the Gastrointestinal Tract of Healthy Individuals. *Gastroenterology* 2019; 157: 1081-1092.e3.
180. Malaty HM, Graham DY, Isaksson I, et al. Are genetic influences on peptic ulcer dependent or independent of genetic influences for Helicobacter pylori infection? *Arch Intern Med* 2000; 160: 105–109.
181. Marcos-Pinto R, Carneiro F, Dinis-Ribeiro M, et al. First-degree relatives of patients with early-onset gastric carcinoma show even at young ages a high prevalence of advanced OLGA/OLGIM stages and dysplasia. *Aliment Pharmacol Ther* 2012; 35: 1451–1459.

182. Rokkas T, Sechopoulos P, Pistiolas D, et al. Helicobacter pylori infection and gastric histology in first-degree relatives of gastric cancer patients: A meta-analysis. *Eur J Gastroenterol Hepatol* 2010; 22: 1128–1133.
183. Raymond J, Thiberge J, Chevalier C, et al. Genetic and Transmission Analysis of. *October* 2004; 10: 1–6.
184. Tindberg Y, So M, Casswall TH, et al. Concordance of. 2003; 41: 5604–5608.
185. Takahashi M, Yokota S -i., Mino E, et al. Five-Year Follow-Up Study of Mother-to-Child Transmission of Helicobacter pylori Infection Detected by a Random Amplified Polymorphic DNA Fingerprinting Method. *J Clin Microbiol* 2005; 43: 2246–2250.
186. Li F, Huang C, Li Q, et al. Construction and comprehensive analysis for dysregulated long non-coding RNA (LncRNA)-associated competing endogenous RNA (ceRNA) network in gastric cancer. *Med Sci Monit* 2018; 24: 37–49.
187. Cui X, Huang Q, Li X, et al. Relationship between interleukin-10 gene C-819T polymorphism and gastric cancer risk: Insights from a meta-analysis. *Med Sci Monit* 2016; 22: 2839–2845.
188. Chen B, Luo MX, Zhou X, et al. Correlation between interleukin-1β-511 C/T polymorphism and gastric cancer in Chinese populations: A meta-analysis. *Med Sci Monit* 2016; 22: 1742–1750.

LIST OF PUBLICATIONS

Publications related to the results of dissertation

1. Spiegelhauer MR, Kupcinskas J, Johannesen TB, **Urba M**, Skieceviciene J, Jonaitis L, et al. Transient and Persistent Gastric Microbiome: Adherence of Bacteria in Gastric Cancer and Dyspeptic Patient Biopsies after Washing. J Clin Med. 2020;9(6):1882. (Impact factor: 4.241 (2020)).
2. Vilchez-Vargas R, Skieceviciene J, Lehr K, Varkalaite G, Thon C, **Urba M**, et al. Gut microbial similarity in twins is driven by shared environment and aging. eBioMedicine [Internet]. 2022;79:104011. Available from: https://www.sciencedirect.com/science/article/pii/S2352396422001955. (Impactor factor: 8.143 (2020)).
3. **Urba M**, Skieceviciene J, Janciauskas D, Jonaitis L, Kupcinskas L, Fassan M, et al. Gastritis Stages in Monozygotic and Dizygotic Dyspeptic Twins. Gastroenterol Res Pract. 2020;2020. (Impact factor: 2.26 (2020)).

List of presentations at scientific conferences

The results of dissertation were presented in the scientific conferences:

1. Jonaitis Laimas; **Urba Mindaugas**; Kupčinskas Juozas; Jančiauskas Dainius; Kupčinskas Limas. OLGA and OLGIM in twins with Helicobacter pylori gastritis: A pilot study // Helicobacter: Special Issue: XXX International Workshop on Helicobacter and Microbiota in Inflammation and Cancer: 7–9 September 2017, Bordoux, France September 2017: Abstracts/ European Helicobacter and group; Issue edited by: Francis Megraud, Peter Malfertheiner. Oxford: Willey-Blackwell. ISSN 1083-4389. 2017, vol. 22, iss Suppl. S1, September, p. 104–104, no. P07.12. DOI: 10.1111/hel.12416.
2. **Urba Mindaugas**; Jonaitis Laimas; Kupcinskas Juozas; Jančiauskas Dainius; Kupčinskas Limas. OLGA and OLGIM in twins with Helicobacter pylori gastritis // Helicobacter: Special Issue: XXXIst International Workshop on Helicobacter and Microbiota in Inflammation and Cancer 13-15 September 2018, Kaunas Lithuania Semptember 2018: Abstracts/ European Helicobacter and Microbiota Study group. Oxford: Willey-Blackwell. ISSN 1083-4389. 2018, vol. 23, iss Suppl. S1, September, p. 66-66, no. P05.07. DOI: 10.1111/hel.12525.
3. **Urba Mindaugas**; Jonaitis Laimas; Jančiauskas Dainius; Kupčinskas Limas; Kupčinskas Juozas. High concordance rates of premalignant gastric lessions assessed by OLGA and OLGIM in monozygotic and dizygotic twins // United European gastroenterology journal: 27th United

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Article

Transient and Persistent Gastric Microbiome: Adherence of Bacteria in Gastric Cancer and Dyspeptic Patient Biopsies after Washing

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Abstract: *Helicobacter pylori* is a common colonizer of the human stomach, and long-term colonization has been related to development of atrophic gastritis, peptic ulcers and gastric cancer. The increased gastric pH caused by *H. pylori* colonization, treatment with antibiotics or proton pump inhibitors (PPI) may allow growth of other bacteria. Previous studies have detected non-*Helicobacter* bacteria in stomach biopsies, but no conclusion has been made of whether these represent a transient contamination or a persistent microbiota. The aim of this study was to evaluate the transient and persistent bacterial communities of gastric biopsies. The washed or unwashed gastric biopsies were investigated by cultivation and microbiota analysis (16S rRNA gene-targeted amplicon sequencing) for the distribution of *H. pylori* and other non-*Helicobacter* bacteria. The number of cultured non-*Helicobacter* bacteria decreased in the washed biopsies, suggesting that they might be a transient contamination. No significant differences in the bacterial diversity were observed in the microbiome analysis between unwashed and washed biopsies. However, the bacterial diversity in biopsies shown *H. pylori*-positive and *H. pylori*-negative were significantly different, implying that *H. pylori* is the major modulator of the gastric microbiome. Further large-scale studies are required to investigate the transient and persistent gastric microbiota.

Keywords: gastric microbiota; transient; persistent; culture; microbiome; sequencing; *Helicobacter pylori*

1. Introduction

1.1. Helicobacter Pylori *Colonization of the Stomach*

Helicobacter is a group of Gram-negative, curved or spiral-shaped bacteria, of which *Helicobacter pylori* is the most commonly known species [1]. *H. pylori* is able to colonize the human stomach by moving through the gastric mucin layer, and long-term colonization may increase

the risk for development of atrophic gastritis, peptic ulcers and ultimately gastric cancer [2–5]. Approximately 30% of adults in developed countries and 80% of adults in developing countries are colonized with *H. pylori*, and of these, 1–3% further develop gastric cancer [6,7].

H. pylori has only been shown as a natural colonizer in humans, but another *Helicobacter* species capable of gastric colonization in humans is *Helicobacter heilmanii* [8,9]. The prevalence of infection with *H. heilmanii* is much lower than of *H. pylori* and it has been described less than 0.5% of patients undergoing upper gastric endoscopy [10].

Due to gastric peristalsis, mucus thickness, low pH and secretion of bile and acid, it was initially believed that no bacteria could survive in the inhospitable stomach environment [2,3,11]. Later, studies detected *H. pylori* as the only bacterium in stomachs with a healthy low pH, while more bacteria were detected at higher pH [12]. The mucus thickness and viscosity are pH-dependent, and a decreased acidity caused by *H. pylori* colonization, atrophic gastritis, treatment with antibiotics or proton pump inhibitors (PPI) may reduce the acidic protection of the stomach, leading to bacterial overgrowth and a higher diversity [13–16]. An alteration of the gastric bacterial composition has also been reported in cases of gastric cancer, with an increase of both oral and intestinal bacterial groups [16]. Recent studies have described the presence of a non-*Helicobacter* gastric microbiota, suggesting that other bacteria are able to live in the strict environment of the stomach [13].

1.2. Previous Studies of the Gastric Microbiota

Initial studies on gastric bacterial communities were reported in the 1980s [17]. Since then, several studies have investigated the presence of *H. pylori* and non-*Helicobacter* bacteria by cultivation methods and DNA-based methods [16,17]. Zilberstein et al. cultured gastric biopsies in aerobic and anaerobic conditions and found *Veillonella, Lactobacillus* and *Clostridium* to be the predominant bacterial groups [18]. They concluded that species of these bacterial groups may be transiently present [18]. Li et al. performed 16S rRNA gene amplicon sequencing on gastric biopsies from healthy people and patients with gastritis and observed a community dominated by species of *Prevotella, Streptococcus, Veillonella, Rothia* and *Haemophilus* [14]. Similar results were obtained by Bik et al. by 16S rRNA gene amplicon sequencing, where the dominating genera were found to be *Streptococcus, Prevotella, Rothia, Fusobacterium* and *Veillonella* [2]. A study by Delgado et al. on healthy patients identified *Lactobacillus* as one of the most abundant genera in the stomach as well as *Streptococcus* and *Propionibacterium* by 16S rRNA gene amplicon pyrosequencing [19]. A study by Dicksved et al. detected a bacterial community of *Streptococcus, Lactobacillus, Veillonella* and *Prevotella*, with a low abundance of *Helicobacter* [20]. The study found no significant differences in the gastric bacterial communities of patients with gastric cancer or dyspepsia [8]. Maldonado-Contreras et al. investigated the microbial composition by 16S rRNA gene amplicon hybridization and found non-*Helicobacter* bacteria such as *Proteobacteria, Firmicutes, Actinobacteria* and *Bacteroidetes* to be dominating [21]. Yu et al. identified similar phyla in the gastric stomach area and other body sites [22]. The authors performed a functional profiling of the stomach microbiota and concluded that though similar phyla were present in these areas, the microbiota of the different areas presented different functions [22]. A systematic review by Rajilic-Stojanovic et al. compared the results of papers investigating the gastric microbiota by next-generation sequencing (NGS) [16]. Approximately 2/3 of the described papers detected species of *Prevotella, Streptococcus, Veillonella, Neisseria, Fusobacterium* and *Haemophilus* and have described that the microbiota is subject-specific and differ between individuals [16]. More than 65% of the bacterial groups found in the stomach have also been identified in the human oral cavity, and many bacteria identified in the stomach may originate from the oral cavity or as reflux from the intestine [3]. Previous studies have not been conclusive about whether the detected non-*Helicobacter* bacteria represent a transient contamination of the stomach or if they belong to the persistent gastric microbiota.

1.3. H. pylori *and the Non-Helicobacter Microbial Community*

The human stomach has been described to contain a core microbiome mainly consisting of *Prevotella, Streptococcus, Veillonella, Rothia and Haemophilus* spp., influenced by diet, inflammation and medication [3]. However, the effect of *H. pylori* colonization on the bacterial diversity has not been established yet. Andersson et al. investigated the bacterial composition with 16S rRNA gene amplicon pyrosequencing and found complex microbial communities with a great diversity in the absence of *H. pylori* [23]. When present, *H. pylori* accounted for 90% of the bacteria, and diversity was decreased [23]. This agrees with other findings of a large variation in the bacterial community depending on the presence or absence of *H. pylori* [4,12,16,21]. The study by Maldonado-Contreras associated *H. pylori* colonization with an increase of *Proteobacteria*, *Spirochetes* and *Acidobacteria* and decrease of *Actinobacteria*, *Bacteroidetes* and *Firmicutes* [21]. Other studies have opposed the suggestion that the diversity of bacteria in the stomach is negatively affected by the presence of *H. pylori* [2,22,24]. As such, the significance of *H. pylori* on the presence of other bacteria in the gastric environment remains unknown. In a study by Sanduleanu et al., the non-*Helicobacter* bacteria in the stomach were found to contaminate both the gastric juices and the mucosa during gastric acid inhibition [15]. A study by Li et al. investigated the effect of washing on the bacterial content of gastric biopsies [14]. The majority of bacteria remained in the biopsies even after several washing steps, and in particular, *Streptococcus* were not removed [14]. Colonization with *H. pylori* has been associated with increased inflammation and development of gastric atrophy, which may cause overgrowth of other bacteria as the environment turns less acidic [25]. The presence of a complex microbial community in an atrophic stomach may further promote inflammation, malignancies and cancer development [25].

1.4. *Microbial Effects on the Development of Gastric Cancer*

H. pylori is classified as a class I carcinogen, and colonization with this species is associated with an increased risk for gastric cancer [26]. The precise mechanism for this development is not known in detail, but it may be affected by diet and interactions with the gastric microbiota [27]. It has been hypothesized that the presence of a specific microbiota promotes inflammation and that other bacteria in addition to *H. pylori* may further promote cancer development [26]. The pH is usually increased in the stomachs of gastric cancer patients, resulting in atrophic gastritis and subsequent loss of *H. pylori*, as the environment becomes less acidic [28]. Several studies of the gastric microbiota in the settings of gastric cancer have been performed. A study by Dicksved et al. compared the microbiota of biopsies from patients with gastric cancer and dyspepsia by terminal restriction fragment length polymorphism [20]. They identified complex compositions of bacteria in gastric cancer biopsies but with no significant differences in the microbial compositions compared to dyspepsia patient biopsies [20]. A low abundance of *H. pylori* and a dominance of species from *Streptococcus, Lactobacillus, Veillonella* and *Prevotella* were observed in biopsies from gastric cancer patients [20]. Another study by Ferreira et al. investigated the bacterial composition in biopsies from patients with atrophic gastritis and gastric cancer [29]. They detected a reduced microbial diversity in gastric cancer patients and found an abundance of less than 5% *H. pylori* in most gastric cancer patients [29]. Based on calculations of a microbial dysbiosis index, the presence of a dysbiotic microbiota in the gastric cancer patients was suggested, compared to the microbiota of individuals with atrophic gastritis [29]. In particular, the microbiota of gastric cancer biopsies showed increased abundances of the groups *Actinobacteria* and *Firmicutes*, while the presence of the groups *Bacteroidetes* and *Fusobacteria* were decreased [29]. Yu et al. analyzed the microbiota of gastric cancer biopsies and identified *H. pylori* as the most abundant bacterial species present, followed by bacteria associated with the oral environment [22]. A decrease in *Proteobacteria* and increase in *Bacteroidetes, Firmicutes* and *Spirochetes* were observed in gastric cancer tissue, but comparisons of microbiota in the corpus and antrum areas of the stomach showed no differences [22]. An altered bacterial composition has also been reported in cases of gastric cancer, with an increase of both oral and intestinal bacterial groups [16].

1.5. Definition of Persistent and Transient Microbiota and Microbiome

In this study, the gastric microbiota is defined as the persistent bacteria that adhere to the gastric mucosa. The gastric microbiome is defined as the collection of microbial genes in the stomach, which also include the community of non-adherent, transient bacteria [30]. Previous studies have not been able to agree on whether the bacteria identified in the stomach represent a true microbiota or are contamination from the oral cavity. It is therefore not clear which bacteria belong to the true gastric microbiota.

The main aim of this study was to investigate the presence of persistent and transient bacterial communities by comparing the changes in bacterial composition of washed and unwashed gastric biopsies. It is the first comprehensive attempt to distinguish between the transient and resident bacteria of the stomach using 16S rRNA gene sequencing.

The second aim of this study was to investigate and compare the gastric microbiota of gastric cancer patients and dyspepsia patients. The results will contribute to the ongoing debate about whether the process of gastric carcinogenesis is mediated through a microbial shift caused by *H. pylori*. Biopsies from both cancerous and non-cancerous tissue were included, and dyspepsia patients were involved as controls of a healthy microbiota.

The hypothesis of our experimental setup is that *H. pylori* is the only true gastric microbiota of the stomach and will be the predominant bacterium that is able to remain in the washed biopsies. Although other studies have shown the presence of non-*Helicobacter* bacteria in the stomach, these will be expected to be present at least in the unwashed biopsies, as transient contamination from other niches [3].

2. Experimental Section/Materials and Methods

2.1. Sampling of Gastric Biopsies

Twenty-two patients with dyspepsia and twelve patients with gastric cancer were included in the study. The distribution was 68% female (n = 23 patients), 32% male (n = 11 patients) and an age range of 22–91 years (median 53 years). Clinical information about the patients is listed in Table 1. The exclusion criteria for the patients were age below 18 years, a previous history of *H. pylori* eradication, use of PPI within the last 3 months, use of antibiotics within the last 3 months, and previous treatment of gastric cancer. Three antral biopsies were taken about 4 cm from pylorus from patients with dyspepsia (n = 22 patients) by gastroscopy. From patients with gastric cancer (n = 12 patients), three antrum biopsies were taken about 4 cm from pylorus, and three biopsies were taken from the cancer area in the corpus. The biopsies were obtained between November 2017 and June 2019. The first biopsy was immediately used for histology to examine the presence of *H. pylori*. Histological slides were stained with haematoxylin/eosin, and a Giemsa stain was used to confirm the presence or absence of *H. pylori*. Figure 1 shows a histological sample positive for *H. pylori* infection.

Table 1. Clinical data of patients included in the study.

Diagnosis	Mean Age (years)	Gender	*H. pylori* Status (Histology)	Lauren Classification	G Stage	Proximal/Distal Part
Gastric adenocarcinoma (n = 12)	62.2	Female (n = 5)	Negative (n = 9)	Diffuse (n = 6)	2 (n = 5)	Proximal (n = 3)
		Male (n = 7)	Positive (n = 3)	Intestinal (n = 4)	3 (n = 7)	Distal (n = 9)
				Mixed (n = 2)		
Dyspepsia (n = 22)	48.1	Female (n = 18)	Negative (n = 11)	NA	NA	NA
		Male (n = 4)	Positive (n = 11)			

NA: Not applicable.

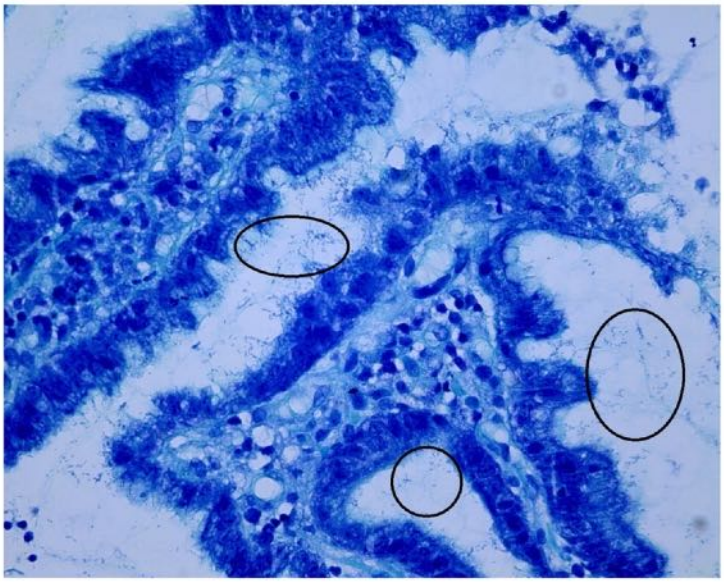


Figure 1. The positive results of *Helicobacter pylori* infection by Giemsa stain. Black circles indicate stained *H. pylori* (blue) that are attached to the gastric epithelial cells.

All patients participating in the study have signed an informed consent form. The study protocol has been approved by Kaunas Regional Bioethics Committee (Protocol No: BE-2-10; P1-BE-2-31).

The second biopsy was immediately transferred to the transport medium Portagerm pylori (bioMérieux, Marcy L'Etoile, France) and stored at −80 °C. The third biopsy was placed in a tube with sterile 4 °C PBS, collected by sterile forceps and washed for 15 s in 4 °C PBS and transferred to a new sterile tube containing 4 °C PBS. This step was repeated twice, after which the biopsy was placed in the transport medium Portagerm pylori (bioMérieux, Marcy L'Etoile, France) and stored at −80 °C. These biopsies were then transported to the Copenhagen University Hospital (Rigshospitalet) for culture.

2.2. Culture of Gastric Biopsies and Identification of Single Colonies

Biopsies were cultured on 7% defibrinated horse blood agar plates (SSI Diagnostica A/S, Hillerød, Denmark), placed in serum bouillon with 10% glycerol (SSI Diagnostica, Hillerød, Denmark), and frozen at −80 °C. The agar plates were incubated 6 days at 37 °C in microaerobic conditions (10% CO_2, 5% O_2); the observed growth was noted, and single colonies of each morphology were isolated on new 5% horse blood agar plates (SSI Diagnostica A/S, Hillerød, Denmark). The plates with the isolated cultures were incubated at 37 °C in microaerobic conditions until visible growth was seen (1–3 days). Each isolate was transferred to a MALDI-TOF target plate, treated with 1µL HCAA matrix (Bruker Daltonics, Billerica, MA, USA), analyzed with MALDI-TOF Mass Spectrometry (Bruker Daltonics, Billerica, MA, USA), and the protein profile was compared to the database Compass (Bruker Daltonics, Billerica, MA, USA) to identify the species. The biopsies were then transported to Statens Serum Institute for microbiome analysis (16S rRNA gene amplicon sequencing).

2.3. Microbiome Analysis (16S rRNA Gene Amplicon Sequencing)

DNA was extracted from the biopsies using a QIAamp DNA mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction for tissues. For each batch of DNA extraction, a negative

control without sample material was included for downstream analysis. DNA was amplified using a two-step PCR using a modified version of the published universal prokaryotic primers 341F (ACTCCTAYGGGRBGCASCAG) and 806R (ACTCCTAYGGGRBGCASCAG) targeting the V3-V4 regions of the 16S rRNA gene. Amplicons were sequenced on the Illumina MiSeq desktop sequencer (Illumina Inc., San Diego, CA, USA), using the V2 Reagent Kit.

2.3.1. Library Preparation

Purified genomic DNA from each sample was initially amplified in a 25 µL reaction, using the REDExtract-N-Amp PCR ReadyMix (Sigma-Aldrich, St Louis, MO, USA) with 0.4 µM of each 16S rRNA gene primer and 2 µL template. The 16S PCR conditions were the following: an initial denaturation at 95 °C for 2 min, 20 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 30 s and final elongation at 72 °C for 7 min. This PCR run is referred to as PCR1. The product from PCR1 was prepared for sequencing by a second PCR (referred to as PCR2), using the same PCR protocol as described above. PCR2 attached an adaptor A, an index i5 and a forward sequencing primer site (FSP) in the 5′ end of the amplicons and an adaptor B, an index i7 and a reverse sequencing primer site (RSP) to the 3′ end of the amplicons. DNA was quantified using the Quant-IT^{TM} dsDNA High Sensitive Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and PCR2 products were pooled in equimolar amounts between samples. Agencourt AMPure XP bead (Beckman Coulter, Brea, CA, USA) purification was performed to remove undesirable DNA amplicons from the pooled amplicon library (PAL) in a two-step process. First, DNA fragments below 300 nucleotides length were removed by a PAL AMPure beads 10:24 ratio, following the manufacturer's instructions, and eluted in 40 µL TE buffer (AM1). Secondly, large DNA fragments above 1kbp were removed by AM1 to AMPure beads 10:16 ratio as previously described. The resulting AMPure beads purified PAL (bPAL) was diluted to a final concentration of 11.5 pM DNA in a 0.001 N NaOH and used for sequencing on the Illumina MiSeq desktop sequencer (Illumina Inc., San Diego, CA, USA). The library was sequenced with the 500-cycle MiSeq Reagent Kit V2 in a 2 × 250 nt setup (Illumina Inc., San Diego, CA, USA). The sequencing was performed at Statens Serum Institute (SSI).

2.3.2. Bioinformatics

BION-META (http://box.com/bion), a newly developed analytical semi-commercial open-source package for 16S rRNA gene and other reference gene analysis classifying mostly species was used, and the data were processed following the previously described automated steps [31,32]. After BION-META analysis, taxonomy tables were made with the identified phylum, class, order, family, genus, species and number of reads. The data from the sequencing were submitted to European Nucleotide Archive with the accession number PRJEB38558.

2.3.3. Statistics of Microbiome Analysis Results

The ten most abundant genera across all samples are shown in staggered bar plots in which samples are ordered according to a hierarchical clustering based on Bray Curtis dissimilarities and ward-linkage. The difference in the distribution of bacterial groups between biopsies was analyzed with an unpaired *t*-test using the graphing and statistics program GraphPad Prism version 5.01 (San Diego, CA, USA).

Analysis of the microbiome diversity was performed in the statistical computing program R, version 3.5.0 (R Foundation for Statistical Computing, Vienna, Austria) using the packages phyloseq v.1.24.3 and vegan v. 2.5-2. Figures were created using the packages ggplot2 v.3.2.0 and plotly v. 4.8.0. Alpha-diversity of samples as well as relative abundance of individual genera were compared pairwise between groups with Mann–Whitney rank sum tests and adjusted for multiple testing using Bonferroni correction. Principal coordinate analysis (PCoA) of samples was performed based BIONs species-level classification on Bray Curtis dissimilarity. Within-group similarities were compared to between-group similarities with analysis of similarities (ANOSIM test) using 1000 random permutations to estimate *p*-value.

3. Results

3.1. Comparison of Bacterial Composition in Unwashed Biopsies and Washed Biopsies

3.1.1. A Decrease in Cultured Bacteria was Observed for the Washed Biopsies

The overall number of cultured non-*Helicobacter* species decreased in the washed biopsies compared to the unwashed biopsies, suggesting that many bacteria do not adhere to the tissue. A total of 27 biopsy pairs showed reduced or no growth in the washed biopsy compared to the growth observed in the unwashed biopsy (Table 2). Only 5 biopsy pairs showed unchanged growth, while 3 showed increased growth.

Table 2. Change in growth of bacterial species cultured from the biopsy pairs.

Growth Result	Dyspepsia Patients	Gastric Cancer Patients
No bacterial growth in either biopsy	8 biopsy pairs	3 biopsy pairs
Growth in unwashed but not in washed biopsy	4 biopsy pairs	5 biopsy pairs
Reduced growth in the washed biopsy	9 biopsy pairs	9 biopsy pairs
Unchanged growth	1 biopsy pairs	4 biopsy pairs
Increased growth in the washed biopsy	0 biopsy pairs	3 biopsy pairs

The total number of colonies of cultured *Streptococcus* spp. decreased, but its relative abundance was higher in the washed biopsies compared to the unwashed biopsies (Figure 2, Table 3). The cultured bacteria in the biopsies were dominated by *Streptococcus* spp., followed by *Rothia* spp. and *Actinomyces* spp. (Table 3).

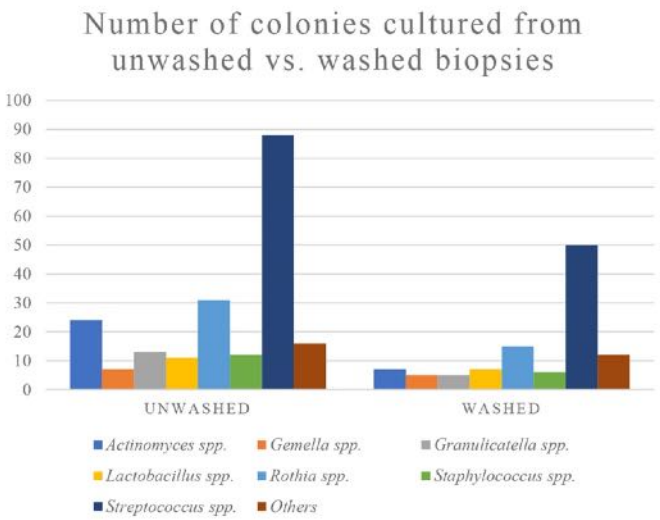


Figure 2. The number of bacterial species isolated from culture of gastric cancer and dyspepsia patient biopsies. "Others" include *Bacillus* spp., *Corynebacterium* spp., *Enterobacter* spp., *Enterococcus* spp., *Haemophilus* spp, *Micrococcus* spp., *Neisseria* spp., and *Stenotrophomonas* spp.

Table 3. Distribution of total cultured bacterial groups.

	Dyspepsia		Gastric Cancer	
	Unwashed	**Washed**	**Unwashed**	**Washed**
Streptococcus spp.	40%	43%	47%	49%
Rothia spp.	16%	14%	15%	14%
Actinomyces spp.	16%	9%	9%	6%
Staphylococcus spp.	8%	6%	4%	6%
Granulicatella spp.	7%	6%	6%	4%
Lactobacillus spp.	4%	3%	7%	8%
Gemella spp.	2%	9%	5%	3%
Enterococcus spp.	1%	3%	6%	8%
Micrococcus spp.	2%	3%	2%	0%
Corynebacterium spp.	2%	3%	-	-
Stenotrophomonas spp.	1%	0%	-	-
Neisseria spp.	1%	0%	-	-
Enterobacter spp.	0%	3%	-	-
Bacillus spp.	-	-	1%	0%
Haemophilus spp.	-	-	0%	3%

3.1.2. Microbiome Analysis

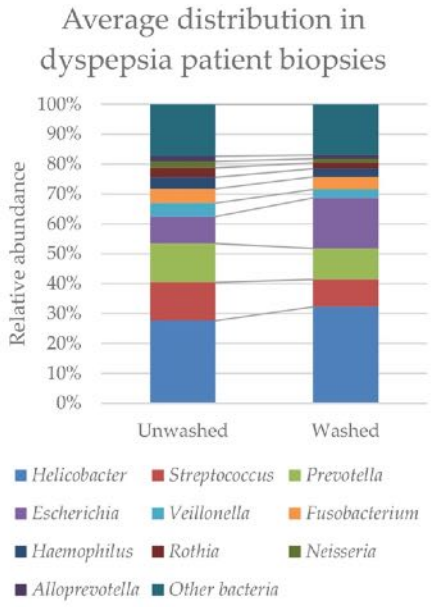
Bacterial DNA was detected in all biopsies, even in those were no growth was observed. The ten most prevalent groups in the microbiome analysis were *Helicobacter* spp., *Streptococcus* spp., *Prevotella* spp., *Escherichia* spp., *Veillonella* spp., *Fusobacterium* spp., *Haemophilus* spp., *Rothia* spp., *Neisseria* spp., and *Alloprevotella* spp. (Figure 3). The average relative abundance of *H. pylori* increased in some of the washed biopsies compared to the unwashed, but this was not always observed in the individual samples (Table 4). The increase in *H. pylori* was therefore not significant. The "other bacteria" belong to over 100 different bacterial groups, of which some species were only present in few biopsies. The bacterial groups that were found in several of the biopsies are among the genera: *Abiotrophia, Aggregatibacter, Atopobium, Campylobacter, Capnocytophaga, Catonella, Corynebaccterium, Dialister, Eubacterium, Filifacter, Flavobacterium, Gemella, Granulicatella, Lachnoanaerobaculum, Lactobacillus, Leptotrichia, Megasphaera, Oribacterium, Parvimonas, Peptostreptococcus, Porphyromonas, Propionibacterium, Selenomonas, Solobacterium, Staphylococcus, Stenotrophomonas, Stomatobaculum* and *Treponema* (Table 5). The relative abundance of other non-*H. pylori* bacteria was similar between unwashed and washed samples, and none of the listed groups showed a significant change in relative abundance in either dyspepsia patients (Figure 3a) or gastric cancer patients (Figure 3b). No significant differences were observed in the bacterial distribution between the patient groups. Comparison of the bacterial diversity within the samples showed no significant differences between the unwashed and washed biopsies (Figure 3c), and similar bacterial species were clustered in both groups (Figure 3d).

Table 4. Mean and standard error of the 10 most common bacterial groups as percentage of total bacterial reads in the microbiome analysis.

	Dyspepsia Biopsiy Pairs (n = 22)		Gastric Cancer Biopsy Pairs (n = 24)	
	Unwashed (% ± SEM)	Washed (% ± SEM)	Unwashed (% ± SEM)	Washed (% ± SEM)
Helicobacter spp.	28.3 ± 7.9	31.2 ± 8.4	11.8 ± 4.4	12.7 ± 5.0
Streptococcus spp.	12.6 ± 1.8	9.6 ± 1.8	21.3 ± 4.3	20.1 ± 4.3
Prevotella spp.	13.9 ± 2.7	11.0 ± 2.5	6.4 ± 1.1	6.1 ± 1.3
Escherichia spp.	7.0 ± 2.9	10.4 ± 3.8	9.5 ± 3.6	11.9 ± 3.6
Veillonella spp.	5.3 ± 0.9	3.4 ± 0.9	3.5 ± 1.0	3.7 ± 0.7
Fusobacterium spp.	4.8 ± 1.0	4.3 ± 1.5	4.2 ± 1.4	3.7 ± 1.2
Haemophilus spp.	4.6 ± 1.3	3.2 ± 0.9	2.4 ± 0.5	2.0 ± 0.5
Rothia spp.	3.5 ± 0.8	1.9 ± 0.6	2.2 ± 0.4	2.0 ± 0.7
Neisseria spp.	2.2 ± 0.7	1.5 ± 0.6	6.5 ± 2.7	6.8 ± 2.5
Alloprevotella spp.	1.5 ± 0.5	1.4 ± 0.4	1.7 ± 0.7	1.8 ± 0.7
Other bacteria	17.4 ± 2.7	17.0 ± 3.1	30.57 ± 5.8	29.3 ± 5.2

Table 5. Mean and standard error of the most common bacterial groups belonging to "other bacteria" as percentage of total bacterial reads in the microbiome analysis.

	Dyspepsia Biopsy Pairs (n = 22)		Gastric Cancer Biopsy Pairs (n = 24)	
	Unwashed (% ± SEM)	Washed (% ± SEM)	Unwashed (% ± SEM)	Washed (% ± SEM)
Abiotrophia spp.	0.013 ± 0.007	0.007 ± 0.009	0.104 ± 0.055	0.128 ± 0.047
Aggregatibacter spp.	0.139 ± 0.043	0.134 ± 0.044	0.103 ± 0.038	0.187 ± 0.086
Atopobium spp.	0.359 ± 0.113	0.31 ± 0.128	0.328 ± 0.064	0.335 ± 0.086
Campylobacter spp.	0.507 ± 0.141	0.452 ± 0.104	0.423 ± 0.121	0.314 ± 0.116
Capnocytophaga spp.	0.455 ± 0.19	0.308 ± 0.123	0.329 ± 0.087	0.274 ± 0.079
Catonella spp.	0.311 ± 0.098	0.212 ± 0.056	0.093 ± 0.027	0.119 ± 0.061
Corynebacterium spp.	0.214 ± 0.112	0.257 ± 0.175	0.047 ± 0.017	0.08 ± 0.033
Dialister spp.	0.304 ± 0.089	0.199 ± 0.083	0.31 ± 0.132	0.242 ± 0.101
Eubacterium spp.	0.123 ± 0.03	0.181 ± 0.059	0.186 ± 0.07	0.277 ± 0.147
Filifactor spp.	0.172 ± 0.076	0.262 ± 0.139	0.043 ± 0.012	0.08 ± 0.044
Flavobcaterium spp.	1.235 ± 0.975	1.27 ± 0.927	0.164 ± 0.063	0.575 ± 0.375
Gemella spp.	0.827 ± 0.228	0.82 ± 0.313	0.861 ± 0.211	1.446 ± 0.433
Granulicatella spp.	1.046 ± 0.219	1.093 ± 0.206	1.333 ± 0.405	1.603 ± 0.431
Lachnoanaerobaculum spp.	0.296 ± 0.066	0.271 ± 0.106	0.457 ± 0.156	0.435 ± 0.137
Lactobacillus spp.	0.013 ± 0.007	0.012 ± 0.006	1.925 ± 1.245	10.5 ± 8.878
Leptotrichia spp.	0.459 ± 0.196	0.967 ± 0.516	0.728 ± 0.362	0.42 ± 0.177
Megasphaera spp.	0.554 ± 0.188	0.448 ± 0.143	0.219 ± 0.062	0.196 ± 0.059
Oribacterium spp.	0.432 ± 0.09	0.336 ± 0.11	0.329 ± 0.113	0.632 ± 0.327
Parvimonas spp.	0.229 ± 0.076	0.31 ± 0.188	1.478 ± 0.833	2.508 ± 1.555
Peptostreptococcus spp.	0.135 ± 0.045	0.13 ± 0.066	2.517 ± 1.448	3.319 ± 1.892
Porphyromonas spp.	1.765 ± 0.52	1.607 ± 0.653	0.666 ± 0.198	1.241 ± 0.566
Propionibacterium spp.	0.058 ± 0.031	0.082 ± 0.026	0.141 ± 0.056	0.256 ± 0.107
Selenomonas spp.	0.167 ± 0.059	0.231 ± 0.082	0.087 ± 0.022	0.088 ± 0.031
Solobacterium spp.	0.281 ± 0.062	0.282 ± 0.103	0.674 ± 0.29	0.603 ± 0.217
Staphylococcus spp.	0.403 ± 0.168	0.627 ± 0.153	0.292 ± 0.077	0.728 ± 0.351
Stenotrophomonas spp.	0.679 ± 0.54	1.128 ± 0.769	0.156 ± 0.052	0.495 ± 0.325
Stomatobaculum spp.	0.328 ± 0.12	0.365 ± 0.115	0.561 ± 0.332	0.775 ± 0.483
Treponema spp.	0.211 ± 0.076	0.391 ± 0.218	0.067 ± 0.031	0.056 ± 0.019



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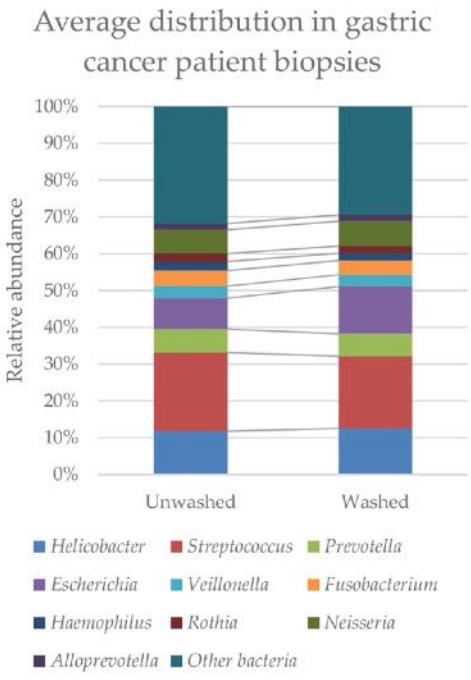

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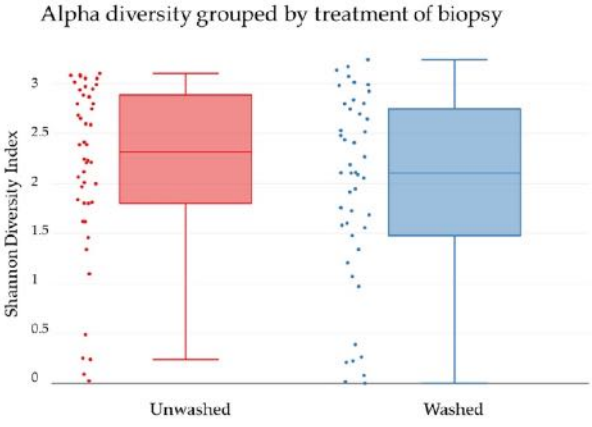

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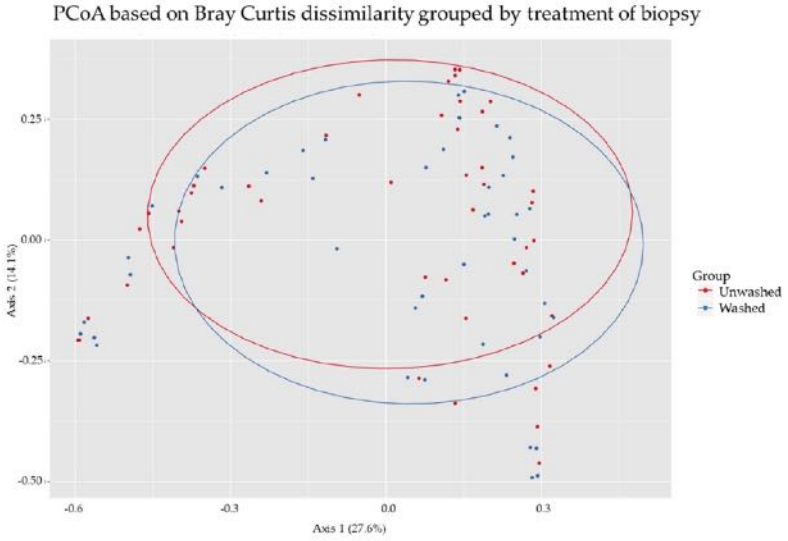


(d)

Figure 3. Comparison of the bacterial reads from microbiome analysis of unwashed and washed biopsies from (**a**) dyspepsia patients and (**b**) gastric cancer patients (percentages are listed in Table 4). Comparison of (**c**) the alpha-diversity shown by a Shannon Index, $p = 0.22581$ and (**d**) the beta-diversity between the two groups shown by PCoA plot, $p = 0.801199$, in unwashed biopsies (red) and washed biopsies (blue).

3.2. *Comparison of Biopsies from Gastric Cancer Patients and Dyspepsia Patients*

3.2.1. Cultured Bacteria were Dominated by *Streptococcus* spp.

The biopsies from dyspepsia patients and gastric cancer patients were dominated by similar cultured bacteria (Figure 4). Species of *Lactobacillus* were cultured from several cancer patient biopsies but only from one dyspepsia patient biopsy (Table 2). The distribution of cultured bacteria in biopsies from gastric cancer patients and dyspepsia patients showed an increase in the relative abundance of *Streptococcus* and a decrease in *Actinomyces* spp. (Table 3, Figure 4).

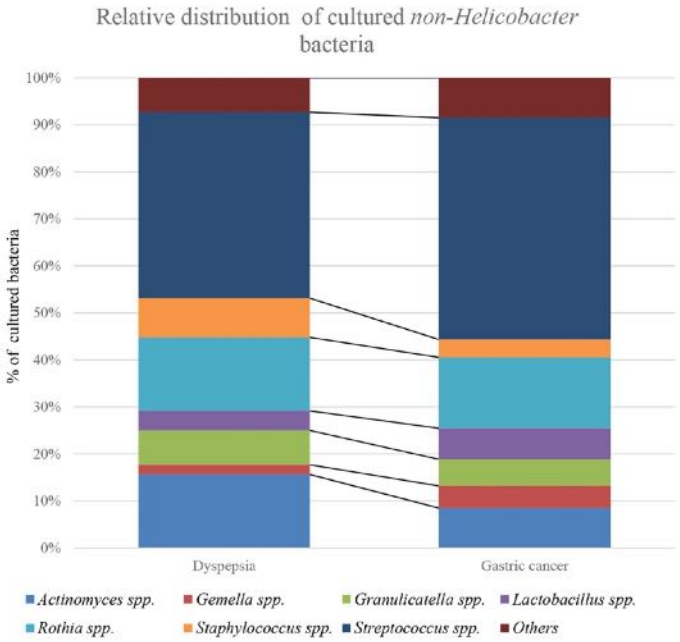
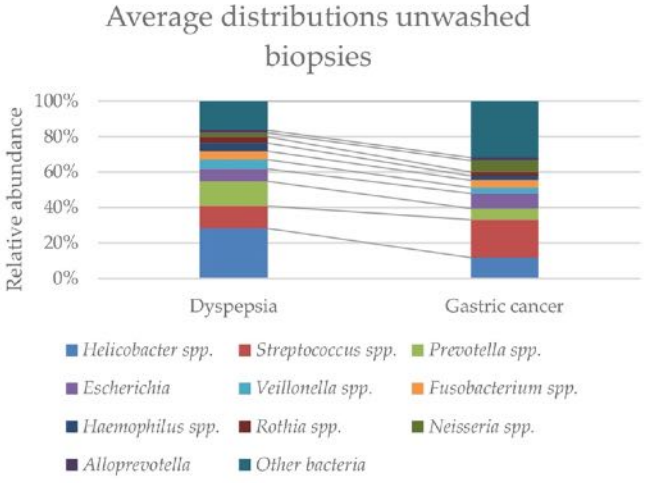


Figure 4. The relative distribution of non-*Helicobacter* bacteria cultured from the unwashed biopsies showed that *Streptococcus* spp. were predominant in both dyspepsia patients and gastric cancer patients. The percentages can be found in Table 3. "Others" include *Bacillus* spp., *Corynebacterium* spp., *Enterobacter* spp., *Enterococcus* spp., *Haemophilus* spp, *Micrococcus* spp., *Neisseria* spp., and *Stenotrophomonas* spp.

3.2.2. Microbiome Analysis Revealed Similar Distributions of Bacteria in Dyspepsia Patients and Gastric Cancer Patients

The average relative abundance of *H. pylori* was not significantly different in untreated biopsies untreated dyspepsia patients and gastric cancer patients (Figure 5a). For most bacterial groups, no significant difference was observed in the distribution of bacteria between dyspepsia patient and gastric cancer patient biopsies. However, a significant increase in the presence of *Prevotella* spp. was observed in dyspepsia patients ($p = 0.0109$) and of "other bacteria" in gastric cancer patients ($p = 0.0349$). This increase in other bacteria may be explained by the dominance of *Enterococcus* spp. in one biopsy pair, where more than 95% of the bacterial reads were identified as this. If this biopsy pair was removed from the data, the difference in "other bacteria" between the patient groups was not significant. The bacterial diversity in biopsies from dyspepsia patients and gastric cancer patients showed no significant differences within the groups (Figure 5b) or between the distribution of species in the groups (Figure 5c).



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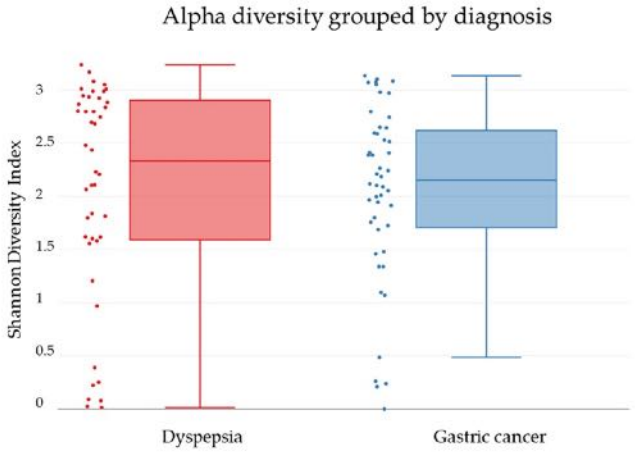

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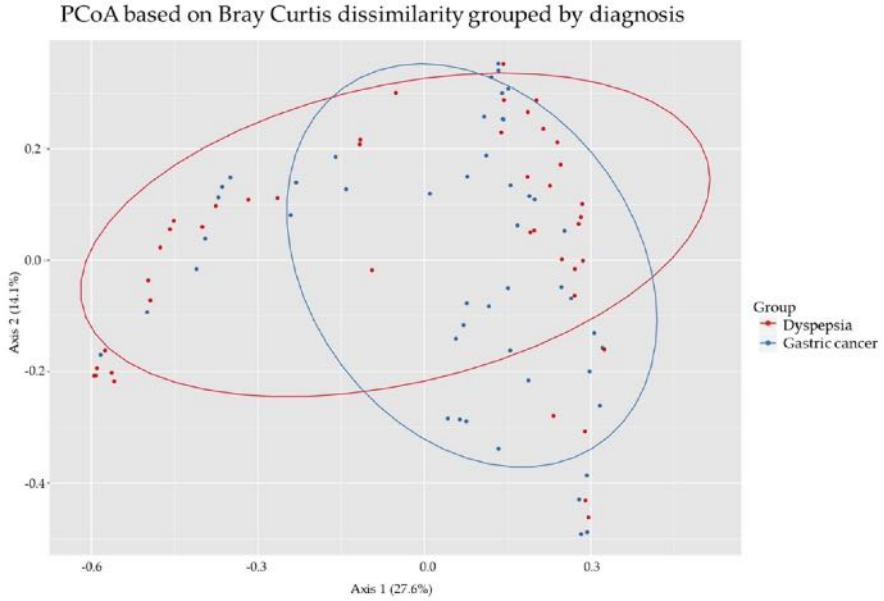


Figure 5. (**a**) The average relative abundance of the ten most common bacterial genera of untreated biopsies from dyspepsia patients and gastric cancer patients. Comparison of (**b**) the alpha-diversity shown by a Shannon Index, $p = 0.556831$ and (**c**) the beta-diversity between the two groups shown by PCoA plot, $p = 0.052947$, in biopsies from dyspepsia patients (red) and gastric cancer patients (blue).

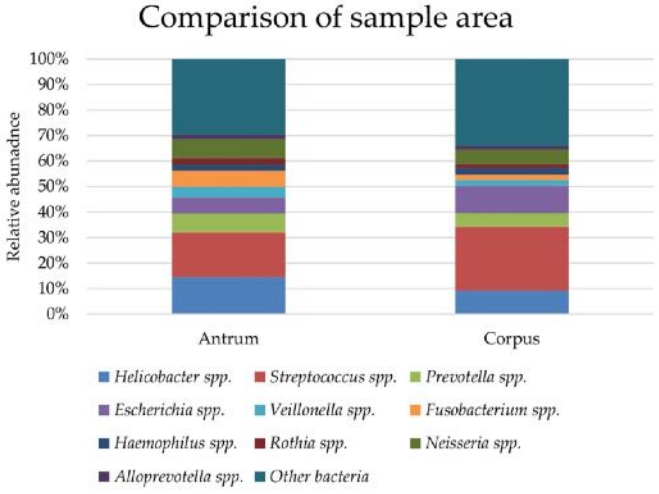
A low percentage of the bacterial reads in biopsies from dyspepsia patients were identified as *Lactobacillus* spp. (<0.1% of total bacterial reads). Most biopsy pairs from gastric cancer patients (30 pairs) did not show presence of *Lactobacillus* spp.; 6 biopsy pairs showed a relative abundance of 0–0.5% of total bacterial reads, and 7 biopsy pairs showed a relative abundance of 0.5–2% *Lactobacillus* spp., while three biopsy pairs showed a high relative abundance of *Lactobacillus* spp., which both increased (from 11 to 27%) and decreased (from 23% to 0.4% and from 29% to 5%) between the unwashed and washed biopsies. This difference in *Lactobacillus* spp. abundance was not significant between dyspepsia and gastric cancer patients.

The biopsies from antrum and cancer area of gastric cancer patients showed no significant differences in the distribution of bacterial groups (Figure 6a, Table 6).

The diversity of bacteria in biopsies from antrum and corpus did not show differences in the bacterial diversity within the sample areas (Figure 6b) or between the two sample areas (Figure 6c).

Table 6. Mean and standard error as percentage of the 10 most common bacteria as percentage of total bacterial reads in the microbiome analysis of biopsies from gastric cancer patients (n = 12).

	Antrum Area (% ± SEM)	Cancer Area (% ± SEM)
Helicobacter spp.	14.5 ± 7.3	9.1 ± 4.9
Streptococcus spp.	17.6 ± 5.1	25.1 ± 6.9
Prevotella spp.	7.4 ± 1.6	5.3 ± 1.5
Escherichia spp.	6.1 ± 2.7	12.9 ± 6.6
Veillonella spp.	4.3 ± 1.8	2.7 ± 2.0
Fusobacterium spp.	6.2 ± 2.6	2.1 ± 0.8
Haemophilus spp.	2.2 ± 0.7	2.6 ± 0.9
Rothia spp.	2.8 ± 0.8	1.6 ± 0.5
Neisseria spp.	7.3 ± 4.4	5.6 ± 3.2
Alloprevotella spp.	1.9 ± 1.0	1.5 ± 0.9
Other bacteria	29.6 ± 7.6	34.1 ± 8.8



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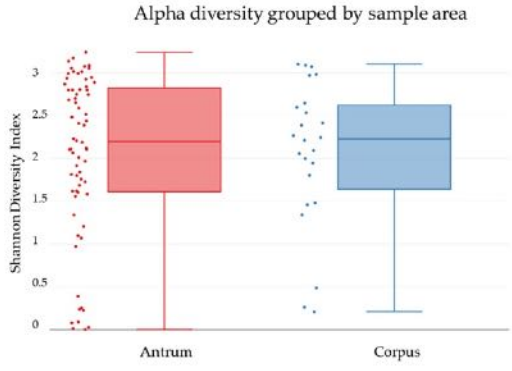

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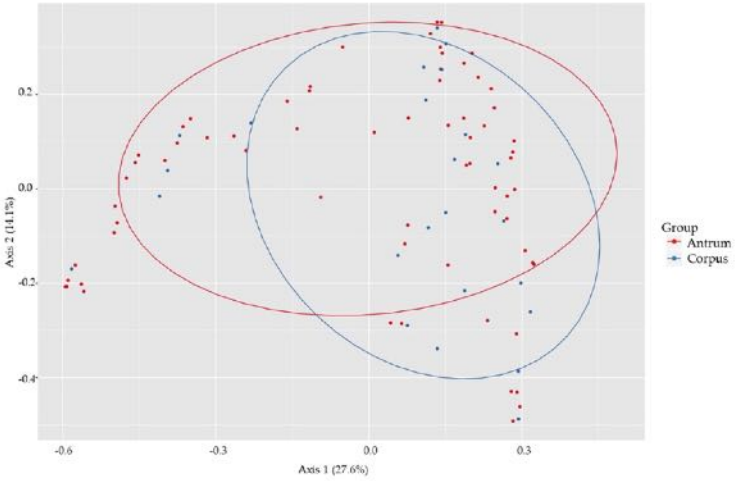
Figure 6. *Cont.*



(c)

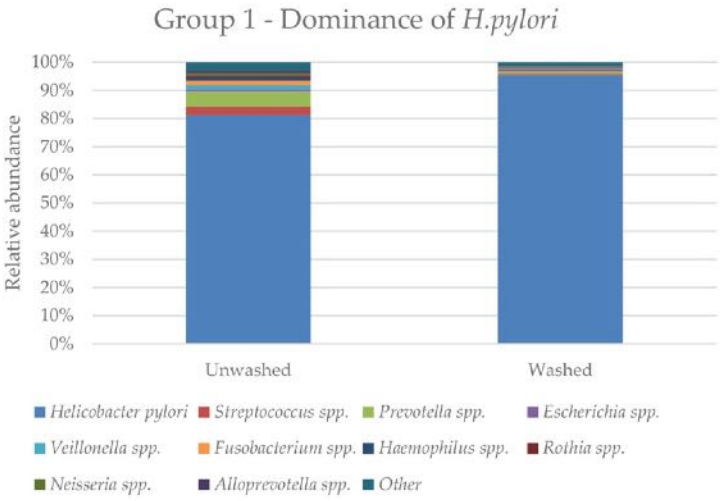
Figure 6. (**a**) The relative abundance of bacteria in untreated biopsies from the antrum and corpus area of gastric cancer patients. No significant differences were observed in the distributions of bacteria between the two groups. (Percentages are listed in Table 6). Comparison of (**b**) the alpha-diversity shown by a Shannon Index, $p = 0.960995$ and (**c**) the beta-diversity between shown by PCoA plot, $p = 0.111888$, in biopsies from antrum area (red) and corpus area (blue) in the stomach.

3.3. *Presence of* H. pylori

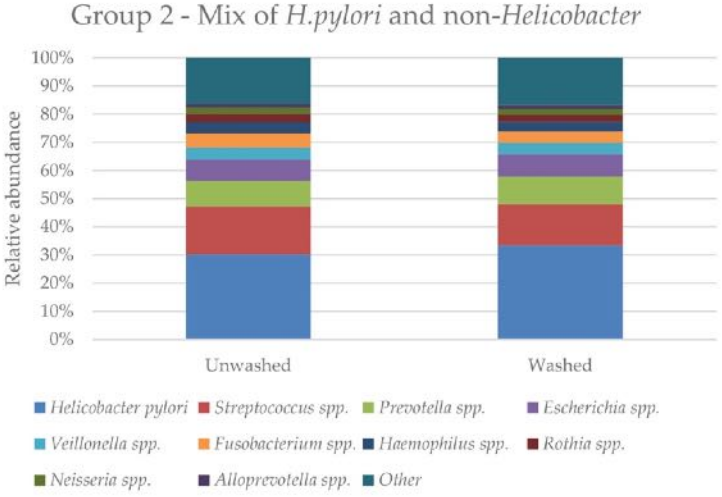
H. pylori was detected in 14 of 34 patients (41%) by histology. Culture of *H. pylori* was only successful for 4 biopsies from 2 patients, despite incubation for additional days. Microbiome analysis identified DNA from *H. pylori* in 16 of 34 patients (47%), and *H. pylori* was identified as the only species of *Helicobacter* in the biopsies. The difference between culture and 16S rRNA gene amplicon sequencing may be explained by the fastidious nature of *H. pylori*, which may be difficult to culture after storage.

Three different distribution types of *H. pylori* and non-*Helicobacter* were observed; 4 biopsy pairs showed almost complete dominance of *H. pylori* (>90% of total bacterial reads in at least one of the biopsies) (Figure 7a), 16 biopsy pairs showed a mixed relative abundance of *H. pylori* and other bacteria (Figure 7b), and 26 biopsy pairs showed none or less than 1% of total bacterial reads identified as *H. pylori* (Figure 7c).

The biopsies determined positive or negative for *H. pylori* by histology showed significant differences in the bacterial diversity. The *H. pylori*-negative biopsies showed a significantly higher diversity than *H. pylori*-positive biopsies (p-value = 0.004353) (Figure 8a). In addition, a significant difference in the bacterial diversity was observed between biopsies determined positive or negative for *H. pylori* (p-value = 0.0009999), indicating that the presence of *H. pylori* may change the bacterial community to allow for a unique composition (Figure 8b).

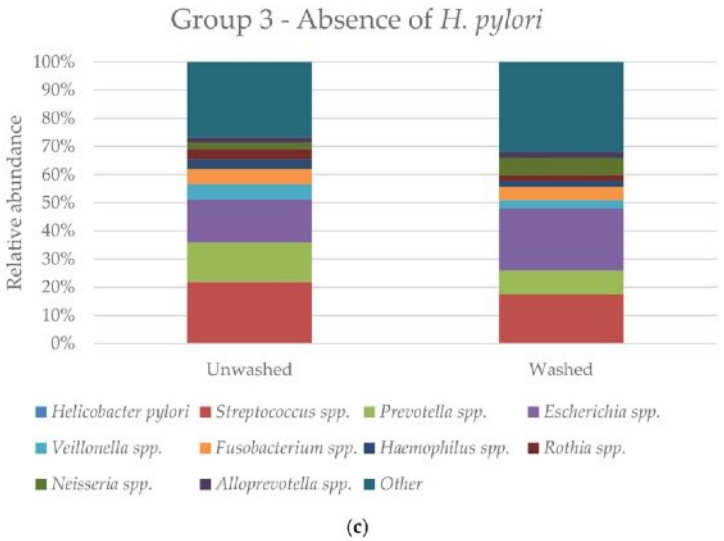


(a)



(b)

Figure 7. *Cont.*



(c)

Figure 7. The average relative abundance divided into three groups based on their distribution of *H. pylori* and other bacteria by (**a**) dominance of *H. pylori*, (**b**) mixed presence of non-*Helicobacter* and *H. pylori*, and (**c**) absence of *H. pylori*.

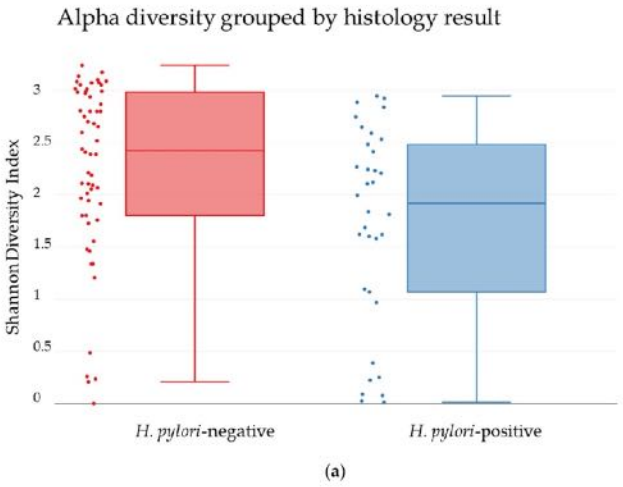
4. Discussion

4.1. Washed Biopsies vs. Unwashed Biopsies

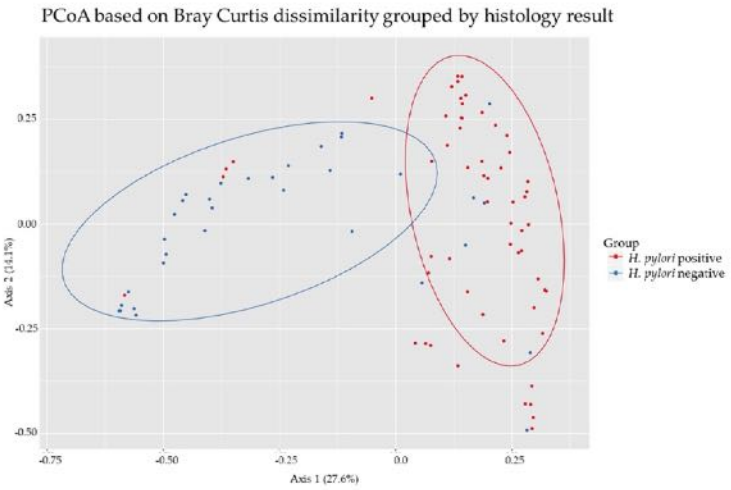
We observed a decreased bacterial growth from the washed biopsies compared to the unwashed biopsies in this study (Figure 2, Table 2). Based on our hypothesis, this might suggest that the bacteria removed by washing were not adhering to the biopsy and should not be considered a part of the gastric microbiota. *Streptococcus* spp. was the most dominant non-*Helicobacter* bacterial group in the biopsies, which may either be attributed to a higher starting concentration in the unwashed biopsy or an increased ability to adhere to the tissue, compared to the other bacteria in the biopsies.

The non-*Helicobacter* species identified in this study have previously been described as commensals of the oral cavity, upper airways and intestinal tract, including species of the dominant groups *Streptococcus*, *Rothia* and *Actinomyces* [11,16,20,21,25]. Intestinal bacteria such as *Enterococcus* spp. and *Escherichia* spp. were also identified with both culture and microbiome analysis and may be a sign of overgrowth from the intestines. Species of *Staphylococcus* were cultured from several biopsies, but the group was not among the 10 most common in the microbiome analysis, where between 0% and 4% of the reads were identified as *Staphylococcus* spp. The high detection in culture compared to microbiome analysis may be explained by selection of growth of *Staphylococcus*, as species of this group are facultative aerobic with a fast growth rate.

Our expectation was that only *H. pylori* would remain in the washed biopsies. This was not the case, as we observed the presence of many bacteria, of which the average relative abundance and diversity within the biopsies did not significantly change in the washed biopsies (Figure 3). This may suggest that some bacterial groups may remain in the biopsies and should be further investigated.



(a)



(b)

Figure 8. Comparison of the bacterial diversity from biopsies of patients shown negative for *H. pylori* (red) or positive for *H. pylori* (blue). (**a**) The alpha-diversity shown by a Shannon Index, $p = 0.004353$. (**b**) The beta-diversity between the two groups shown by PCoA plot, $p = 0.000999.4$.

4.2. Gastric Cancer Patients vs. Dyspepsia Patients

Comparison of the bacterial composition did not reveal significant differences in the bacterial diversity between biopsies from dyspepsia patients or gastric cancer patients. *Lactobacillus* was cultured, and DNA was detected in a higher abundance in biopsies from gastric cancer patients than in biopsies from dyspepsia patients, although not significantly. Development of gastric cancer has been described as a result of dysbiosis, and it is believed that a change in the gastric bacteria towards oral and intestinal bacteria may contribute to this process [19]. However, previous studies have not detected specific bacterial genera in only one of the patient groups [8,19]. It may be discussed if the presence of cancer cells causes alterations in the environment, which leads to changed microbial growth, or if the development is inversed. The increased presence of cultured *Lactobacillus* spp. in gastric cancer

patient biopsies may also be an important difference between these patient groups, although the exact function is unknown.

The expected result from this comparison was that a different bacterial composition would be observed in the cancer tissue compared to non-cancer patients, which was not confirmed by our results (Figure 6). To date, no studies have shown a connection between non-*Helicobacter* bacteria and development of pathologies such as gastric cancer in humans [11]. We observed an increased presence of *Lactobacillus* spp. in gastric cancer patient biopsies compared to dyspepsia patient biopsies in both the culture and microbiome analysis but cannot with certainty conclude that this bacterial group is involved in the development of gastric cancer. Four biopsies were positive for culture of *Lactobacillus* spp., where no reads were found in the microbiome analysis. However, 16 biopsies were negative for growth of *Lactobacillus* spp. but positive in the microbiome analysis. 16S rRNA-based analysis is more sensitive, and it is possible to detect the presence of bacteria in small quantities. An explanation for the difference in culture and microbiome results may be that 16S rRNA gene amplicon sequencing is not genus-specific, and as such, the 16S rRNA genes from several bacteria are amplified simultaneously. If *Lactobacillus* spp. are present in small quantities, the presence of a high amount of other DNA may drown it out, and it will not be detected. Combined with a selection in the culture for microaerobic-growing bacteria, the abundance of *Lactobacillus* spp. may appear higher in this experiment setting. This discrepancy between methods should also be considered in future culture- and sequencing-based studies.

A study by Blaser and Atherton discussed whether *H. pylori* is a main driver of gastric cancer development, or if its presence is enough to change the stomach environment and allow for growth of other bacteria, which in turn increase the risk of gastric cancer [28]. The authors agree that long-term colonization causes inflammation and damage to the host tissues but that the mechanism leading to development of gastric cancer is not well defined [28]. In order to fully understand the pathological changes of other non-*Helicobacter* species in the stomach, infection assays should be performed on human cell cultures or model organisms with gastrointestinal tracts more similar to humans.

4.3. Presence of H. pylor in the Biopsies

H. pylori was only cultured from the biopsies of two patients after extended culture, even though its presence in other patients was confirmed by histology and microbiome analysis. Further, microbiome analysis identified *H. pylori* in two biopsies, where the histology result was negative for *H. pylori*. This comparison of methods demonstrates that culture, histology and 16S rRNA gene sequencing are not always in accordance, and investigation of bacterial communities using several methods is preferred. During colonization or prolonged incubation, *H. pylori* take a coccoid form which may be difficult or impossible to culture in vitro [25]. Based on its slow growth and fastidious nature, culture of this species from clinical specimens is expected to be challenging. The culture results are still considered to be valid, as other species were cultivated, and thus, the medium and growth conditions must have been sufficient for bacterial growth. However, in order to culture all the present bacteria in the biopsies, several other medium types and atmospheres might have been considered. The relative distribution of *H. pylori* was expected to increase after wash, along with the removal of contaminating bacteria. This was not always observed (Figure 3). Studies have shown that it is possible to detect *H. pylori* with sensitive DNA-based methods in individuals previously shown negative for *H. pylori* with conventional methods [19]. This may also be the reason why a higher prevalence of *H. pylori* was observed with microbiome analysis compared to histology. The results from microbiome sequencing showed a variable distribution of *H. pylori* and non-*H. pylori* bacteria in the gastric environment. Three overall types of bacterial distribution were observed, suggesting that the stomach is more complex than previously thought (Figure 7). Natural variations in the microbiota caused by the geographical origin of the patients may also be of importance for this. The results from this study contribute to the agreement of a dynamic relationship between *H. pylori* and non-*Helicobacter* bacteria in the stomach, and previous discrepancies between studies may be caused by natural differences

between patients. The ten most prevalent groups with microbiome analysis fits with the groups detected in other reported NGS-studies [19].

The biopsies showing positive or negative for *H. pylori* by histology analysis displayed significant differences in the bacterial diversity within and between the groups (Figure 8), suggesting that *H. pylori* may cause changes in the environment to allow for survival of other bacteria as suggested by Blaser and Atherton [28]. Our findings are also in agreement with the study by Maldonado-Contreras, which discovered a difference in the bacterial diversity based on *H. pylori*-status [21]. Patients negative for *H. pylori* presented a higher relative abundance of *Actinobacteria* and *Firmicutes*, whereas *H. pylori*-positive patients presented a higher abundance of *Proteobacteria* and *Acidobacteria*.

The most common bacterial groups identified in the culture and microbiome analysis shows few similarities, and only two genera in the 10 most abundant groups from the microbiome analysis were cultured. However, both *Gemella* and *Granulicatella* were detected in several biopsies but not in high enough reads to be included in the top 10. The difference between the methods may be caused by selection of certain bacteria in the microaerobic incubation. Four of the 10 most common groups have been described as anaerobes or strict anaerobes (*Prevotella*, *Veillonella*, *Fusobacterium*, *Neisseria*), preventing them from growing in the microaerobic environment used in this study. *H. pylori* was detected in 47% of the biopsies by microbiome analysis but only cultured from two biopsy pairs. The biopsies were stored in the transport medium at −80 °C, which might have decreased the viability of *H. pylori*. It may be suggested that other bacteria identified by microbiome analysis were also present in the biopsies but were inhibited by the storage/freezing, and therefore unable to be cultured.

4.4. Limitations

It has been described that 80% of the bacteria that are identified with molecular methods in the human gut cannot successfully be cultured in vitro [16]. As we only cultured the biopsies in microaerobic environment, growth was selected for the microaerobically growing bacteria. The presence of these appeared higher than with microbiome sequencing. Several types of growth conditions and culture media would be required to select growth for all bacteria present in the biopsy.

Culture-independent methods such as 16S rRNA gene amplicon sequencing or microbiome analysis may provide detailed information about the bacterial composition. One disadvantage may be that it does not differentiate between live and dead bacteria and between residents and contamination [2,10,19]. Other methods with the ability to distinguish between active and inactive bacteria, such as immunostaining or analysis of the metabolic activity, may also be considered for future investigations [22].

The results of this study are new as both culturing and sequencing are included as methods of detection, and the effect of washing or presence of *H. pylori* on the bacterial composition and diversity are investigated. Our results showed a decrease in the growth of some bacterial groups from washed biopsies, which are also known oral commensal bacteria. The species that remain in the tissue after wash must thus contain mechanisms for adhesion to avoid being removed.

We present the first comprehensive paper attempting to distinguish between transient and resident bacteria in the stomach using a 16S rRNA gene amplicon sequencing approach and washing of biopsies. One other study has investigated the bacterial content of biopsies with a similar approach [14]. However, the study included only a small number of samples and used a taxon-specific quantitative PCR to define the taxa [14]. Future investigations in gastric microbiota should consider the presence of other bacteria in the stomach that may only be a transient contamination.

5. Conclusions

In conclusion, the number of cultured non-*Helicobacter* bacteria decreased in the washed biopsies, suggesting that they might be a transient contamination from oral cavity; however, in the microbiome analysis, no significant differences in the bacterial diversity were observed between unwashed and

washed biopsies. The bacterial diversity in biopsies that were *H. pylori*-positive and *H. pylori*-negative was significantly different, implying that *H. pylori* is the major modulator of gastric microbiome. Further large-scale studies are required to investigate the transient and persistent gastric microbiota. This may include an increased number of samples, investigation of dysbiosis, a wider range of culture conditions and growth media, metatranscriptomic analysis, immunological assays or additional treatment of biopsies.

Author Contributions: Conceptualization, J.K., L.K. and L.P.A.; methodology, J.K., L.K. and L.P.A.; validation, M.R.S., T.B.J., T.H.F., K.F. and L.P.A.; formal analysis, M.R.S., T.B.J. and K.F.; investigation, M.R.S., M.U., J.S. and L.J.; resources, J.K., J.S., K.F. and L.P.A.; data curation, M.R.S., T.B.J. and M.U.; writing—original draft preparation, M.R.S.; writing—review and editing, M.R.S., J.K. and L.P.A.; visualization, M.R.S., T.B.J. and K.F.; supervision, J.K., K.F., L.K. and L.P.A.; project administration, J.K.; funding acquisition, J.K. and L.K. All authors have read and agreed to the published version of the manuscript.

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References

1. Dewhirst, F.E.; Fox, J.G.; On, S.L.W. Recommended minimal standards for Helicobacter. *Int. J. Syst. Evol. Microbiol.* **2000**, *50*, 2231–2237. [CrossRef]
2. Bik, E.M.; Eckburg, P.B.; Gill, S.R.; Nelson, K.E.; Purdom, E.A.; Francois, F.; Peres-Perez, G.; Blaser, M.J.; Relman, D.A. Molecular analysis of the bacterial microbiota in the human stomach. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 732–737. [CrossRef]
3. Nardone, G.; Compare, D. The human gastric microbiota: Is it time to rethink the pathogenesis of stomach diseases? *United Eur. Gastroenterol. J.* **2015**, *3*, 255–260. [CrossRef] [PubMed]
4. Llorca, L.; Perez-Perez, G.; Urruzuno, P.; Martinez, M.J.; Iizumi, T.; Gao, Z.; Sohn, J.; Chung, J.; Cox, L.; Simón-Soro, A.; et al. Characterization of the Gastric Microbiota in a Pediatric Population According to *Helicobacter Pylori* Status. *Pediatri. Infect. Dis. J.* **2017**, *36*, 173–178. [CrossRef] [PubMed]
5. Jonaitis, L.; Pellicano, R.; Kupcinskas, L. *Helicobacter pylori* and nonmalignant upper gastrointestinal diseases. *Helicobacter* **2018**, *23* (Suppl. 1), e12522. [CrossRef]
6. Pérez-Pérez, G.I.; Sack, R.B.; Reid, R.; Reid, R.; Santosham, M.; Croll, J.; Blaser, M.J. Transient and Persistent *Helicobacter pylori* Colonization in Native American Children. *J. Clin. Microbiol.* **2003**, *41*, 2401–2407. [CrossRef] [PubMed]
7. Li, J.; Perez, G.I.P. Is There a Role for the Non-*Helicobacter pylori* Bacteria in the Risk of Developing Gastric Cancer ? *Int. J. Mol. Sci.* **2018**, *19*, 1353. [CrossRef]
8. Cover, T.L.; Blaser, M.J. *Helicobacter pylori* in health and disease. *Gastroenterology* **2009**, *136*, 1863–1873. [CrossRef]
9. Smet, A.; Flahou, B.; Mukhopadhya, I.; Ducatelle, R.; Pasmans, F.; Haseobrouck, F.; Hold, G.L. The Other Helicobacters. *Helicobacter* **2011**, *16*, 70–75. [CrossRef]
10. Solnick, J.V.; Schauer, D.B. Emergence of Diverse *Helicobacter* Species in the Pathogenesis of Gastric and Enterohepatic Diseases. *Clin. Microbiol. Rev.* **2001**, *14*, 59–97. [CrossRef]
11. O'Hara, A.M.; Shanahan, F. The gut flora as a forgotten organ. *EMBO Rep.* **2006**, *7*, 688–693. [CrossRef] [PubMed]
12. Ianiro, G.; Molina-Infante, J.; Gasbarrini, A. Gastric Microbiota. *Helicobacter* **2015**, *20*, 68–71. [CrossRef] [PubMed]
13. Engstrand, L.; Lindberg, M. *Helicobacter pylori* and the gastric microbiota. *Best Pract. Res. Clin. Gastroenterol.* **2013**, *27*, 39–45. [CrossRef] [PubMed]
14. Li, X.X.; Wong, G.L.H.; To, K.F.; Wong, V.W.S.; Lai, L.H.; Chow, D.K.; Lau, J.Y.W.; Sung, J.J.Y.; Ding, C. Bacterial microbiota profiling in gastritis without *Helicobacter pylori* infection or non-steroidal anti-inflammatory drug use. *PLoS ONE* **2009**, *4*, e7985. [CrossRef]

15. Sanduleanu, S.; Jonkers, D.; Bruine ADe Hameeteman, W.; Stockbrügger, R.W. Non-*Helicobacter pylori* bacterial flora during acid-suppressive therapy: Differential findings in gastric juice and gastric mucosa. *Aliment. Pharmacol. Ther.* **2001**, *15*, 379–388. [CrossRef]
16. Rajilic-Stojanovic, M.; Figueiredo, C.; Smet, A.; Hansen, R.; Kupcinskas, J.; Rokkas, T.; Andersen, L.; Machado, J.; Ianiro, G.; Gasbarrini, A.; et al. Systematic review: Gastric microbiota in health and disease. *Aliment. Pharmacol. Ther.* **2020**, *51*, 582–602. [CrossRef]
17. Schulz, C.; Schütte, K.; Malfertheiner, P. *Helicobacter pylori* and Other Gastric Microbiota in Gastroduodenal Pathologies. *Dig. Dis.* **2016**, *34*, 210–216. [CrossRef]
18. Zilberstein, B.; Quintanilha, A.G.; Santos, M.A.A.; Pajecki, D.; Moura, E.G.; Alves, P.R.A.; Filho, F.M.; Souza, J.A.U.; Gama-Rodriguez, J. Digestive tract microbiota in healthy volunteers. *Clinics* **2007**, *62*, 47–54. [CrossRef]
19. Delgado, S.; Cabrera-Rubio, R.; Mira, A.; Suarez, A.; Mayo, B. Microbiological Survey of the Human Gastric Ecosystem Using Culturing and Pyrosequencing Methods. *Microb. Ecol.* **2013**, *65*, 763–772. [CrossRef]
20. Dicksved, J.; Lindberg, M.; Rosenquist, M.; Enroth, H.; Jansson, J.K.; Engstrand, L. Molecular characterization of the stomach microbiota in patients with gastric cancer and in controls. *J. Med. Microbiol.* **2009**, *58*, 509–516. [CrossRef]
21. Maldonado-Contreras, A.; Goldfarb, K.C.; Godoy-Vitorino, F.; Karaoz, U.; Contreras, M.; Blaser, M.J.; Brodie, E.L.; Dominguez-Bello, M.G. Structure of the human gastric bacterial community in relation to *Helicobacter pylori* status. *ISME J.* **2011**, *5*, 574–579. [CrossRef]
22. Yu, G.; Torres, J.; Hu, N.; Medrano-Guzman, R.; Herrera-Goepfert, R.; Humphrys, M.S.; Wang, L.; Wang, C.; Ding, T.; Ravel, J.; et al. Molecular Characterization of the Human Stomach Microbiota in Gastric Cancer Patients. *Front. Cell. Infect. Microbiol.* 2017. [CrossRef] [PubMed]
23. Andersson, A.F.; Lindberg, M.; Jakobsson, H.; Bäckhed, F.; Nyrén, P.; Engstrand, L. Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLoS ONE* **2008**, 3. [CrossRef]
24. Monstein, È.R.G.; Tiveljung, A.; Kraft, C.H.; Borch, K.Ä.; Jonasson, J. Profiling of bacterial flora in gastric biopsies from patients with *Helicobacter pylori*-associated gastritis and histologically normal control individuals by temperature gradient gel electrophoresis and 16S rDNA sequence analysis. *J. Med. Microbiol.* **2000**, *49*, 817–822. [CrossRef]
25. Lofgren, J.L.; Whary, M.T.; Ge, Z.; Muthupalani, S.; Taylor, N.S.; Mobley, M.; Potter, A.; Varro, A.; Eiback, D.; Suerbaum, S.; et al. Lack of Commensal Flora in *Helicobacter pylori*—Infected INS-GAS Mice Reduces Gastritis and Delays Intraepithelial Neoplasia. *Gastroenterology* **2011**, *140*, 210–220. [CrossRef]
26. Abreu, M.T.; Peek, R.M. Gastrointestinal Malignancy and the Microbiome. *Gastroenterology* **2014**, *146*, 1534–1546. [CrossRef]
27. Amieva, M.; Peek, R.M. Pathobiology of *Helicobacter pylori*-induced Gastric Cancer. *Gastroenterology* **2016**, *150*, 64–78. [CrossRef]
28. Blaser, M.J.; Atherton, J.C. *Helicobacter pylori* persistence: Biology and disease. *J. Clin. Investig.* **2004**, *113*, 321–333. [CrossRef]
29. Ferreira, R.M.; Pereira-Marques, J.; Pinto-Ribeiro, I.; Costa, J.L.; Carneiro, F.; Machado, J.C.; Figueiredo, C. Gastric microbial community profiling reveals a dysbiotic cancer-associted microbiota. *Gut* **2018**, *67*, 226–236. [CrossRef]
30. Marchesi, J.R.; Ravel, J. The vocabulary of microbiome research: A proposal. *Microbiome* **2015**, *3*, 31. [CrossRef]
31. Mcdonald, J.E.; Larsen, N.; Pennington, A.; Connoly, J.; Wallis, C.; Rooks, D.J.; Hall, N.; McCarthy, A.J.; Allosin, H.E. Characterising the Canine Oral Microbiome by Direct Sequencing of Reverse-Transcribed rRNA Molecules. *PLoS ONE* **2016**, *11*, e0157046. [CrossRef] [PubMed]
32. Ring, H.C.; Thorsen, J.; Saunte, D.M.; Lilje, B.; Bay, L.; Riis, P.T.; Larsen, N.; Andersen, L.O.; Nielsen, H.V.; Miller, I.M.; et al. The Follicular Skin Microbiome in Patients With Hidradenitis Suppurativa and Healthy Controls. *JAMA Dermatol.* **2017**, *153*, 897–905. [CrossRef] [PubMed]

Gut microbial similarity in twins is driven by shared environment and aging

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Summary

Background Human gut microbiome composition is influenced by genetics, diet and environmental factors. We investigated the microbial composition in several gastrointestinal (GI) compartments to evaluate the impact of genetics, delivery mode, diet, household sharing and aging on microbial similarity in monozygotic and dizygotic twins.

Methods Fecal, biopsy and saliva samples were obtained from total 108 twins. DNA and/or RNA was extracted and the region V1-V2 of the 16S rRNA gene was amplified and sequenced. Bray-Curtis similarity was used for further microbiome comparisons, Mann-Whitney test was applied to evaluate the significant differences between groups and Spearman test was applied to reveal potential correlations between data.

Findings The global bacterial profiles were grouped into two clusters separating the upper and lower GI. The upper GI microbiome composition was strictly dependent on the *Helicobacter pylori* status. With a positivity rate of 55%, *H. pylori* completely colonized the stomach and separated infected twins from non-infected twins irrespective of zygosity status. Lower GI microbiome similarity between the twins was defined mainly by household-sharing and aging; whereas delivery mode and host genetics had no influence. There was a progredient decrease in the bacterial similarity with aging. Shared vs. non-shared phylotypes analysis showed that in both siblings the shared phylotypes progressively diminished with aging, while the non-shared phylotypes increased.

Interpretation Our findings strongly highlight the aging and shared household as they key determinants in gut microbial similarity and drift in twins irrespective of their zygotic state.

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Keywords: Microbiome; 16S rRNA sequencing; Aging; Shared household; Stomach; *Helicobacter pylori*; Equality

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Research in context

Evidence before this study

Previous studies in twins have suggested host genetics as a determinant factor for the gut microbiome profile. More recent evidence suggests an important contributing role of environmental factors on the gut microbiota structure in twins.

Added value of this study

We provide the evidence of the key impact of *Helicobacter pylori* on composition of stomach microbiome in twins subjects, which was independent of zygosity status. We show that similarity of gut microbiome in twins was dependent of several factors. Shared household and aging were among the most important factors in defining the gut microbiome similarity in mono- and dizygotic twins. The increasing shift of microbiome concordance with age suggests a dynamic age-and environment-related process.

Implications of all the available evidence

Our study support the increasing evidence that shared household, including environment and diet, as well as aging are the key dynamic factors in shaping microbiome in twins' subjects. The data from this work may be applicable to microbiome dynamics in household members irrespective of genetic similarity.

Introduction

The gut microbiome plays a crucial role in human health, and dysbiosis is related to a variety of diseases.[1] Gut microbiome is considered an intestinal dynamic organ where its composition evolves throughout the lifetime,[2,3] and reduction and alteration of microbial diversity is linked to gastrointestinal (GI) and non-GI diseases.[4] Individual microbiome is variable and multiple factors contribute to bacterial composition in humans. Delivery mode,[5,6] diet,[6,7] physical activity[8,9] and shared household[10] are among the factors that have been most consistently linked with microbiome diversity.

Recently, an interplay between host genetics and microbiome has caused great interest.[11] Several studies have analyzed the link between gut microbiome composition and host genetic variations, using a genome-wide association study (GWAS).[10,12–14] Although a number of loci identified were associated with microbial taxonomies, microbial pathways, and measures on difference in the composition of microorganisms, most of these findings were population specific and difficult to replicate. In general, GWAS studies suggest that only up to 10% of microbial diversity might be associated with heritable factors. However, study design, population cohort and methodological approach may vary, therefore, making direct comparison challenging. Hence, additional studies are needed to determine the interplay of genetics and microbiome as it remains considered that heritable factors may determine the diversity of human microbiome.[15]

Studies in twins provide an excellent model to explore the influence of heritable factors in relation to other factors in particular to environmental factors.[16] Several studies with focus on microbiome in twins have been completed so far.[17–20] Analysis of fecal specimens from monozygotic (MZ) and dizygotic (DZ) twins and their mothers revealed similarity in microbiome pattern in family members which was associated with either leanness or obesity.[17] Another study including 416 twin pairs suggested a link between host genetics to several bacterial taxa, although the overall degree of similarity between MZ and DZ twins might be of the marginal significance.[19] The largest cohort so far with 1,126 UK twin pairs observed an association between fecal microbiome taxa and genes related to diet, metabolism and olfaction.[18] Nevertheless, the exact role of environmental factors is relatively unexplored in twin cohorts, and the impact of shared household and aging, although suggested, has not been proven so far. Furthermore, gastric community has been increasingly studied, but the role of genetic factors has been not explored yet. *Helicobacter pylori (H. pylori)* Infection, which is key pathogen for peptic ulcer development and gastric cancer and which has a profound influence on the microbial composition of the stomach as *H. pylori* infection kind of suppress other bacterial communities.[21] But also, *Fusobacterium nucleatum* (*F. nucleatum*) has been frequently found in stomach of patients with gastric cancer and was associated with prognosis of gastric cancer.[22] Microbial community has not been systematically studied taken to account the genetics in twin cohorts.

The aim of our study was to comprehensively analyze the role of genetics, delivery mode, diet, household sharing and aging on microbial similarity in saliva, and gastric mucosa, with specific attention to *Helicobacter pylori* and most specifically fecal specimens in MZ and DZ twins.

Material and methods

Study population

Twins from the Twin Registry Center at Lithuanian University of Health Sciences were invited to take part in the study during the years 2016-2018. Detailed characteristics of the study participants are provided in Supplementary file 1. The initial study cohort included 115 twin pairs (230 subjects). 108 out of 115 twin pairs were included in the final analysis: 50 pairs of monozygotic and 58 pairs of dizygotic (29 same-sex pairs and 29 mixed-sex pairs) twins. None of the study participants has previously undergone anti-*H. pylori* treatment, and did not use PPIs or antibiotics at least one month prior

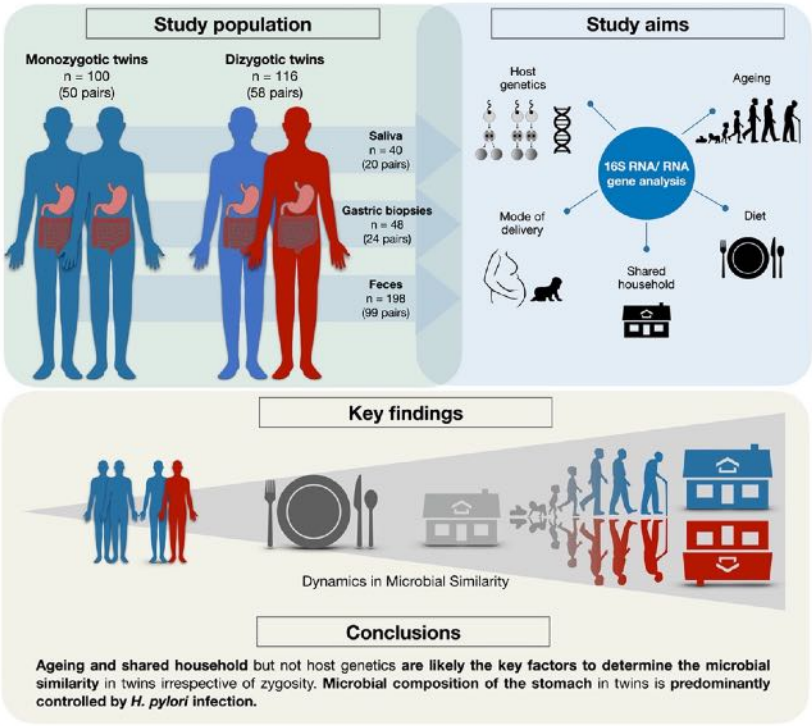

Figure 1. Graphical abstract. Design of the study.

to inclusion. If both twins had any potential clinical symptoms, they were offered to undergo upper gastrointestinal endoscopy. *H. pylori* presence/absence was identified after histological evaluation (haematoxylin/eosin and Giemsa staining). Demographic and diet data were collected using questionnaires (detailed description is provided in the Supplementary file 2). The study protocols were approved by Kaunas regional ethics committee (Protocol No: BE-2-10 and P1-52/2005). All participants provided a written informed consent to take part in the study. Graphical Abstract on the study design is shown in Figure 1.

Sample collection

EDTA blood samples for DNA analysis were collected from all twins (108 paired twins, 216 samples) within this study in order to confirm their zygosity. Feces from 99 paired twins (198 samples) were collected in stool collection containers with stabilizer (Stratec molecular), delivered to laboratory within 24 h period, aliquoted and stored at -80°C until DNA extraction. Saliva samples were collected from 20 twins (40 samples) before gastroscopy procedure (08:00 am–12:00 pm). Participants were not exposed to food for at least 8 h or more and beverages for at least 4 h. Each subject rinsed their mouth with water and saliva was collected in standard 50 ml sterile conical polypropylene tube (Falcon) using passive drool collection method (collected volume 3-5 ml). After collection, the saliva samples were immediately centrifuged and pellet was stored -80C before extraction. Two biopsies from the antrum and two biopsies from the corpus were collected during upper GIi endoscopy in twins, snap frozen in liquid nitrogen and stored at -80°C until DNA (from 19 twin pairs (38 samples) for corpus and 20 from antrum) or RNA (22 twin

pairs from corpus and 24 from antrum) extraction. Detailed list of biological samples collected from each twin is shown in Supplementary file 3.

Determination of zygosity

Zygosity testing (MZ versus DZ) was performed on blood DNA samples. Short tandem repeat polymorphic DNA markers were amplified by PCR using AmpFLSTR® Identifiler® Plus PCR Amplification Kit (Thermo Fisher Scientific, USA), labeled with fluorescent markers and separated by capillary electrophoresis to distinguish different alleles at each of 15 different loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TROX, D18S51, D5S818, and FGA).

Nucleic acids extraction

Bacterial genomic DNA from stool was extracted using QIAamp Fast DNA Stool Mini Kit (Qiagen), DNA from saliva was extracted using QIAamp DNA mini kit (Qiagen) and DNA and RNA from gastric biopsy samples were extracted using Allprep DNA/RNA mini kit (Qiagen) according to the manufacturer's instructions. On-column DNA digestion during RNA purification was performed using RNase-Free DNase Set (Qiagen). RNA was reverse transcribed to cDNA using Superscript IV First-Strand Synthesis System (Thermo Fisher Scientific).

Library construction and sequencing analysis

Library construction and sequencing of the samples were performed at the Otto-von-Guericke University Hospital of Magdeburg. Amplicon libraries were generated by amplification of the V1-V2 region of the 16S rRNA, taking as template either the DNA (16S rRNA gene) or the cDNA (16S rRNA) after 20 cycles PCR reaction, using the 27F and 338R primers, and sequenced on a MiSeq (2 × 250 bp, Illumina, Hayward, California, USA).[23,24]

FastQ files were analyzed using the dada2 package,[25] version 1.10.1, in R. Overall, 11 770 078 paired-end reads were obtained, with a minimum of 39 and average of 23 446 per sample. Samples that did not reach 4 000 reads were discarded from further analysis. All samples were resampled to equal sequencing depth of 4838 reads using the phyloseq package,[26] referring to 16 421 phylotypes (see online Supplementary file 4). Phylotypes were annotated to a taxonomic affiliation based on the naïve Bayesian classification[27] with a pseudo-bootstrap threshold of 80%. Microbial communities were analyzed at all taxonomy ranks (from phylum to genus) and phylotype taxonomy ranks. The relative abundances (expressed as percentages) were used for downstream analyses.

Dendrograms and PCoA were built using the sample-similarity matrix by Bray-Curtis algorithm (1000 bootstrap) at phylotype level and plotted using Past,[4] Mega7 software or iTol Interactive Tree of Life website.[28] Samples were analyzed for outliers in their Bray-Curtis-Similarity, using the ROUT method (Q = 1%) in Prism 7 (GraphPad Software, La Jolla, CA). Mann-Whitney test was applied to evaluate the significant differences between groups defined a priori and correlations were performed using Spearman test using Prism 7 (GraphPad Software, La Jolla, CA). PERMANOVA was calculated using Primer-e (Primer 7, Version 7.0.17, Add on: Permanova+).[29] P value < 0.05 was considered statistically different. For comparison of paired twins, we considered only the samples where both twins from the pair were successfully sequenced (198 samples out of 222 samples).

Heritability calculations

Heritability of bacteria showing similarity between the twins on genus level was estimated as previously described.[18] Bacteria had to be present in more than 50% of individuals. Relative abundances were transformed using Box-Cox transformation (PowerTransform command implemented in the R package 'car' was used to calculate λ) and regressed using multiple linear regression to eliminate influence from the number of sequencing reads per sample, age, gender, household sharing status. The residuals from this regression were then used for the heritability estimates. Heritability estimates were calculated by a twin-based ACE model using the R package 'OpenMx'. ACE, CE, AE, and E models were calculated and p values were determined by using the likelihood ratio test in order to evaluate the significance of additive genetic (ACE vs. CE), common environment (ACE vs. AE) and unique environment (ACE vs. E) components. Multiple testing correction for 15 traits was applied for the heritability analysis using the Benjamini-Hochberg algorithm in R. Intraclass correlation coefficients (ICCs) were calculated using 'icc' command from the R package 'irr'.

Role of funding source

Funders had no role in study design, data collection, data analyses, interpretation, or writing of report.

Results

General cohort

Bacterial communities of a total of 408 samples were characterized from 108 paired twins. The general cohort included 198 fecal samples from 99 paired twins and 210 samples from 19 to 24 twins of the upper GI, including samples from saliva (DNA), corpus (DNA and RNA) and antrum (DNA and RNA). After sequencing and rarefying library size to the minimum sequencing depth, 16 421 phylotypes, belonging to 21 different phyla and 385 genera, were retrieved and taxonomically

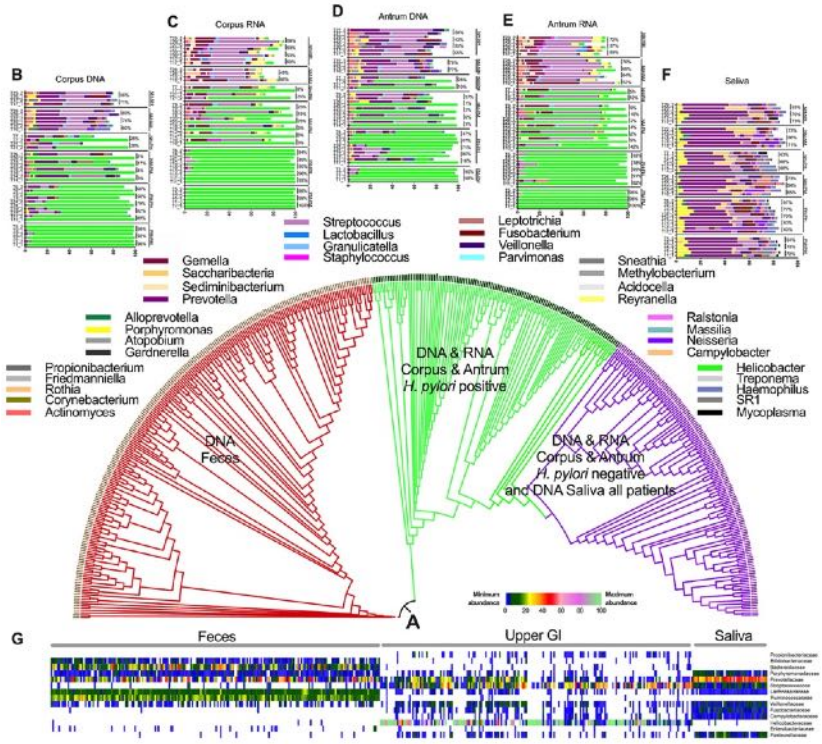

Figure 2. A. Group-average agglomerative hierarchical clustering of studied samples, based on the global bacterial profile at phylotype-level along the upper GI (saliva, corpus and antrum) and fecal samples. B to F: Most abundant genera detected in corpus, antrum and saliva in H. pylori negative (N) and positive (P) individuals as well as in dizygotic (DZ) and monozygotic (MZ) twins. Percentages shown the Bray-Curtis similarities between twin pairs. G: Heatmap at family-level with the most abundant taxa representing the microbiome in saliva, antrum, corpus and feces.

annotated (Supplementary file 4). The global bacterial profiles were grouped into two clusters based on their % of Bray-Curtis similarities (Figure 2A), clearly showing differences between lower GI (feces) and upper GI (biopsies from corpus or antrum and saliva). The upper GI (corpus and antrum biopsy samples) microbiome composition was defined by *H. pylori* status. *H. pylori* completely colonized the stomach (Figure 2A–F) and thereby, infected twins were grouped separately from *H. pylori* negative twins. Global bacteria composition analysis along the GI indicates that saliva mainly is colonized by Prevotellaceae while stomach is colonized by Streptococcaceae and lower GI is colonized by Lachnospiraceae and Ruminococcaceae (Figure 2G).

Helicobacter pylori status in twins

Overall, 55% of the twins with available gastric tissue were *H. pylori* positive (Figure 2B to 2E). Most of the twin pairs were concordant (both twins in the pair) for the *H. pylori* infection and eight twin pairs, incl. T4_2, T8_1 or T9_1, were discordant. As previously described, *H. pylori* was solely detected in the stomach samples.[10] Principal coordinates analysis showed *H. pylori* to be the major determinant for differentiating twins based on their bacterial composition in the stomach (Figure 3A). Moreover, *H. pylori* infection status was similar both in MZ or DZ twins. When comparing the percentage of Bray-Curtis similarities between paired twins, no significant differences were found between MZ and DZ twins

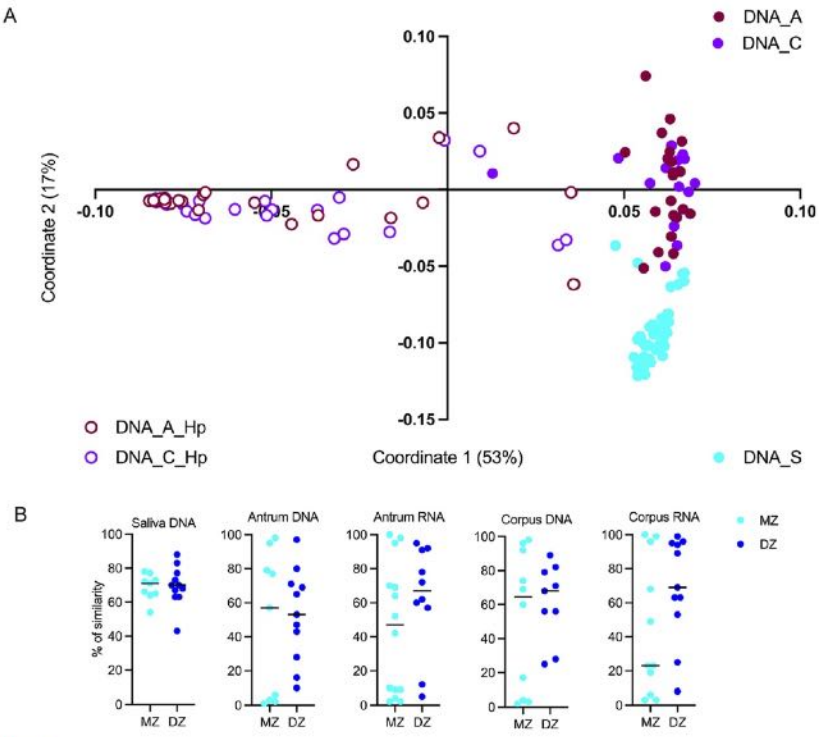

Figure 3. A. Principal Coordinates Analysis (PCoA) of the bacterial communities in the upper GI (DNA_S for saliva, DNA_C or RNA_C for corpus DNA_A or RNA_A for antrum) at phylotype-level based on the Bray-Curtis similarity matrix. Samples from corpus and antrum of patients infected by *H. pylori* are denoted as Hp. B: Percentage of Bray-Curtis similarities of the bacterial communities in twin pairs in saliva corpus and antrum between monozygotic twins (MZ) and dizygotic twins (DZ).

(Figure 3B, Supplementary file 5), suggesting that the genetic background does not affect the stomach bacterial community structure, although, due to the small cohort size, this statement might not be generally correct.

Heritability of the predominant microbes in the gastrointestinal tract

Following the previous studies in twins, we calculated heritability of the most abundant taxa detected in our cohort as well as the shared environmental influence and the nonshared environmental influence applying the ACE model (Supplementary file 6).[18,19] In order to eliminate influence from the known cofounding factors, taxa abundances were regressed on covariates (number of sequences reads per sample, age, gender, household sharing status). Results of the regression analysis are presented in the Supplementary file 7. The residuals from this regression were then used for the heritability estimates. Contrary to what was previously published, none of the fifteen more abundant genera (including *Bacteroides, Blautia, Prevotella, Bifidobacterium* among others) appeared to be significantly heritable, based on the additive genetic influence from the ACE model, although similar values were obtained for *Blautia, Faecalibacterium, Dialister* among others (Supplementary file 8). In the same manner, no influence of the shared environment on any of the genera was detected. However, regarding the nonshared environment, *Bacteroides, Blautia, Collinsella* and *Holdemanella* were strongly influenced (p value < 0.001) and in less extent also *Alistipes, Ruminococcus, Prevotella* and *Catenibacterium* (p

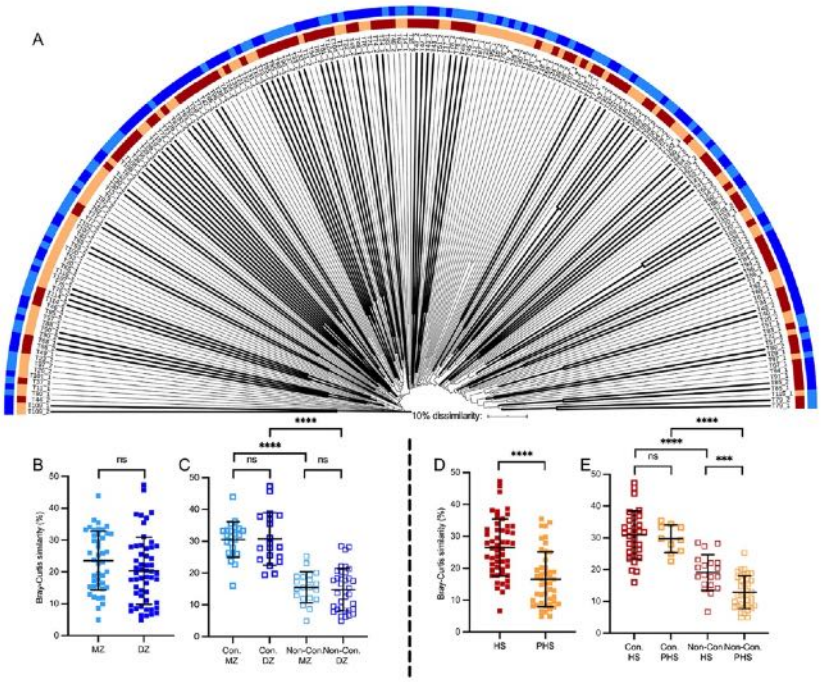

Figure 4. A. Group-average agglomerative hierarchical clustering of 198 fecal samples at phylotype-level. Concordant twins (Con) are denoted in bold in contrast to non-concordant (Non-Con). Light blue and dark blue denoted monozygotic (MZ) and dizygotic (DZ) twins, respectively as well as light brown and dark brown denoted shared and non-shared household twins, respectively. B to E: Bray-Curtis similarities of the bacterial communities in twin pairs and a priori defined groups (one dot represents two twins). Statistically differences are shown as *** if p value < 0.001 and **** if p value < 0.0001 and ns denotes no statistical differences.

value < 0.05). These results might suggest a stronger influence of the environment rather than the genetic background in certain taxa.

Bacterial community assemblages in the GUT of twins

All possible paired analyses between fecal samples (total 19 503 possible comparisons) of twins (198 samples from 99 paired-twins) revealed an average of 11±5 % of Bray-Curtis similarity, suggesting a low similarity between samples. Nonetheless, roughly 50% of the twins from a pair were grouping together and showed a higher similarity (higher than roughly 20% of similarity) to each other than with the other twin pairs. Those twins were named "concordant twins". In contrast, twin pairs with lower similarity values (lower than roughly 20% of similarity) and not clustering together - "non-concordant twins". Further, the possible factors affecting bacterial community structure were evaluated (Figure 4A). It was observed that 76% of concordant twins were sharing the household (HS group vs. 24% past HS, p value < 0.0001) (Figure 4D), while 55% of the concordant twins were MZ vs. 45% DZ (p > 0.05) (Figure 4B and C). This suggests that environmental factors in particular HS have much stronger influence on the similarity of the bacterial communities rather than genetic background (Figure 4A). Moreover, concordant twins living together had the highest similarity of bacterial communities (mean of similarity 31%), whereas not-concordant twins living apart – the lowest (mean of similarity 12.9%) (Figure 4E).

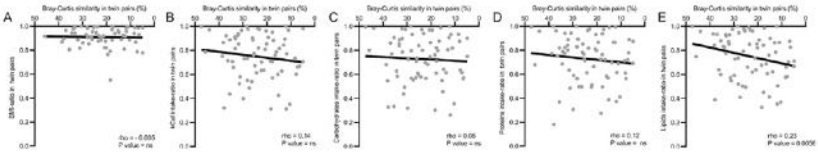

Figure 5. Spearman correlations (rho) between Bray-Curtis similarities of the bacterial communities in twins pairs (one dot represents two twins) and the body mass index ratio (BMI-ratio) (A), Kilocalories (Kcal) intake ratio (B), and carbohydrates, proteins (C) and lipid (D) intake ratios, respectively.

Influence of nutrition on bacterial communities in the paired-twins

Having shown the relevance of the shared household for similarities of the bacterial communities in paired twins, we next evaluated the potential impact of diet. Out of the 99 twin pairs, records of nutritional information were available for 73 twin pairs (74% of the cohort). Based on the Spearman correlation index, diet was not the major determinant defining the bacterial community differences between the paired twins. There were no correlations between Bray-Curtis similarity and body mass index-ratio (Figure 5A), as well as kilocalories intake-ratio (Figure 5B). However, the nutrient analysis revealed that lipid intake had influence on bacterial community similarity (Figure 5E). In twins with similar intake of lipids, the bacterial communities were similar in both siblings ($p < 0.01$). Neither carbohydrate or protein intake showed statistically significant influences on the Bray-Curtis similarity although the trends were similar to the lipid's intake (Figure 5C to E).

The body mass index did not show statistically significant differences with the similarities of the bacterial communities between twins. The similar intake of total calories resulted in more similar communities within twins.

Influence of aging on the bacterial composition between twins

Limited knowledge exists on the role of aging in twin cohorts as it has not been sufficiently addressed in previous twin studies. Our cohort consists of 198 twins with age ranging from 9 to 72 years (aver. age 29 ± 14 years), therefore our twin-cohort covered the broadest age-range with the highest standard deviation of all cohorts published so far (Figure 6A).

Analysis of age influence on similarities of the bacterial communities between twins revealed a strong negative correlation (Figure 6B), thereby indicating that bacterial communities diverge with increasing age in the twins ($p < 0.0001$). In addition, the analysis of concordant and non-concordant twin groups showed that concordant twins were much younger (Figure 6C), indicating the age as one of the major determinant factors in the bacterial community development. Analysis results revealed that 21 years could be considered as the age when bacterial communities diverge within paired twins. Further, shared vs. non-shared phylotype analysis showed that phylotypes shared by both siblings progressively diminished (rho = - 0.42, $p < 0.0001$) with the age, while the richness of the non-shared phylotypes strongly increased between 10 and 20-year-old twins and afterwards the increase was slower (rho = 0.46, $p < 0.0001$) (Figure 6D). In addition, when considering age as a co-variable of household sharing, age showed a p value of 0.0001 while household sharing showed a p value of 0.04 (Supplementary file 9).

GUT taxonomy profile in twins

The abundances of the most predominant genera detected in the GUT of paired twins are shown in Figure 7. Overall, eighteen genera were broadly colonizing the GUT of our cohort, *Prevotella* or *Bacteroides* being the most abundant ones. These eighteen genera made up more than 50% of the bacterial communities in most of the individuals. Colonization of the GUT by *Bacteroides* and *Prevotella* seems to be aleatory, which might suggest that either *Prevotella* or *Bacteroides*, but never both together, colonized the GUT. Interestingly, MZ twins showed a higher correlation with several genera compared to DZ twins, including *Bacteroides* (p value in MZ < 0.001), *Prevotella* (p value in MZ < 0.001), *Parasutterella* (p value in MZ < 0.01), among others (Supplementary file 8). It is worth mentioning that no correlations have been observed with any of the genera regarding delivery mode and breastfeeding (Supplementary file 10).

Discussion

It has been hypothesized that host genetics may determine the composition of the gut microbiome,[18] even though the genetic inheritance of microbial composition has been recently questioned.[10] In this work, we performed systematic analysis of microbial composition of MZ and DZ twins to delineate the interplay between genetic and various concomitant factors including shared environment, delivery mode and diet in various compartments of the gut. The analysis of the stomach microbiota between MZ and DZ twins highlights the

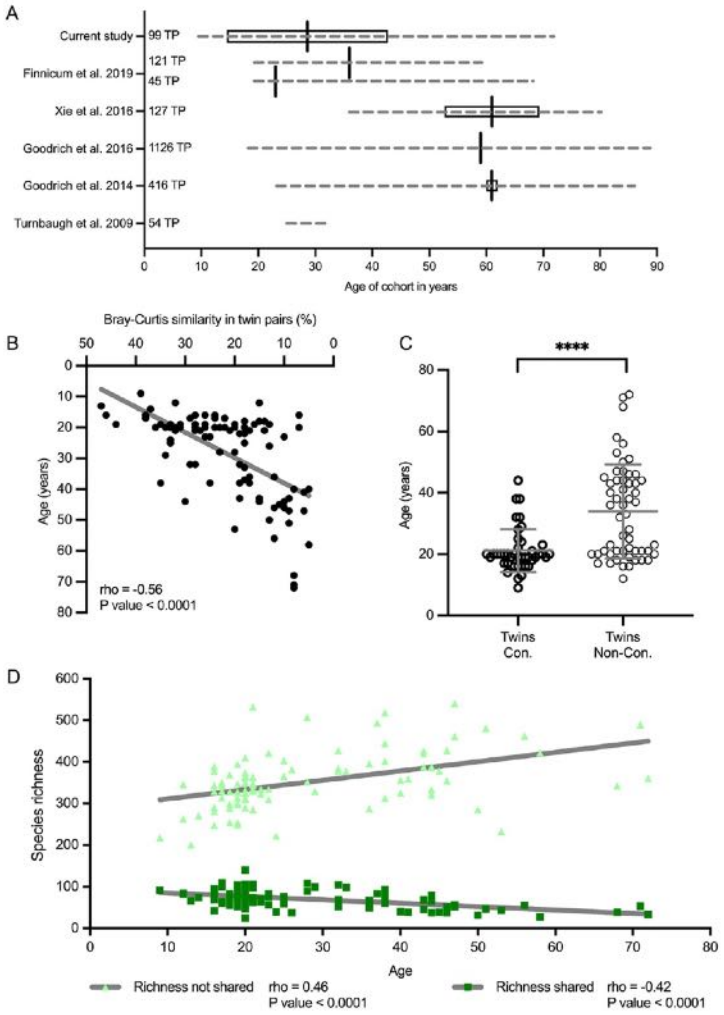

Figure 6. A. Overview of the age on the different cohort of twins published so far compared to this study. If it was published, for each study is shown the minimum and the maximum of age, as well as the standard deviation and the median. B: Spearman correlation (rho) in twin pairs (one dot represents two twins) between their age and their percentage of similarity of the bacterial communities. C: Differences on the age between concordant (Con) twins and non-concordant (Non-Con) twins according to the Bray-Curtis similarities shown in Fig. 4. **** denotes *p* value < 0.0001. D: Spearman correlation (rho) between the number of phylotypes shared (light green) and non-shared (dark green) between twins paired (TP).

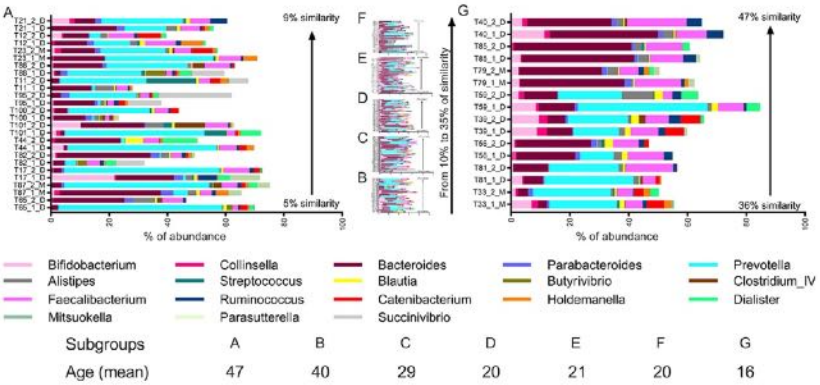

Figure 7. Abundances of the most predominant genera detected in fecal samples. Samples are sorted in increasing percentage of Bray-Curtis similarity and in twin pairs side-by-side, as well as divided in 7 groups due to space limitation, showing magnified the first group (A) and the last group (G) and in small sizes groups B to F.

role of *H. pylori* in defining bacterial composition of the stomach mucosa. Evaluation of the fecal microbiome revealed a very strong impact of shared household and age as the main determinants of the microbial concordance among twins, which was in addition independent of the zygosity, diet and delivery mode at birth.

The role of host genetics in shaping microbiome has been suggested earlier[29]; however, more recent evidence emphasizes the predominant role of environmental factors, (such as age, sugar consumption, toothbrushing habits) over the host factors in defining the bacterial composition in twins.[31–33] The concept of gastric microbiome is still evolving,[34,35] with an ongoing discussion about what constitutes a gastric microbiome.[36] While the largest studies related to twins have been dealing with easily accessible specimens like saliva and feces, only limited knowledge was available for systematic characterization of mucosal microbiome in other body niches including stomach. In the only available study at present, the authors evaluated four pairs of twins with very heterogeneous results.[37] In current work, we evaluated microbiome in the largest cohort (24 twin pairs) that were representative for various combinations of MZ and DZ including concordant and discordant for *H. pylori* status. Even though larger cohorts are needed to evaluate the minor effects in concordant groups, we clearly show that *H. pylori* status had a dominant impact on the microbiome composition in twins irrespective of zygosity status. Our findings are in line with previous studies where *H. pylori* is the major gastric microbiome shaping species.[21,30] In earlier high-quality studies, familial environment and *H. pylori* status were considered as the most important risk factors for peptic ulcer development which is supported by our data.[38] In addition, *H. pylori* status in the stomach had no impact on oral microbiome as shown for the stomach.

Twin studies offer a unique opportunity to evaluate the effect of host genetics in defining microbial profile. Initial studies indicated that host genetics may influence microbiome for instance by the means of metabolic regulation.[17,19,39] Contrary to those results, none of the most abundant genera (including *Bacteroidetes, Prevotella, Bifidobacterium*, etc.) seems to be significantly heritable in our cohort using ACE model, although some effect was visible taking into consideration the nonshared environment (*Bacteroides, Blautia, Collinsella* and *Holdemanella*). These studies indicated that although MZ twin pairs generally have more similar microbiomes compared to DZ twin pairs or unrelated individuals, MZ twins can display a large range of within-twin-pair microbiome diversity.[19] These data suggest that environmental factors play the predominant role rather than host genetics in shaping fecal microbiome.

As of today, only a modest link between microbiome and heritability has been shown; therefore; it is likely that other factors including shared household, delivery mode or even aging might be responsible for microbial similarity in twins. A twin study from the TwinsUK registry showed that the Bray-Curtis distance between twin pairs did not associate with age.[39] On the other hand, this study found that Bray-Curtis distance negatively correlated with the age when twins started living apart and to a lesser extent positively correlated with years the twins lived apart.[39] Rothshild et al. have recently re-evaluated the impact of genetics and environment in a cohort of 1046 healthy Israelis and re-analyzed the link between microbiome, genetics and environment in the

2252 twins from the TwinsUK cohort.[10] The authors show that gut microbiome composition is predominantly shaped by environmental factors while the association with individual SNPs that was previously reported could not be replicated.

In our study, we took the advantage of the wide age distribution (age range: 9–72 years) and detailed characterization of the twins and therefore were able to have an in-depth view on potential factors influencing microbiome. Neither the delivery mode, nor the breastfeeding were associated with microbial similarity in MZ and DZ twins. However, shared household was one of the most important factors that was associated with bacterial concordance, which was furthermore independent of zygosity. Our observation is also indirectly supported by a recent UK twin registry microbiome study looking at socioeconomic impacts on microbiome.[40] The study showed that higher discordance in the index of social deprivation was associated with greater dissimilarity of twin microbiomes. Several studies have now reported that household sharing may at least partially determine microbiome similarity among relatives, while living apart reduces this similarity.[10,39]

It is important to point out that sharing a household is interlinked with other co-founding variables including diet that may additionally contribute to the microbial signature. Within our study, analysis of individual differences in the diet did not reveal any potential differences that could partially be related to common preferences of the twins. The similarity in nutritional behavior is very likely to be prone to similarity during the early life sequence. Previous analyses estimated that diet and lifestyle may be responsible for the up to 20% variance in microbiome.[10,14] Furthermore, host lifestyle clearly affects the microbiota on the daily timescale, which is relevant for twins that share the same social and family lifestyle niche.[41]

The aging has been considered, but has only received suboptimal attention in twin cohorts' due to difficulties to assess the lifetime frame. Our cohort consisted of MZ and DZ twins in the range of 9 to 72 years with a mean of 29.5 years, whereas, as shown on Figure 5A,the previously studied cohorts included a rather adult population with limited possibility to assess the landscape of microbial changes in twin subjects. While common in young twin pairs, Bray-Curtis similarity showed a clear shift apart in association with aging. Indeed, aging is the process that includes various steps starting from delivery mode, feeding, diseases in childhood etc. It is likely that leaving the common environment (shared household) may be a crucial step, but the drift does not stop here and shows a clear progression during the life time course.[10,42] Our data supports recent studies showing that age, menopausal status, and prior disease were also the top factors defining the microbial urobiome diversion in twins.[43]

This work provides several important aspects on the microbiome dynamics in twins; however, various additional aspects need more accurate evaluation. Even though this work is so far the largest to assess the stomach microbiota in twins, we were unable to address the microbial similarity in depth for the substantial effect of *H. pylori* on gastric microbiota. Further studies in larger cohorts of MZ, DZ and also non-twin siblings would likely help to estimate the dynamics in microbial patterns; however, it is questionable if upper GI endoscopy in asymptomatic population may be ethically justified. This cohort included only a single time point, whereas multiple time points would be needed to assess. The dietary information was acquired using self-reported food frequency questionnaires, thereby providing possible bias in reporting certain food intake. Furthermore, this work is based on 16S rRNA; therefore, whole genome sequencing including metatranscriptomic would likely provide a much more comprehensive view on the similarity and function of the GI microbiome. A recent study in twins looked beyond the microbiome and evaluated concordance for microbiome and virome in 21 adult MZ twins showing that microbiome-discordant twins display more divergent viromes compared to microbiome-concordant twins.[44] The results of this work were highly robust for the identified factors; therefore, we believe the data are representative also for other populations. Nevertheless, further studies with larger samples size will be necessary to delineate all compartments of microbiota, their possible functional interaction and also the way to precisely modulate microbiome in beneficial way.

Conclusions

In conclusion, in-depth analysis of various microbial comparts of twins strongly suggest the major role of non-hereditary factors in defining the microbial similarity in twins. Among those household sharing and aging are likely the most crucial determinants of fecal microbial dynamics in twins while *H. pylori* is the key factor in defining the stomach microbiota composition.

Declaration of interest

JS and JK were supported by the grant of the Research Council of Lithuania (Project no. APP-2/2016). PM received either speakers of consulting fees from Aboca, Bayer, Biocodex, Malesci, Mayoly-Spindler, Menarini, Synlab, Danone, Phathom during the conduct of the study. AL received research funding from EFRE (Project ID: ZS/2018/11/95324). All other authors have nothing to disclose.

Contributors

RVV, JS, KL, GV, CT, EM, KB, AL and JK contributed to data curation. RVV, JS, KL, JK and AL did the formal analysis. RVV, JS and KL contributed on the

methodology. KL, GV contributed on the visualization. LK, MU, CT provided resources and clinical data. DS and MZ contributed to the sequencing resources. RVV, JS, KL, JK and AL wrote, reviewed, and edited he manuscript. GV, CT, EM, KB, DS, MZ, PM edited and reviewed the final version. JK, PM, AL contributed on conceptualization. AL, JK were involved in funding acquisition and project administration, study supervision and share responsibility. All authors have had full access to the data, read and approved the manuscript.

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Data sharing statement

All sequences data and the taxonomic annotations are available in Supplementary file 4. All other data are available from the corresponding authors upon reasonable request.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.104011.

References

1 Lynch SV, Pedersen O. The human intestinal microbiome in health and disease. *N Engl J Med*. 2016;375:2369–2379.
2 Odamaki T, Kato K, Sugahara H, et al. Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. *BMC Microbiol*. 2016;16:90.
3 O'Toole PW, Jeffery IB. Gut microbiota and aging. *Science*. 2015;350:1214–1215. (80-).
4 Gilbert JA, Quinn RA, Debelius J, et al. Microbiome-wide association studies link dynamic microbial consortia to disease. *Nature*. 2016;535:94–103.
5 Hill CJ, Lynch DB, Murphy K, et al. Evolution of gut microbiota composition from birth to 24 weeks in the INFANTMET Cohort. *Microbiome*. 2017;5:4.
6 Bokulich NA, Chung J, Battaglia T, et al. Antibiotics, birth mode, and diet shape microbiome maturation during early life. *Sci Transl Med*. 2016;8:343ra82.
7 David LA, Maurice CF, Carmody RN, et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature*. 2014;505:559–563.
8 Barton W, Penney NC, Cronin O, et al. The microbiome of professional athletes differs from that of more sedentary subjects in composition and particularly at the functional metabolic level. *Gut*. 2018;67:625–633.
9 Scheiman J, Luber JM, Chavkin TA, et al. Meta-omics analysis of elite athletes identifies a performance-enhancing microbe that functions via lactate metabolism. *Nat Med*. 2019;25:1104–1109.
10 Rothschild D, Weissbrod O, Barkan E, et al. Environment dominates over host genetics in shaping human gut microbiota. *Nature*. 2018;555:210–215.
11 Kurilshikov A, Wijmenga C, Fu J, Zhernakova A. Host genetics and gut microbiome: challenges and perspectives. *Trends Immunol*. 2017;38:633–647.
12 Bonder MJ, Kurilshikov A, Tigchelaar EF, et al. The effect of host genetics on the gut microbiome. *Nat Genet*. 2016;48:1407–1412.
13 Turpin W, Espin-Garcia O, Xu W, et al. Association of host genome with intestinal microbial composition in a large healthy cohort. *Nat Genet*. 2016;48:1413–1417.
14 Wang J, Thingholm LB, Skiecevičienė J, et al. Genome-wide association analysis identifies variation in vitamin D receptor and other host factors influencing the gut microbiota. *Nat Genet*. 2016;48:1396–1406.
15 Weissbrod O, Rothschild D, Barkan E, Segal E. Host genetics and microbiome associations through the lens of genome wide association studies. *Curr Opin Microbiol*. 2018;44:9–19.
16 Polderman TJC, Benyamin B, de Leeuw CA, et al. Meta-analysis of the heritability of human traits based on fifty years of twin studies. *Nat Genet*. 2015;47:702–709.
17 Turnbaugh PJ, Hamady M, Yatsunenko T, et al. A core gut microbiome in obese and lean twins. *Nature*. 2009;457:480–484.
18 Goodrich JK, Davenport ER, Beaumont M, et al. Genetic Determinants of the Gut Microbiome in UK Twins. *Cell Host Microbe*. 2016;19:731–743.
19 Goodrich JK, Waters JL, Poole AC, et al. Human genetics shape the gut microbiome. *Cell*. 2014;159:789–799.
20 Gomez A, Espinoza JL, Harkins DM, et al. Host genetic control of the oral microbiome in health and disease. *Cell Host Microbe*. 2017;22:269–278. e3.
21 Schulz C, Schütte K, Koch N, et al. The active bacterial assemblages of the upper GI tract in individuals with and without Helicobacter infection. *Gut*. 2018;67:216–225.
22 Boehm ET, Thon C, Kupcinskas J, et al. Fusobacterium nucleatum is associated with worse prognosis in Lauren's diffuse type gastric cancer patients. *Sci Rep*. 2020;10:16240.
23 Camarinha-Silva A, Jáuregui R, Chaves-Moreno D, et al. Comparing the anterior nare bacterial community of two discrete human populations using Illumina amplicon sequencing. *Environ Microbiol*. 2014;16:2939–2952.
24 Chaves-Moreno D, Plumeier I, Kahl S, et al. The microbial community structure of the cotton rat nose. *Environ Microbiol Rep*. 2015;7:929–935.
25 Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods*. 2016;13:581–583.
26 McMurdie PJ, Holmes S. Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*. 2013;8:e61217.
27 Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol*. 2007;73:5261–5267.
28 Letunic I, Bork P. Interactive tree of life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res*. 2019;47:W256–W259.
29 Anderson MJ. *GRN & CKR. PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods*. Plymouth, UK.: PRIMER-E; 2008.
30 Vasapolli R, Schütte K, Schulz C, et al. Analysis of transcriptionally active bacteria throughout the gastrointestinal tract of healthy individuals. *Gastroenterology*. 2019;157:1081–1092. e3.
31 Freire M, Moustafa A, Harkins DM, et al. Longitudinal study of oral microbiome variation in twins. *Sci Rep*. 2020;10:7954.
32 Wu H, Zeng B, Li B, et al. Research on oral microbiota of monozygotic twins with discordant caries experience - *in vitro* and *in vivo* study. *Sci Rep*. 2018;8:7267.
33 Mukherjee C, Moyer CO, Steinkamp HM, et al. Acquisition of oral microbiota is driven by environment, not host genetics. *Microbiome*. 2021;9:54.
34 Rajilic-Stojanovic M, Figueiredo C, Smet A, et al. Systematic review: gastric microbiota in health and disease. *Aliment Pharmacol Ther*. 2020;51:582–602.
35 Kupcinskas J, Hold GL. Other Helicobacters and the gastric microbiome. *Helicobacter*. 2018;23(Suppl 1):e12521.
36 Spiegelhauer MR, Kupcinskas J, Johannesen TB, et al. Transient and persistent gastric microbiome: adherence of bacteria in gastric

cancer and dyspeptic patient biopsies after washing. *J Clin Med.* 2020;9:1–23.
37 Dong Q, Xin Y, Wang L, et al. Characterization of gastric microbiota in twins. *Curr Microbiol.* 2017;74:224–229.
38 Malaty HM, Graham DY, Isaksson I, Engstrand L, Pedersen NL. Are genetic influences on peptic ulcer dependent or independent of genetic influences for Helicobacter pylori infection? *Arch Intern Med.* 2000;160:105–109.
39 Xie H, Guo R, Zhong H, et al. Shotgun metagenomics of 250 adult twins reveals genetic and environmental impacts on the gut microbiome. *Cell Syst.* 2016;3:572–584. e3.
40 Bowyer R, Jackson M, Le Roy C, et al. Socioeconomic status and the gut microbiome: a TwinsUK cohort study. *Microorganisms.* 2019;7:17.
41 David LA, Materna AC, Friedman J, et al. Host lifestyle affects human microbiota on daily timescales. *Genome Biol.* 2014;15:1–15.
42 Koo H, Hakim JA, Crossman DK, Lefkowitz EJ, Morrow CD. Sharing of gut microbial strains between selected individual sets of twins cohabitating for decades. *PLoS One.* 2019;14.
43 Adebayo AS, Ackermann G, Bowyer RCE, et al. The urinary tract microbiome in older women exhibits host genetic and environmental influences. *Cell Host Microbe.* 2020;28:298–305. e3.
44 Moreno-Gallego JL, Chou SP, Di Rienzi SC, et al. Virome diversity correlates with intestinal microbiome diversity in adult monozygotic twins. *Cell Host Microbe.* 2019;25:261–272. e5.

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Research Article

Gastritis Stages in Monozygotic and Dizygotic Dyspeptic Twins

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Background. The progression of *Helicobacter pylori*-associated gastritis towards atrophic gastritis is modulated by host-related and environmental factors. Studies that explore the possible involvement of host-related versus environmental factors in the development of gastritis phenotype induced by *H. pylori* are highly needed. *Aims*. Our study was aimed at investigating the phenotype of *H. pylori*-associated gastritis in two cohorts of monozygotic and dizygotic twins, using the OLGA/OLGIM gastritis staging system. *Methods*. Two cohorts of monozygotic (14 pairs) and dizygotic (15 pairs) dyspeptic twins prospectively underwent endoscopy with biopsy sampling based on Sydney protocol. *H. pylori* status and OLGA/OLGIM stages were assessed and compared. *Results*. The mean age of monozygotic and dizygotic twins was 40.4 and 38.6 years, respectively ($p = 0.623$). The overall prevalence of *H. pylori* infection was 51.7%. Among the 14 monozygotic twin pairs, five pairs were *H. pylori*-positive, four were *H. pylori*-negative, and five were *H. pylori*-discordant. Among the 15 dizygotic twin pairs, five pairs were *H. pylori*-positive, five were *H. pylori*-negative, and five were *H. pylori*-discordant. Concordance for antrum atrophy in monozygotic twins was 78.6% (11/14 pairs) and in dizygotic twins 73.3% (11/15 pairs) ($p = 0.742$). Concordance for corpus atrophy in monozygotic versus dizygotic twins was 92.9% (13/14 pairs) and 86.7% (13/15 pairs), respectively ($p = 0.584$). Concordance for antrum intestinal metaplasia (IM) in monozygotic twins was 85.7% (12/14 pairs) and in dizygotic 73.3% (11/15 pairs) ($p = 0.411$). Concordance for corpus IM in monozygotic twins was 85.7% (12/14 pairs) and in dizygotic 86.7% (13/15 pairs) ($p = 0.941$). Among monozygotic and dizygotic subjects, the stage of gastritis was concordant in both *H. pylori*-positive and *H. pylori*-negative subjects. *Conclusions*. In conclusion, histological gastric mucosa alterations in monozygotic and dizygotic twins showed high rates of concordance. Furthermore, OLGA/OLGIM gastritis stages were not modulated by the zygosity of the twins.

1. Introduction

Gastric carcinogenesis is a multistep process, including a stepwise sequence of phenotypic modifications of the native gastric tissue from healthy gastric mucosa towards atrophic gastritis (AG), intestinal metaplasia (IM), and gastric cancer (GC) [1]. Within this spectrum of lesions, gastric mucosa atrophy is considered the elective "field of cancerization" prone to GC development [1]. *Helicobacter pylori* (*H. pylori*) is by far the most common etiological agent of gastric atrophy, and, all over the world, the prevalence of the *H. pylori* infection is consistently linked with both, AG and GC [1–3].

In some peculiar epidemiological contexts, however, a high prevalence of bacterial infection is associated with a low prevalence of gastric precancerous/cancer lesions, or equivalent rates of bacterial infection are associated with significantly different risk of gastric malignancy [4, 5]. These unexpected findings would support the hypothesis that etiological factors, other than *H. pylori*, may be involved in the modulation of the oncogenetic "cascade." Among these

factors, both host-related (genetic variations, noncoding RNAs, methylation, etc) and/or other environmental factors have been considered [6–14].

To explore the potential host-related factors in the pathogenesis of gastric atrophy, this study compares two populations of dyspeptic monozygotic and dizygotic Lithuanian twins. This is the first study in the field that comprehensively evaluates gastric mucosa alterations in twins.

2. Materials and Methods

2.1. Study Population. Two cohorts of twins were selected from the Twin Registry Center at Lithuanian University of Health Sciences (years 2016-2018). The twins were interviewed by phone calls, and those that reported clinical symptoms of dyspepsia were referred for upper gastrointestinal endoscopy. Zygosity of twins (mono- versus dizygosity) was confirmed by automated analysis of DNA microsatellite markers. The study included 29 twin pairs (58 subjects) older than 18 years: 14 pairs of monozygotic and 15 pairs (10 same-sex pairs and 5 mixed-sex pairs) of dizygotic twins. None of the considered patients had previously undergone anti-*H. pylori* treatment. All study participants did not use PPIs or antibiotics at least one month prior to inclusion.

2.2. Information on Approval of the Local Ethical Committee and Informed Consent. The study protocol was approved by the Ethical Committee of Lithuanian University of Health Sciences (BE-2-10). Informed consent was given for all patients before endoscopy.

2.3. Endoscopy and Protocol of Gastric Biopsy Sampling. All the endoscopic procedures were performed by the same trained gastroenterologist at the Department of Gastroenterology of Hospital of Lithuanian University of Health Sciences Kaunas Clinics in order to meet requirements for OLGA and OLGIM histological scoring [14–16]. In all patients, the biopsy protocol included 5 biopsy samples, according to the Sydney recommendations [17]. Biopsy specimens were fixed in formalin (10% solution), embedded in paraffin, and stained with hematoxylin and eosin and Giemsa stain for *H. pylori*. Gastric biopsy specimens were histologically assessed (Sydney score) by a trained pathologist, blinded to any clinical information. In all cases, both the OLGA and OLGIM staging systems were applied, according to the defined criteria [1, 18, 19].

2.4. Statistical Analysis. Statistical analysis was performed using IBM SPSS software (v 22.0). Age is presented as mean and median values, while age differences between the groups were evaluated comparing mean values using a T-test. The Pearson Chi-squared test to compare sample proportion was applied. The exact Pearson Chi-squared test was used to assess concordance of OLGA and OLGIM stages between monozygotic and dizygotic twins. $p < 0.05$ was considered significant.

3. Results

3.1. Characteristics of Subjects. Demographics and major clinical findings of twins included within the study are shown in Table 1. The mean and median age (years) of monozygotic and dizygotic twins was 40.3 (39.5) and 38.6 (36), respectively ($p = 0.623$).

3.2. H. pylori Status in Twins. The overall prevalence of *H. pylori* infection was 51.7%. Concordance rates for *H. pylori* infection prevalence among monozygotic and dizygotic twins was 35.7% and 33.3%, respectively ($p = 0.891$).

Among monozygotic twins, the *H. pylori* status (*Hp*-status) was as the following: five pairs were *Hp*-positive, four pairs were *Hp*-negative, and five pairs were *Hp*-discordant. Among dizygotic twins, five pairs were *Hp*-positive and five pairs were *Hp*-negative, and in five pairs, the *Hp*-status was discordant. Concordant *Hp*-status (both *Hp*-positive and *Hp*-negative) in monozygotic and dizygotic twins was determined in 9/14 (64.3%) and in 10/15 (66.7%) twin pairs, respectively ($p = 0.893$) (Table 1).

3.3. Histological Alterations of Gastric Mucosa in Twins. Concordance of topographical extension of atrophic lesions among monozygotic and dizygotic twins is shown in Table 2.

The distribution of monozygotic and dizygotic twins according to OLGA and OLGIM gastritis stages is shown in Table 3. The prevalence of low-risk OLGA and OLGIM stages among mono- and dizygotic twins was 100% and 93.3%, respectively, whereas the prevalence of high-risk stages was 0% and 6.7%, respectively. Concordance according to OLGA and OLGIM stages between monozygotic and dizygotic twins did not reach statistical significance ($p = 0.097$ and $p = 0.175$, respectively).

Tables 4 and 5 show the prevalence of similar or different gastritis stages (grouped in low versus high risk) in monozygotic and dizygotic twins distinguished by their *Hp*-status (*Hp*-positive pair, *Hp*-negative-pair, and *Hp*-discordant pair). Low-risk stages (stages 0-I-II) largely prevailed among both mono- and dizygotic twins (only two cases featured a high-risk stage (stage III, by OLGA and OLGIM). By applying both OLGA and OLGIM staging, no differences emerged in the distribution of twins by stage after performing a comparison of low- versus high-risk stages.

4. Discussion

Our study has investigated the phenotype of *H. pylori*-associated gastritis in two cohorts of monozygotic (14 pairs) and dizygotic (15 pairs) twins. The results of our study revealed high concordance rates of gastric mucosal alterations both in monozygotic and dizygotic twins. To our best knowledge, to date, there have been no previous reports, which have assessed the importance of shared genetic influences on susceptibility and phenotype of chronic *H. pylori* gastritis and premalignant gastric alterations on a well-defined twin cohort.

Epidemiological contexts, case-controls, and twins studies have addressed the issue of the possible interaction between different host-related and environmental risk factors

Table 1: Demographics and *H. pylori* status distinguishing monozygotic versus dizygotic twins.

Twin pairs	Monozygotic n (14)			Dizygotic n (15)			p value
Gender of twins within a pair	MM n (3)	FF n (11)	MF —	MM n (1)	FF n (9)	MF n (5)	
Mean (median) age	40.3 (39.5)			38.6 (36)			0.623
Concordant *Hp*-positive status	5			5			
Concordant *Hp*-negative status	4			5			0.893
Discordant *Hp*-negative status	5			5			

MM: both twins of male gender; FF: both twins of female gender; MF: twin pair comprised of a male and a female twin.

Table 2: Concordance of topographical extension of atrophy and IM in monozygotic and dizygotic twins.

Twin pairs	Monozygotic		Dizygotic		p value
	n (14)	%	n (15)	%	
Concordance according to antrum athrophy	11	78.6%	11	73.3%	0.742
Concordance according to corpus athrophy	13	92.9%	13	86.7%	0.584
Concordance according to antrum IM	12	85.7%	11	73.3%	0.411
Concordance according to corpus IM	12	85.7%	13	86.7%	0.941

IM: intestinal metaplasia.

Table 3: Gastritis stage (OLGA and OLGIM systems) in monozygotic and dizygotic twins.

Stage	OLGA staging				p value	OLGIM staging				p value
	Monozygotic twins		Dizygotic twins			Monozygotic twins		Dizygotic twins		
	n (28)	%	n (30)	%		n (28)	%	n (30)	%	
0	18	64.3%	24	80%		19	67.9%	24	80%	
I	9	32.1%	3	10%		8	28.6%	3	10%	
II	1	3.6%	1	3.3%	0.097	1	3.6%	1	3.3%	0.175
III	—	—	2	6.7%		—	—	2	6.7%	
IV	—	—	—	—		—	—	—	—	

OLGA: Operative Link on Gastritis Assessment; OLGIM: Operative Link on Gastritis/Intestinal-Metaplasia Assessment.

in the promotion of gastric precancerous lesions and gastric mucosal atrophy. These factors have been extensively studied among the patients with precancerous gastric lesions and included virulence factors of *H. pylori* strains, environmental factors (smoking, salted food, etc.), or genetic predisposition of the host [20, 21]. To date, however, the exact factors that trigger malignant transformation from normal gastric mucosa towards gastric cancer are not completely understood.

A previous study conducted in Sweden involving monozygotic (36 pairs) and dizygotic (88 pairs) twins reared apart revealed that the concordance rate for *H. pylori* infection was significantly higher among monozygotic twins (82%) than among dizygotic (66%) twins [22]. Slightly higher concordance rates for *H. pylori* infection prevalence among monozygotic (35.7%) versus dizygotic (33.3%) have also been observed in the present study. The difference, however, did not reach statistical significance, probably due to a small number of twin pairs. Based on case-control studies in different geographical regions, which evaluate the prevalence of *H. pylori* infection and the development of gastric atrophy and IM, first-degree relatives of GC patients are associated with an increased risk for GC. A Marcos-Pinto et al. [21] study showed that first-degree relatives of early-onset gastric carcinoma patients have significantly higher prevalence of *H. pylori*, AG, and advanced stages of OLGA. *H. pylori* was present in 82% of cases and in 59% of controls ($p = 0.001$); AG was diagnosed in 70% of cases and in 32% of control individuals, respectively ($p < 0.001$), while OLGA stages III and IV were present in 10% and 9% in both groups, respectively ($p < 0.001$). The results of Rokkas et al. [23] meta-analysis showed similar results: their pooled OR of *H. pylori* infection, AG, and IM between individuals with family history of GC were 1.92-fold ($p \leq 0.001$), 2.2-fold ($p = 0.005$), and 1.98-fold ($p \leq 0.001$) higher, respectively, in comparison with controls. These results can be influenced by the exposition of shared environmental factors (alcohol, smoking, salted and smoked food, and hygiene) between family members, circulation of intrafamilial *H. pylori* strains [24–26], and genetic susceptibility [27–29].

Our study showed that there was no difference in concordance rates for chronic *H. pylori* gastritis among monozygotic twins (64.3%, 9/14 pairs) as compared to dizygotic twins 66.7% (10/15 pairs) ($p = 0.893$). This could partly relate to the fact that twins share the same environment in early childhood and genetic predisposition for *H. pylori*

Table 4: Gastritis OLGA-stage according to the *H. pylori* status: twins are distinguished according to zygosity.

Group of stages	Twin pair *Hp*-positive (10 pairs)		Twin pair *Hp*-negative (9 pairs)		Twin pair *Hp*-discordant (10 pairs)	
	Monozygotic	Dizygotic	Monozygotic	Dizygotic	Monozygotic	Dizygotic
Similar low-risk stage (0-I-II)	5	4	4	5	5	4
Similar high-risk stage (III-IV)	—	—	—	—	—	—
Discordant stage (0-I-II versus III-IV)	—	1	—	—	—	1

OLGA: Operative Link on Gastritis Assessment; *Hp*: *H. pylori*.

Table 5: Gastritis OLGIM-stage according to the *H. pylori* status distinguished by zygosity.

Group of stages	Twin pair *Hp*-positive (10 pairs)		Twin pair *Hp*-negative (9 pairs)		Twin pair *Hp*-discordant (10 pairs)	
	Monozygotic	Dizygotic	Monozygotic	Dizygotic	Monozygotic	Dizygotic
Similar low-risk stage (0-I-II)	5	4	4	5	5	4
Similar high-risk stage (III-IV)	—	—	—	—	—	—
Discordant stage (0-I-II versus III-IV)	—	1	—	—	—	1

OLGIM: Operative Link on Gastritis/Intestinal-Metaplasia Assessment; *Hp*: *H. pylori*.

acquisition might be less important. Concordance of topographical extension of atrophy and IM was higher among monozygotic twins but did not reach statistical significance. There were no statistical differences in concordance rates according to OLGA and OLGIM stages between monozygotic and dizygotic twins ($p = 0.097$ and 0.175, respectively). It is very important to point out that 11 of 14 monozygotic twin pairs and 10 out of 15 dizygotic twins had absolute concordance for OLGA stages. Similarly, 10 out of 14 monozygotic twin pairs and 10 out of 15 dizygotic twins had absolute concordance for OLGIM stages. These findings suggest that if one twin is identified with high-risk premalignant gastric lesions, the other twin should also be assessed for these alterations.

A relatively small number of the study population represent the main limitation of the present study. The numbers of individual twins within our study group were not very large, and certain effects might have been biased. The design of the study also did not allow us to evaluate as to what extent gastric mucosal alterations in twins are related to the common shared genetic factors per se or shared environmental factors during childhood and later on in lifetime. There might also have been a certain selection bias, because our study included only twins with dyspeptic symptoms.

5. Conclusions

In conclusion, histological gastric mucosa alterations in monozygotic and dizygotic twins showed high rates of concordance. Furthermore, OLGA and OLGIM gastritis stages were not modulated by the zygosity of the twins.

Data Availability

The authors can make research data available on request after institutional review board approval through contacting the corresponding author by juozas.kupcinskas@lsmuni.lt

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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References

[1] R. M. Genta and M. Rugge, "Gastric precancerous lesions: heading for an international consensus," *Gut*, vol. 45, Supplement 1, pp. 15–18, 1999.

[2] F. Farinati, R. Cardin, V. M. Russo, G. Busatto, M. Franco, and M. Rugge, "Helicobacter pylori CagA status, mucosal oxidative damage and gastritis phenotype: a potential pathway to cancer?," *Helicobacter*, vol. 8, no. 3, pp. 227–234, 2003.

[3] H. R. Ghasemi Basir, M. Ghobakhlou, P. Akbari, A. Dehghan, and M. A. Seif Rabiei, "Correlation between the intensity of Helicobacter pylori colonization and severity of gastritis," *Gastroenterology Research and Practice*, vol. 2017, 5 pages, 2017.

[4] V. V. Tsukanov, N. N. Butorin, A. S. Maady et al., "Helicobacter pylori infection, intestinal metaplasia, and gastric cancer risk in Eastern Siberia," *Helicobacter*, vol. 16, no. 2, pp. 107–112, 2011.

[5] V. V. Tsukanov, E. V. Kasparov, J. L. Tonkikh et al., "Peptic ulcer disease and Helicobacter pylori infection in different Siberian ethnicities," *Helicobacter*, vol. 22, no. 1, 2017.

[6] F. Petersson, K. Borch, and L. E. Franzén, "Prevalence of subtypes of intestinal metaplasia in the general population and in patients with autoimmune chronic atrophic gastritis," *Scandinavian Journal of Gastroenterology*, vol. 37, no. 3, pp. 262–266, 2009.

[7] S. K. M. Heinrichs, T. Hess, J. Becker et al., "Evidence for PTGER4, PSCA, and MBOAT7 as risk genes for gastric cancer

on the genome and transcriptome level," *Cancer Medicine*, vol. 7, no. 10, pp. 5057–5065, 2018.

[8] L. Kupcinskas, T. Wex, J. Kupcinskas et al., "Interleukin-1B and interleukin-1 receptor antagonist gene polymorphisms are not associated with premalignant gastric conditions: a combined haplotype analysis," *European Journal of Gastroenterology and Hepatology*, vol. 22, no. 10, pp. 1189–1195, 2010.

[9] V. Petkevicius, V. Salteniene, S. Juzenas et al., "Polymorphisms of microRNA target genesIL12B,INSR,CCND1andIL10in gastric cancer," *World Journal of Gastroenterology*, vol. 23, no. 19, pp. 3480–3487, 2017.

[10] A. Link and J. Kupcinskas, "MicroRNAs as non-invasive diagnostic biomarkers for gastric cancer: current insights and future perspectives," *World Journal of Gastroenterology*, vol. 24, no. 30, pp. 3313–3329, 2018.

[11] D. B. Polk and R. M. Peek, "_Helicobacter pylori_ : gastric cancer and beyond," *Nature Reviews. Cancer*, vol. 10, no. 6, pp. 403–414, 2010.

[12] M. Amieva and R. M. Peek, "Pathobiology of _Helicobacter pylori_ -induced gastric cancer," *Gastroenterology*, vol. 150, no. 1, pp. 64–78, 2016.

[13] K. Abdeljawad, A. Wehbeh, and E. Qayed, "Low prevalence of clinically significant endoscopic findings in outpatients with dyspepsia," *Gastroenterology Research and Practice*, vol. 2017, 7 pages, 2017.

[14] M. Rugge, K. Sugano, C. Scarpignato, D. Sacchi, W. J. Oblitas, and A. G. Naccarato, "Gastric cancer prevention targeted on risk assessment: gastritis OLGA staging," *Helicobacter*, vol. 24, no. 2, article e12571, 2019.

[15] M. Rugge, R. M. Genta, M. Fassan et al., "OLGA gastritis staging for the prediction of gastric cancer risk: a long-term follow-up study of 7436 patients," *American Journal of Gastroenterology*, vol. 113, no. 11, pp. 1621–1628, 2018.

[16] M. Rugge, A. Meggio, C. Pravadelli et al., "Gastritis staging in the endoscopic follow-up for the secondary prevention of gastric cancer: a 5-year prospective study of 1755 patients," *Gut*, vol. 68, no. 1, pp. 11–17, 2018.

[17] M. F. Dixon, R. M. Genta, J. H. Yardley, and P. Correa, "Classification and grading of gastritis," *American Journal of Surgical Pathology*, vol. 20, no. 10, pp. 1161–1181, 1996.

[18] M. Rugge, R. M. Genta, D. Y. Graham et al., "Chronicles of a cancer foretold: 35 years of gastric cancer risk assessment," *Gut*, vol. 65, no. 5, pp. 721–725, 2016.

[19] M. Rugge, G. Pennelli, E. Pilozzi et al., "Gastritis: the histology report," *Digestive and Liver Disease*, vol. 43, SUPPLEMENT 4, pp. S373–S384, 2011.

[20] W. J. den Hollander, I. L. Holster, C. M. den Hoed et al., "Surveillance of premalignant gastric lesions: a multicentre prospective cohort study from low incidence regions," *Gut*, vol. 68, no. 4, pp. 585–593, 2019.

[21] R. Marcos-Pinto, F. Carneiro, M. Dinis-Ribeiro et al., "First-degree relatives of patients with early-onset gastric carcinoma show even at young ages a high prevalence of advanced OLGA/OLGIM stages and dysplasia," *Alimentary Pharmacology and Therapeutics*, vol. 35, no. 12, pp. 1451–1459, 2012.

[22] H. M. Malaty, D. Y. Graham, I. Isaksson, L. Engstrand, and N. L. Pedersen, "Are genetic influences on peptic ulcer dependent or independent of genetic influences for Helicobacter pylori infection?," *Archives of Internal Medicine*, vol. 160, no. 1, pp. 105–109, 2000.

[23] T. Rokkas, P. Sechopoulos, D. Pistiolas, G. Margantinis, and G. Koukoulis, "Helicobacter pylori infection and gastric histology in first-degree relatives of gastric cancer patients: a meta-analysis," *European Journal of Gastroenterology and Hepatology*, vol. 22, no. 9, pp. 1128–1133, 2010.

[24] J. Raymond, J.-M. Thiberge, C. Chevalier et al., "Genetic and transmission analysis of Helicobacter pylori strains within a family," *Emerging Infectious Diseases*, vol. 10, no. 10, pp. 1816–1821, 2004.

[25] M. Kivi, Y. Tindberg, M. Sorberg et al., "Concordance of Helicobacter pylori strains within families," *Journal of Clinical Microbiology*, vol. 41, no. 12, pp. 5604–5608, 2003.

[26] M. Konno, N. Fujii, S.-i. Yokota et al., "Five-year follow-up study of mother-to-child transmission of Helicobacter pylori infection detected by a random amplified polymorphic DNA fingerprinting method," *Journal of Clinical Microbiology*, vol. 43, no. 5, pp. 2246–2250, 2005.

[27] F. Li, C. Huang, Q. Li, and X. Wu, "Construction and comprehensive analysis for dysregulated long non-coding RNA (lncRNA)-associated competing endogenous RNA (ceRNA) network in gastric cancer," *Medical Science Monitor*, vol. 24, pp. 37–49, 2018.

[28] X. Cui, Q. Huang, X. Li et al., "Relationship between interleukin-10 gene C-819T polymorphism and gastric cancer risk: insights from a meta-analysis," *Medical Science Monitor*, vol. 22, pp. 2839–2845, 2016.

[29] B. Chen, M. X. Luo, X. Zhou, Y. Lv, and G. Q. Su, "Correlation between interleukin-1β-511 C/T polymorphism and gastric cancer in Chinese populations: a meta-analysis," *Medical Science Monitor*, vol. 22, pp. 1742–1750, 2016.

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