

Microbiological Survey of the Human Gastric Ecosystem Using Culturing and Pyrosequencing Methods

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Abstract Stomach mucosa biopsies and gastric juices samples of 12 healthy persons were analysed by culturing in selective- and non-selective-rich media. Microbial DNA from four mucosal samples was also amplified by nested PCR using universal bacterial primers, and the 16S rDNA amplicons pyrosequenced. The total number of cultivable microorganisms recovered from the samples ranged from 10^2 to 10^4 cfu/g or ml. The isolates were identified at the species level by PCR amplification and sequencing of the 16S rDNA. Isolates belonged mainly to four genera; *Propionibacterium*, *Lactobacillus*, *Streptococcus* and *Staphylococcus*. A total of 15,622 high-quality 16S rDNA sequence reads were obtained by pyrosequencing from the four mucosal samples. Sequence analysis grouped the reads into 59 families and 69 genera, revealing wide bacterial diversity. Considerable differences in the composition of the gastric

microbiota were observed among the subjects, although in all samples the most abundant operational taxonomic units belonged to *Streptococcus*, *Propionibacterium* and *Lactobacillus*. Comparison of the stomach microbiota with that present in other parts of the human gastrointestinal tract revealed distinctive microbial communities. This is the first study in which a combination of culture and culture-independent techniques has been used to explore the bacterial diversity of the human stomach.

Introduction

Until recently, the human stomach was considered a sterile organ, given its strongly acidic environment and the presence of digestive enzymes. Indeed, the discovery of *Helicobacter pylori* in this ecosystem [1] came as a surprise. *H. pylori*, which is frequently associated with gastritis and peptic ulcers, possesses a biochemical machinery that allows it to thrive under the harsh conditions it must face [2]. Its finding led to the idea that other microbial communities might also be adapted and inhabit the stomach.

Characterisation of the gastric microbiota has traditionally relied on the use of culturing techniques, although recent papers involving them are scarce. These procedures led to several members of the phyla *Firmicutes*, *Proteobacteria*, *Actinobacteria* and *Fusobacteria* [3] being identified. In recent years, culture-independent techniques have broadened the known bacterial diversity of the human stomach. Among these, temporal temperature gradient gel electrophoresis (TTGE) profiling suggested the presence of *Enterococcus*, *Pseudomonas*, *Streptococcus*, *Staphylococcus* and *Stomacoccus* [4]. Ensuing work involving the construction and sequence analysis of 16S rDNA libraries identified 128 phylotypes belonging to eight phyla in the gastric mucosa of 23 North Americans [5]. More recently, next-generation

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sequencing (NGS) approaches [6, 7] detected 130–260 phylotypes from up to 13 phyla; the majority were uncultured bacteria, while many others have not yet been described as members of the gastric microbiota with cultivation-based analyses. *Proteobacteria* (to which *H. pylori* belongs) seems to be the best represented phylum in the normal gastric microbiome, quickly followed by *Firmicutes*, and then by *Bacteroidetes*, *Actinobacteria* and *Fusobacteria* [5–7]. Apart from *Helicobacter*, the genera *Haemophilus*, *Actinobacillus* and *Neisseria* contribute to the dominance of *Proteobacteria*. *Firmicutes* is mainly composed of species from the genera *Streptococcus* and *Bacillus*, while *Prevotella* species are the majority among *Bacteroidetes*, and *Rothia*, *Actinomyces* and *Micrococcus* species are the dominant *Actinobacteria* [5–7].

However, detection of 16S rDNA sequences does not imply that live bacteria are present. For this reason, culturing and culture-independent techniques can provide complementary results allowing a better description of the microbial components of the gastric environment [7, 8]. Moreover, the culturing of gastric microorganisms allows the isolation of strains adapted to this niche that might prove useful as probiotics for counteracting gastric pathologies of microbial origin [9].

The aim of the present work was, therefore, to identify and isolate the bacteria present in the healthy human stomach via a combination of classical culturing, in selective and non-selective-rich media, and pyrosequencing of stomach-derived DNA amplicons. Results of the culturing and culture-independent methods were then compared among themselves and to those in the recent literature.

Material and Methods

This study was approved by the Ethics Committee for Clinical Research of the *Principado de Asturias*.

Sample Collection and Processing

Gastric biopsies and stomach juices were collected from 12 subjects undergoing routine upper gastrointestinal endoscopy for dyspepsia. The participants (six males and six females; mean age, 60.5 years) were examined at the Digestive Unit of Cabueñes Hospital (Asturias, Spain). They had not been treated with any proton-pump inhibitor drugs, antacids, H₂-antagonists or antibiotics during 30 days previous to the exploration. After the gastroscopy and the ultrasonography were performed, ulceration, malignancy or other gastric pathology was ruled out in all the patients. Subjects were also determined to be *H. pylori* negative by a commercial urease test (GOLD Hp dry, Lencomm, Poland). No co-morbidities were encountered, and the routine analytic

tests reflected normal hemograms in all of them. They also reported normal diet. Prior to sampling, they followed a fasting period of 12 h. All biopsies were recovered from the gastric antrum section using an Olympus GIF-Q0165 gastroscope (Olympus Corporation, Tokyo, Japan) and a large (8 Fr) single-use, sterile biopsy forceps with a central bayonet (Boston Scientific Corporation, Natick, MA). Before exploration, the gastroscope was washed and disinfected by immersion in a detergent solution with proteolytic enzymes (at 7 %) and glutaraldehyde (at 2 %). Samples were collected in sterile containers, transported to the laboratory in a reduced sterile solution (0.9 % NaCl, 0.1 % peptone, 0.1 % Tween 80® and 0.02 % cysteine) and processed within 2 h of sampling.

Culturing and Identification of Isolates

Biopsy material was washed several times in phosphate-buffered saline (PBS), drained, weighed and homogenised in Maximum Recovering Diluent (Scharlab, Barcelona, Spain). Dilutions of the samples were spread on the surface of agar plates containing the following media: MRSC (de Man, Rogosa and Sharpe supplemented with 0.25 % cysteine; both MRS and cysteine were purchased from Merck (Darmstadt, Germany)) for the recovery of *Lactobacillales*, and CB (Columbia Blood, containing 5 % defibrinated horse blood (Merck)) and Brain Heart Infusion (BHI; Oxoid, Basingstoke, Hampshire) as non-selective-rich media. Dilutions, inoculations and incubations (37 °C for up to 5 days) were all performed in an anaerobic chamber (Mac500, Down Whitley Scientific, West Yorkshire, UK) containing an anoxic atmosphere (10 % H₂, 10 % CO₂ and 80 % N₂). In order to check for potential contamination, effluents of the gastroscope, transport and diluent solutions, as well as media used were randomly incubated and served as negative controls for bacterial growth.

Representative colonies of all morphotypes and sizes recovered in the different agar plates were selected, purified by subculturing on the same medium, and stored at –80 °C until identification. Total genomic DNA from the recovered isolates was purified from overnight cultures using the GenElute™ Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO) following the manufacturer's recommendations. The isolates were then identified at the species level by a combination of amplified ribosomal DNA restriction analysis (ARDRA), plus sequencing of the 16S rDNA amplicons. ARDRA was accomplished after amplification of the full 16S rDNA with the universal primers 27F (5'-AGAGTTT GATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGT TACGACTT-3') using a Taq-DNA polymerase master mix (75 mM Tris-HCl at pH 8.5, 20 mM (NH₄)₂S₀₄, 1.5 mM MgCl₂, 0.1 % Tween 20®, 0.2 mM of each dNTP and 1.25 units Taq polymerase (Ampliqon ApS, Skovlunde, Denmark))

and 0.2 mM of each primer. The PCR conditions were as follows: one cycle at 95 °C for 5 min, 35 cycles at 94 °C for 30 s, 50 °C for 45 s and 72 °C for 2 min and a final extension step at 72 °C for 10 min. Amplicons were purified using GenElute™ PCR Clean-Up columns (Sigma-Aldrich), digested with the restriction enzymes *Hae*III and *Hinf*I (Invitrogen Ltd., Paisley, UK) and electrophoresed in 2 % agarose gels. Bands were visualised under UV light after staining with ethidium bromide (0.5 µg/ml). The gels were scanned and analysed with a molecular analysis fingerprinting software (Quantity One, Bio-Rad, Richmond, CA). At least two representative amplicons of the different profiles encountered were sequenced by cycle extension in an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA) and the sequences (an average of 800 bp obtained) were compared with those in the GenBank database using the BLASTN program (<http://www.ncbi.nlm.nih.gov/BLAST/>), and to those held by the Ribosomal Database Project (RDP) (<http://rdp.cme.msu.edu/index.jsp>). The identity of the isolates was determined on the basis of the highest scores. Sequences with a percentage similarity of 97 % or higher were assigned to species level. The partial sequences of the 16S rRNA gene of the different species were deposited in the EMBL database under accession numbers HF564643 through HF564655.

Culture-Independent Sequence-Based Analysis (Pyrosequencing)

Four mucosal samples were selected among those positive for bacterial growth, and their bacterial diversity established by nested PCR amplification of the 16S rDNA with universal primers and pyrosequencing of derived amplicons. A nested PCR strategy, with a reduced number of cycles to avoid preferential amplification, was chosen in an attempt to enhance PCR sensitivity and overcome the problem of low yield of bacterial DNA in gastric biopsies.

Total Microbial DNA Extraction

Total microbial DNA extraction from the gastric biopsies was accomplished according to the optimised protocol described by Zoetendal et al. [10] modified with an additional enzymatic lysis step, as follows. Tenfold dilutions of mucosal homogenates were mixed thoroughly with glass beads and centrifuged (2,000×g, 2 min) to remove disrupting tissue debris. Microbial cells from the supernatant were lysed initially by enzymatic treatment consisting on an incubation at 37 °C for 60 min with lysozyme (30 mg/ml) and mutanolysin (20 units; Sigma-Aldrich), followed by treatment with SDS (10 %) and Proteinase K (20 mg/ml) for 60 min at 55 °C. DNA was finally purified by phenol/chloroform extractions as previously reported [10].

Primers and 16S rRNA Gene Amplification Conditions

The 16S rRNA genes were first amplified using the universal primers 27F and 1492R. Amplifications were performed as described above but reducing the number of cycles to 25. A second PCR run using the universal primers Y1 (5'-TGG CTC AGG ACG AAC GCT GGC GGC-3') (position 20–43 on 16S rRNA gene of *Escherichia coli*) and Y2 (5'-CCT ACT GCT GCC TCC CGT AGG AGT-3'; positions 361–338) was then performed to amplify a 348-bp stretch of DNA encompassing the variable V1 and V2 regions of the 16S rDNA. Specific 454 adaptors were included in both forward (5'-CGTATCGCCTCCCTCGCGCCATCAG-3') and reverse (5'-CTATGCGCCTTGCCAGCCCCGCTCAG-3') primers, followed by 10 bp sample-specific barcode sequences (5'-ACGAGTGCGT-3', 5'-ACGCTCGACA-3', 5'-AGACGCACTC-3' and 5'-AGCACTGTAG-3', respectively). One microlitre of the amplicons from the first PCR run was used as a template. The second amplification program consisted of an initial step at 95 °C for 5 min, followed by 20 cycles at 94 °C for 30 s, 52 °C for 40 s, 72 °C for 30 s and a final step at 72 °C for 10 min. No-template controls, consisting of the PCR master mix and sterile molecular biology-grade water (Sigma), were included in the consecutive PCR reactions.

Amplicons from both template and no-template control reactions were purified through GenElute™ columns. DNA concentration and quality was initially evaluated by visual inspection on agarose gels and quantified thereafter using an Epoch microvolume spectrophotometer (BioTek Instruments, Winooski, VT). Unspecific amplifications in the no-template controls were discarded. Equal amounts of DNA from the amplifications from the four mucosal samples were pooled together at a concentration of 100 ng in 100 µl. Pooled DNA was subsequently amplified by emulsion PCR using reagents and protocols of the supplier (Roche, Basel, Switzerland) and sequenced in different lanes of a PicoTiterPlate using a 454 Genome Sequencer 20 (Roche).

Sequences Treatment and Bioinformatic Analysis

Raw sequences were processed through the RDP pyrosequencing pipeline (<http://wildpigeon.cme.msu.edu/pyro/index.jsp>). Sequences were excluded from the analysis if they had low quality (mean quality value of <20), differences in the primer or more than four ambiguities in homopolymeric regions. Chimeric sequences were filtered out using the software Ballerophon [11]. Only reads longer than 200 bp were considered, as it has been shown that taxonomic assignment accuracy decreases dramatically in reads shorter than 200 bp, and the use of short reads inflates rarefaction curves [12]. To obtain the rarefaction curves,

sequences were clustered at 97 % sequence identity employing CD-HIT software [13]; the resulting clusters were then used to generate a predictive rarefaction model using Analytic Rarefaction 1.3 software [14]. High-quality partial 16S rDNA sequences were subjected to the RDP-II classifier using an 80 % confidence threshold to obtain the taxonomic assignment and relative abundances of the different bacterial groups, as reported elsewhere [15]. Data on assigned sequences at the genus level shared between samples were used to generate a Venn diagram. Barcodes were designed to vary by more than two nucleotides so they could be unequivocally assigned to the corresponding sample even in the presence of sequencing errors within the barcode.

The high-quality sequences recorded in this study were uploaded to the MG-RAST server, where they could be accessed under numbers 4487136.3, 4487137.3, 4487138.3 and 4487139.3. These sequences were compared with those available in the server of the same variable region of the 16S rDNA from other positions in the gastrointestinal tract, including oral cavity [16], throat and nose [17] and faeces [18]. All sequences were first clustered as described above and then trimmed and aligned using Infernal software with default settings [19]. A phylogenetic tree was obtained using FastTree software [20] and principal coordinates analysis (PCoA) was then performed using UniFrac software [21].

Results

Microbial Culturing Analysis of Stomach Samples

In 10 of the 12 subjects, the total number of cultivable microorganisms recovered from the stomach samples (mucosa or gastric juices) ranged from 10^2 to 10^4 cfu/g or ml, with similar count numbers obtained on MRSC, CB and BHI media (Table 1). The highest counts were obtained for mucosa and gastric juice samples from subject M11. However, as counts were so different from all other samples, contamination or improper biopsy preparation was suspected. Viable microorganisms from the stomach samples of two subjects (M4 and M9) were not recovered. As compared with the biopsy samples, counts were only obtained from five of the 12 samples of gastric juice (Table 1). Eighty-six isolates from either the mucosa or gastric juice samples were selected for molecular identification by successive ARDRA and sequencing. In total, 16 different microorganisms were identified. Of these, 15 were assigned down to species level with two cases in which differentiation between two closed related species could not be achieved and one additional sequence that was only identified at genus level (Table 2). Propionibacteria and lactobacilli accounted for the majority of the cultivable bacteria. Species of *Propionibacterium* and *Lactobacillus* were

Table 1 Total and lactic acid bacteria counts (in colony-forming units per gramme or millilitre) for stomach mucosa and gastric juice samples provided by healthy humans

Culture medium	Sample M1		Sample M2		Sample M3		Sample M4		Sample M5		Sample M6		Sample M7		Sample M8		Sample M9		Sample M10		Sample M11		Sample M12		
	M	GJ	M	GJ	M	GJ	M	GJ	M	GJ	M	GJ	M	GJ	M	GJ	M	GJ	M	GJ	M	GJ	M	GJ	
MRSC	1.5×10^3	–	1.7×10^3	–	–	1.0×10^4	–	1.0×10^4	–	2.0×10^2	–	4.0×10^2	–	–	6.0×10^2	–	1.0×10^2	–	–	1.0×10^2	–	1.0×10^5	–	–	1.3×10^3
BHI	3.5×10^3	–	1.0×10^3	–	1.2×10^4	–	–	–	–	–	–	–	–	1.0×10^3	–	–	–	–	–	5.0×10^2	–	3.0×10^5	–	–	3.0×10^3
CB	–	–	5.2×10^3	–	1.0×10^4	–	–	–	–	–	–	–	–	1.2×10^3	–	–	–	–	–	–	–	–	–	–	4.0×10^3

MRSC de Man, Rogosa and Sharpe medium with cysteine, BHI Brain Heart Infusion medium, CB Columbia Blood agar medium, M mucosal sample, GJ gastric juice sample

Table 2 Cultivable microorganisms identified in the different mucosa and gastric juice samples

Species	Isolates from the different samples																								Total
	M1		M2		M3		M4		M5		M6		M7		M8		M9		M10		M11		M12		
	M	GJ	M	GJ	M	GJ	M	GJ	M	GJ	M	GJ	M	GJ	M	GJ	M	GJ	M	GJ	M	GJ	M	GJ	
<i>Propionibacterium acnes</i>	9	-	6	-	2	-	2	-	2	-	11	-	6	-	4	-	-	-	-	1	-	-	-	-	41
<i>Lactobacillus gasseri</i>	-	-	-	-	2	-	2	-	2	-	-	-	-	-	-	-	-	-	-	2	-	-	-	3	9
<i>Staphylococcus epidermidis</i>	-	-	3	-	2	-	-	-	-	-	-	-	1	-	1	-	-	-	-	-	-	-	-	-	7
<i>Lactobacillus reuteri</i>	-	-	-	-	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4
<i>Clostridium</i> spp.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	3
<i>Escherichia coli</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	-	-	3
<i>Lactobacillus vaginalis</i>	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	3
<i>Staphylococcus saprophyticus</i>	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3
<i>Aerococcus viridans</i>	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
<i>Brachybacterium paracongolomeratum</i>	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
<i>Lactobacillus fermentum</i>	-	-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	2
<i>Streptococcus salivarius</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	2	-	2
<i>Lactobacillus casei/paracasei</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	1
<i>Propionibacterium granulosum</i>	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
<i>Pediococcus pentosaceus</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1
<i>Staphylococcus pasteurii/warneri</i>	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Total	16	-	10	-	9	2	-	-	6	-	11	-	7	4	-	5	-	-	1	2	6	-	3	4	86

M mucosal sample, GJ gastric juice sample

isolated from eight and five subjects, respectively. *Propionibacterium acnes* was the predominant species, accounting for 49 % (41 isolates) of all isolates. The 19 isolates of lactobacilli came from the mucosa and gastric juice samples of two and four subjects, respectively. They were assigned to five different species (Table 2), of which *Lactobacillus gasseri* was the most common. Members of this species were isolated from four subjects. Staphylococci ranked in a third position with 11 isolates belonging to the following species (*Staphylococcus epidermidis*, *Staphylococcus pasteurii/warneri* and *Staphylococcus saprophyticus*); these were recovered from the stomach of five subjects. Additionally, three isolates identified as *Streptococcus salivarius* were obtained from samples of two subjects. Finally, single isolates of *E. coli* and *Clostridium* spp. were obtained from the mucosal sample of subject M11.

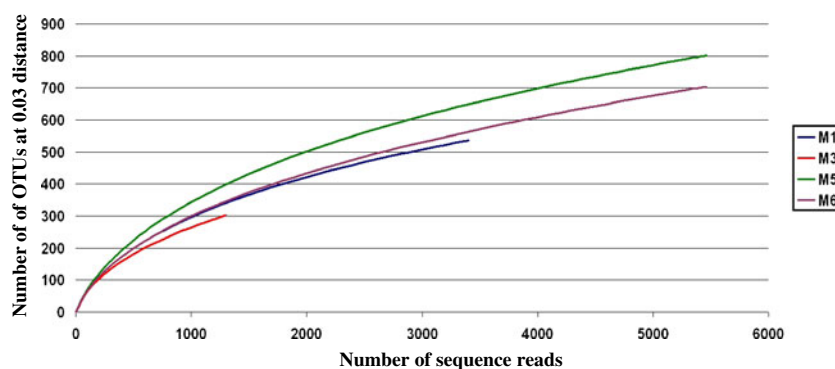
Bacterial Composition of Gastric Mucosa Determined by Pyrosequencing

Total microbial DNA from four mucosal samples (M1, M3, M5 and M6) was subjected to pyrosequencing. Of a total of 56,738 16S rDNA reads obtained, 15,622 proved to be of high quality as previously defined. These included 3,429 sequences from mucosal sample M1, 1,324 from sample M3, 5,457 from sample M5 and 5,412 from sample M6. Rarefaction curves showing estimated number of species (operational taxonomic units (OTUs) at 0.03 distance) for the four samples are represented in Fig. 1. Although the number of reads obtained for each sample was different (and thus the corresponding number of OTUs), the curves showed similar slopes with a good sequenced coverage in general. By the analysis at the RDP server, the bacterial sequences were then taxonomically assigned to nine different phyla, among which *Firmicutes* and *Proteobacteria* were the most abundant in all four samples (38 and 37 % of sequences, respectively, in sample M1; 69 and 22 % in M3; 61 and 26 % in M5; and 47 and 34 % in M6), followed by members of the phylum *Actinobacteria* (23 % in M1, 10 % in M5 and 8 % in samples M3 and M6). Other phyla, such as *Deinococcus-Thermus*, *Bacteroidetes* and

Gemmatimonadetes, were represented in small numbers (less than 3 %). The sequences were grouped into 59 different families, of which *Streptococcaceae* (25 % of sequences in sample M3, 44 % in M5 and 14 % in M6), *Lactobacillaceae* (28 % of sequences of sample M3) and *Propionibacteriaceae* (20 % of M1) were the best represented. Wide diversity at the genus level was seen among the sequences, with up to 69 different genera obtained (Fig. 2). Members of *Streptococcus*, *Propionibacterium*, *Lactobacillus* and *Enterococcus* dominated the different mucosal samples although their relative abundance varied significantly (Fig. 2). *Streptococcus* reads were dominant in sample M5, and significant numbers were seen in the other three samples. Sequences belonging to the genus *Propionibacterium* were a majority in sample M1, while *Lactobacillus* was dominant in sample M3. Different classes of the *Proteobacteria* phylum (*Alpha*, *Beta* and *Gamma*) were found in the different samples. The genus *Methylobacterium* accounted for 3–9 % of the total sequence reads whereas 14 % of the sequences of sample M1 belonged to the genus *Acinetobacter*. Other genera of this phylum, such as *Vibrio* or *Pseudomonas*, represented less than 5 % of the assigned sequences within each sample. To assess the distribution of genera between the different mucosal samples, a Venn diagram was constructed (Fig. 3). This showed a high proportion of sample-specific genera (ranging between 4 and 15) not shared among the different samples, indicating a high degree of inter-subject variability. However, 19 genera were found to be present in all four samples (representing between 35 and 54 % of the total genera detected per sample), including representatives of Gram-positive (*Lactobacillus*, *Lactococcus*, *Propionibacterium*, *Staphylococcus*, *Streptococcus* and *Brevibacterium* among others) and Gram-negative (*Methylobacterium*, *Pseudomonas*, *Serratia*, *Stenotrophomonas*, *Veillonella* and *Vibrio*) bacteria. It should be noted that only five sequences (belonging to mucosal sample M6) were assigned to the genus *Helicobacter*.

The sequences of the gastric microbial community were compared by PCoA with those of other collectives from different positions in the gastrointestinal tract. The results showed the microbial communities of the stomach samples

Fig. 1 Rarefaction curves (97 % similarity level) for the partial sequences of the bacterial 16S rRNA genes obtained from stomach mucosal samples M1, M3, M5 and M6



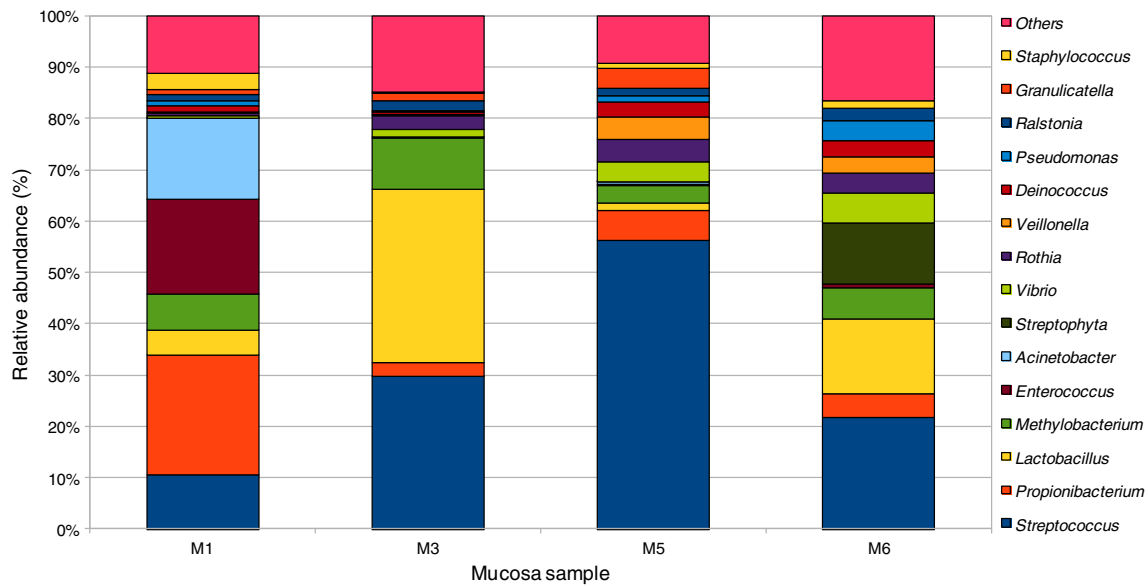


Fig. 2 Relative abundance at the genus level (95 % similarity), based on the classification of the partial 16S rDNA bacterial sequences from mucosal samples M1, M3, M5 and M6, using the RDP classifier. Only

genera appearing at high percentages are included separately in the figure. Those with low percentages are all included under the denomination “others”

to cluster together (Fig. 4). Communities from the oral cavity and throat clustered in the proximity of those from the stomach, indicating that they are close in terms of their bacterial composition and rather more different to those of the nose and distal gut.

Discussion

Despite the potential importance of gastric microbial communities in human health and disease, little information exists on the complexity of this microbiota. The role of *H. pylori* in the development of gastritis and peptic ulcers, and its contribution to adenocarcinoma is well known [22]. However, little is known about the behaviour of other members of the human gastric ecosystem. The advent of culture-independent molecular methods, such as the cloning and sequencing of 16S rRNA genes [5, 7, 23], TTGE of PCR-amplified 16S rDNA fragments [4], have furthered our understanding of this microbiota, identifying taxa that have never been cultured. These molecular approaches are very powerful, but they say nothing about the physiological state of the microorganisms they detect. In fact, they cannot distinguish between dead and alive bacteria. Thus, complementary approaches of conventional culturing and culture-independent methods are more informative. In addition, culturing could allow the isolation of bacteria with antagonistic activity against gastric pathogens, including *H. pylori* [24, 25].

In the present work, conventional culturing and culture-independent methods were combined to characterise the microbial communities of the healthy human stomach.

Sixteen different microorganisms were identified among the cultures. Older studies using traditional culturing techniques have previously described the presence of some of these bacteria, including members of the genera *Streptococcus*, *Staphylococcus* and *Lactobacillus* in the human esophagus and stomach [3, 26, 27]. Conversely, it was surprising to find *P. acnes* as a majority component among our isolates. Recorded as the predominant bacterium of the pilosebaceous follicles [28], *P. acnes* has also been reported as a regular inhabitant of different human mucosa, such as those from the oral cavity, the nostrils, conjunctiva, the external ear canal and the upper respiratory and intestinal tracts [5, 29]. Biochemical and genetic properties of the stomach isolates have recently been shown to be similar to skin-associated *P. acnes* [30]. As concerning the lactobacilli, *L. gasseri* has already been reported as a normal inhabitant of the colonic mucosa [31]. The isolation of *L. gasseri* from gastric juice and mucosa, suggests that this bacterium is likely to be a predominant species all along the human gastrointestinal tract.

The small number of different genera found by culturing (9) contrasts with the number detected by pyrosequencing (69). Pyrosequencing is a NGS technique allowing a more complete view of the overall community composition of a given ecosystem. In spite of this, it is noteworthy the high degree of correspondence between cultivation and the metagenomic approach obtained in this work since, although not at the same proportions, members of the commonest bacterial genera (such as *Propionibacterium*, *Lactobacillus* and *Streptococcus*) were all detected by the two techniques. Indeed, members of these taxonomic groups are alive in the gastric niche.

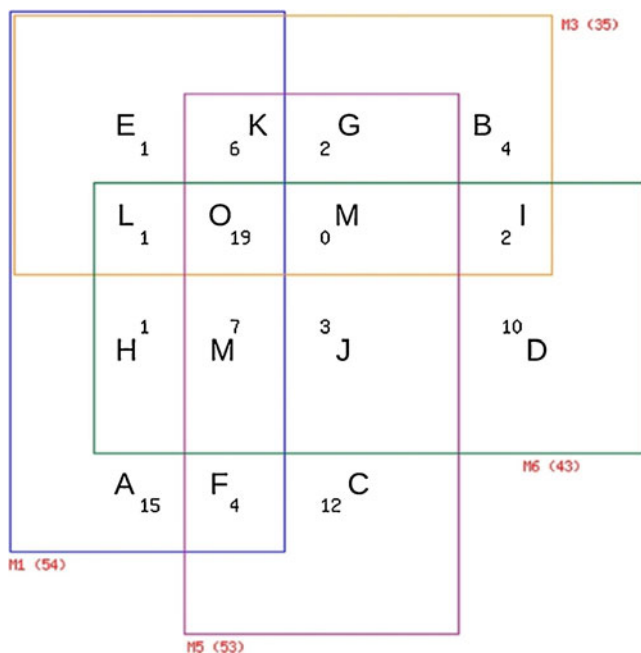


Fig. 3 Venn diagram showing shared and exclusive genera for mucosal samples M1, M3, M5 and M6. The number in parentheses refers to the total number of genera detected per sample. Letter A includes the following genera: *Brachybacterium*, *Bradyrhizobium*, *Campylobacter*, *Cellvibrio*, *Duganella*, *Enterobacter*, *Escherichia/Shigella*, *Janibacter*, *Micrococcus*, *Nesterenkonia*, *Novosphingobium*, *Peptoniphilus*, *Sphingobacterium*, *Sphingobium* and *Sphingomonas*; B, *Aggregatibacter*, *Clostridium*, *Enhydrobacter* and *Paracoccus*; C, *Atopobium*, *Bulleidia*, *Fingoldia*, *Gemmatimonas*, *Hydrogenobacter*, *Kingella*, *Listonella*, *Megasphaera*, *Methylibium*, *Oribacterium*, *Selenomonas* and *Uruburueella*; D, *Abiotrophia*, *Blautia*, *Capnocytophaga*, *Catonella*, *Caulobacter*, *Dyella*, *Helicobacter*, *Leuconostoc*, *Rubrobacter* and *Tetragenococcus*; E, *Shewanella*; F, *Chelonobacter*, *Dialister*, *Flavobacterium* and *Thermoanaerobacterium*; G: *Kocuria* and *Parvimonas*; H, *Peptostreptococcus*; I, *Neisseria* and *TM7_genera_incertae_sedis*; J, *Bacillus*, *Brevibacillus* and *Haemophilus*; K, *Acinetobacter*, *Eubacterium*, *Gemella*, *Granulicatella*, *Meiothermus* and *Solobacterium*; L, *Mogibacterium*; M, *Asticcacaulis*, *Bifidobacterium*, *Corynebacterium*, *Enterococcus*, *Micrococcus*, *Streptophyta* and *Yersinia*; O, *Actinomyces*, *Brevibacterium*, *Deinococcus*, *Geobacillus*, *Lactobacillus*, *Lactococcus*, *Methylobacterium*, *Ochrobactrum*, *Propionibacterium*, *Pseudomonas*, *Ralstonia*, *Rothia*, *Serratia*, *Staphylococcus*, *Stenotrophomonas*, *Streptococcus*, *Thermus*, *Veillonella* and *Vibrio*

Firmicutes and *Proteobacteria* were the most abundant phyla, as indicated in previous pyrosequencing studies of the gastric microbiota of healthy people [6, 11]. The diversity found using this strategy was similar to that reported by Andersson et al. [6] but greater than that recorded by Bik et al. [5] and Dicksved et al. [23]. The inter-subject variability observed has also been reported in other 16S rDNA studies of the gastrointestinal microbiota [5, 6, 16, 32].

Most of the microbial types identified by pyrosequencing have no cultured relatives, while some others have been rarely described in the gastric ecosystem. As an example, 8 % of the sequences of sample M6 were assigned to *Cyanobacteria* and around 3 % of those detected in M5

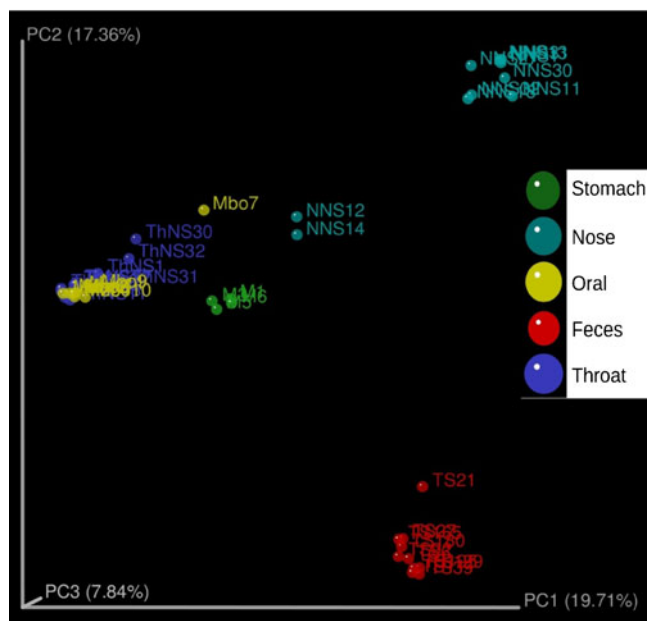


Fig. 4 Scatter plot comparing the bacterial composition (16S rDNA sequence reads) of stomach samples (coded as M (green); this study), nose (coded as NNS (light blue) [17]), oral cavity (coded as Mbo (yellow) [16]), faeces (coded as TS (red) [18]) and throat (coded as ThNS (dark blue) [17]), as generated by PCoA. The percentages of variation are indicated on the axes

and M6 were related to the *Deinococcus-Thermus* phylum. Both these groups of microorganisms are commonly associated with habitats completely different from the human body, though they have been sporadically described as present in the human stomach [5, 6]. A closer examination to our cyanobacteria-related reads showed that they were in fact 99 % identical to chloroplast sequences. Although it is difficult to establish the origin of these sequences and we cannot discard possible sequence artifacts due to PCR errors, they may probable come from amplification of ingested plant material. Hence, the presence of cyanobacteria in 16S rDNA datasets of the stomach should be regarded with caution. Though in low proportions, *Pseudomonas*-related sequences were also detected in all four mucosal samples. The association of this microorganism with the stomach environment is unclear too. *Pseudomonas* has been described in the stomach by Monstein et al. [4] but was not encountered by Bik et al. [5] who reported the presence of 128 gastric phylotypes.

A high proportion of *H. pylori* sequences has been detected in the gastrointestinal microbiota of some individuals, particularly if they had been diagnosed as symptomatic for this pathogen [6, 7, 33]. However, in this work, and in agreement with results of other authors [5, 23], only small numbers of *H. pylori*-related sequences were detected by pyrosequencing. The present results support the idea that the diversity of the microbiota of the human stomach is high when *H. pylori* is absent or present at a low ratio [6].

Whether this diversity is associated with a healthy state, as has been suggested for the large intestine [33], needs to be further explored.

Although comparison of different metagenomic data sets is difficult and differences exist depending on the human population under analysis and the sequencing methodologies employed, PCoA analysis revealed the gastric microbiota to be different from that of other parts of the gastrointestinal tract. The relative proximity of the gastric communities with those of the oral cavity and throat, suggest the existence (at least in part) of a transient microbiota [4, 6, 34]. Thus, it is likely that some of the gastric microbial constituents detected may be swallowed from the mouth and throat. Whether these bacteria are active in the stomach might be determined in the future using metatranscriptomic methods.

In conclusion, the stomachs of healthy people appear to have a highly diverse microbiota, with appreciable inter-subject variability. *Streptococcus*, *Propionibacterium* and *Lactobacillus* are among the main genera, as revealed by both culturing and pyrosequencing. These bacteria might be playing an important role in the microbial homeostasis of this organ. Characterisation of the stomach endogenous microorganisms could allow the future selection of adapted probiotics to the gastric environment; this would be useful to counteract gastric pathogens such as *H. pylori*. In fact, assessment of the probiotic potential of these gastric isolates is currently underway.

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