

Bariatric surgery drives major rearrangements of the intestinal microbiota including the biofilm composition

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1. ABSTRACT

The intestinal microbiota disequilibrium has been associated with obesity, while the role of the gut mucosal biofilms in this pathology is still unknown. We analysed the changes in the intestinal microbiota of obese patients after bariatric surgery with the aim of disclosing the rearrangement of the biofilm configuration. Although the bariatric surgery drives major rearrangements of the gut microbiota, obese patients maintain the *Prevotella* enterotype before and after surgery, as shown by normal weight patients, with an increase of *Bacteroides vulgatus* and *Bacteroides uniformis*. The *Bacteroides* enterotype guarantees the strong ability to form a biofilm which allows a more efficient digestion of polysaccharides than planktonic communities and leads to the production of acetate which is a key player to inhibit enteropathogens. Additionally, the laparoscopic gastric bypass induces an increase of *Hafnia alvei* (*Proteobacteria*), a facultative anaerobic bacterium involved in intestinal and inflammatory disorders. Bariatric surgery influences the microbial composition of gut biofilm. Further studies are needed to elucidate the impact of this variation on recovery after surgery and on weight loss.

2. INTRODUCTION

The human gastrointestinal (GI) tract harbours a collection of bacteria, archaea and eukarya,

termed the 'gut microbiota', which has co-evolved with the host and have formed an intricate and mutually beneficial relationship (1, 2). The development of the gut microbiota is generally believed to begin from birth and even before in the gestational phase, as revealed by studies in which microbes are detected in womb tissues, such as the placenta (3, 4). The mode of delivery appears to affect the gut microbiota composition, with vaginally delivered infants' microbiota containing a high abundance of *Lactobacilli* belonging to mother's vaginal flora (5, 6). In contrast, the gut microbiota of infants delivered by C-section is composed of facultative anaerobes such as *Clostridium* species (7, 8). Although in the early life the composition of the gut microbiota changes, it becomes stable after the age of 2 years and onward into adult life. In adults, Wang *et al.* (9) analysed the bacterial diversity from mucosal biopsies along the human intestinal tract by direct sequencing of 16S rRNA genes and identified six main bacterial phyla: *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, *Verrucomicrobia*, and *Actinobacteria*, showing that the bacterial community in jejunum is remarkably different from that in distal ileum, ascending colon and rectum in terms of diversity indices, phylotype composition and phylogenetic distribution of the 16S rDNA clones and that the major phylogenetic groups are similar from distal ileum to rectum. At the genus and species level, more inter-

individual differences exist, revealing the presence of three enterotypes identifiable by variations in the level of one of three genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3) (10), although the existence of these enterotypes is controversial (11). Enterotype 1 is highly associated with long-term consumption of animal proteins and saturated fat, whereas enterotype 2 is associated with a carbohydrate-based diet (12). Indeed, the main contributor to the composition of the gut microbiota is the diet (13-16) which can account for 57 % of the variations in microbiota compared to genetic variations in the host which can only account for 12 % (17). Nonetheless, the acute diet modifications can induce rapid changes in the gut microbiome but not a complete switch from one enterotype to the other (12).

The human gut microbiome and its role in health and disease have been extensively studied, establishing its positive involvement in human physiology, metabolism, nutrition and immune function, but also in pathological conditions including susceptibility to infections, metabolic syndromes (e.g., diabetes and cardiovascular diseases), allergy and other inflammatory diseases (18). Gut microbiota is crucial in human metabolism, synthesizing unique and essential molecules like vitamins, metabolizing ingested compounds, including dietary and pharmaceutical material, promoting the absorption of glucose, cooperating in the extraction of calories and their storage in host adipose tissue, providing energy and nutrients for microbial growth and proliferation, cooperating in the metabolism of bile acids and is also an important factor for brain development. Moreover, it stimulates the host immune system contributing to its development and competitively inhibits the colonization of the intestine by pathogenic bacteria (19). Life events such as illness, antibiotic treatment and changes in diet, causing a dysbiosis, can disrupt these beneficial mechanisms (4, 20). Indeed, gut microbes adapt to a certain type of lifestyle which influences the features of the biochemical niches available in the gut (21). The species that most rapidly seem to respond to changes, such as the carbohydrate intake, belong to the *Firmicutes* and *Actinobacteria* (22-25). Indeed, decreases of *Firmicutes* and *Actinobacteria* have been observed in response to low-carbohydrate intake (26). Furthermore, bariatric surgery, by drastically altering the anatomy of the gastrointestinal tract and allowing a higher amount of dissolved oxygen in the gut, favours the fast-growing facultative anaerobes, including *Proteobacteria*, which are able to use available oxygen as an electron acceptor compared with most obligate anaerobes, except *Bacteroides* spp. (27) and *Faecalibacterium prausnitzii* (28), which can also use oxygen. Nonetheless, *Bacteroides* and *Faecalibacterium*, owing to the oxygen sensitivity of these anaerobes, can stand at a low oxygen concentration (29).

Several studies have demonstrated the existence of a link between intestinal microbiota and obesity. Obesity is a complex multifactorial chronic disease characterized by chronic low-grade inflammation and predisposition to develop metabolic syndromes with consequent comorbidities such as cardio-vascular disease, diabetes, and some cancers in both adults and children. In obese subjects an intestinal microbiota dysregulation has been documented with changes mainly in the proportion of *Firmicutes* and *Bacteroidetes* in favour of an excess of *Firmicutes* and with a reduction in terms of diversity, although the dysregulation of many other bacteria has been associated to this metabolic disease (30). Recently, the changes in the *Firmicutes/Bacteroidetes* ratio have been linked to comorbidities, such as diabetes, rather than to the obesity (31).

Nowadays, bariatric surgery is the most effective approach in adults to deal with morbid obesity and its associated cardio metabolic risks, better than dietary interventions (32). As it is expected that in a relatively short time period the prevalence of obesity will dramatically increase among children, also minors will be eligible for this surgical approach (33).

Two of the most commonly performed surgical procedure are the laparoscopic sleeve gastrectomy (SG) and laparoscopic gastric bypass (LGB). The SG consists in a large stomach resection which is carried out in order to create a tube of about 60 ml, leaving the pyloric sphincter intact. The LGB provides the creation of a gastric pouch (~30 ml) directly linked to the distal jejunum by the Roux limb. The distal stomach, duodenum and proximal part of the jejunum are subsequently anastomosed 1.5. cm below the gastrojejunal anastomosis (34).

The SG is a purely restrictive operation that reduces the size of the gastric reservoir to 60–100 mL, allowing the intake of only small amounts of food and inducing a feeling of satiety earlier during a meal. Nonetheless, it has been suggested that attenuation of endogenous ghrelin levels, which is a hunger-regulating peptide hormone, may also contribute to the success of SG (35). Indeed, by resecting the stomach fundus in SG, the majority of ghrelin-producing cells are removed. The LGB is believed to exert its effect by mean of a threefold mechanism including the reduction in food intake, malabsorptive feature and the shifts in the dietary preferences of the patients. The average weight loss is 50% of the initial excess weight during the first year after surgeries. For both surgery, surgical complications rates are similar with mortality rates about 0.1.% and serious early complication rates of 5% (36, 37).

To date, limited data are available concerning the microbiota composition after bariatric surgery and its possible influence on the restoring of a gut

permanent “healthy” condition. For example, Zhang *et al.* (38) showed that Firmicutes were dominant in normal weight and obese individuals but were markedly reduced in post-gastric bypass patients. Although it has been suggested that bariatric surgery affects the composition of the intestinal microbiota, its role in modulating intestinal biofilm is not yet clear. Recent data suggest that multiple components of the mucosal barrier are capable of distinguishing and responding to the colonization of specific microbial species by means of a dynamic interplay between the mucosal immune system and mucous gel layer (39). Specifically, the mucine secreted by the intestinal epithelium protects from bacterial hydrolytic enzymes and simultaneously acts as an energetic source providing a nutrient-rich habitat for the microbiota (40). Furthermore, secretory immunoglobulins A (IgA) contribute to biofilm formation having a critical role in excluding pathogens and maintaining a mutually beneficial relationship with commensals (41).

The presence of biofilm in the gastrointestinal tract (GIT) has long been a controversial topic due to the inaccessibility of the gut and the astonishing rate of growth of the human intestinal mucosa (42). Today many studies have demonstrated the presence of biofilm in the GIT, most of which have focused on pathological conditions such as Barrett’s oesophagus (43), colon cancer (44) and inflammatory bowel disease (IBD) (45). It has been observed that in patients with Barrett’s oesophagus there is greater species diversity and higher numbers of bacteria in the distal oesophagus, compared with healthy people, and that there can be extensive micro colony formation on the epithelial surface. Many of these species are nitrate reducers, and may ultimately be responsible for DNA damage through the production of *N*-nitrous compounds, substances that have long been implicated as carcinogens in the lower oesophagus and stomach (43). In colorectal cancer biofilm presence has been associated with bacterial tissue invasion and changes in tissue biology with enhanced cellular proliferation, a basic feature of oncogenic transformation occurring even in colons without evidence of cancer (44). Furthermore, it has been seen that a break of the delicate balance between biofilm composition, barrier function and the immune system is associated with IBD, a chronic disease that comprises of Crohn’s disease (CD) and ulcerative colitis (UC). More recently, attention has been focused on the physiological role of biofilm in maintaining the health of the host and its presence has been detected both on the mucus layer lining mucosal surfaces (46) and on dietary residues in the gut lumen (47). The inner mucus layer that overlies the gut epithelium forms a viscoelastic gel that acts as the first line of defence against both commensal and invading microbes, while the outer mucus of the large intestine forms a unique microbial niche with distinct bacterial communities under physiological conditions (48), facilitating

beneficial functions including nutrient exchange and induction of host innate immunity (49). It is plausible that obesity, being the result of an imbalance between the accumulation of body fat and the expenditure of energy orchestrated by the gut microbiota, influences the composition of mucosal bacterial biofilm.

In order to implement data on the relationship between obesity, bariatric surgery and gut microbiota, the aim of this study was to determine if bariatric surgery shaped the gut microbial composition, influencing the gut mucosal biofilm and if this event may enhance a positive and long-lasting outcome.

3. MATERIAL AND METHODS

3.1. Patients selection

In this study twenty obese subjects eligible for bariatric surgery were enrolled, in accordance with the international guidelines. Subjects had to comply with stringent inclusion criteria: motivated and well-informed patients with acceptable operative risks, declared compliance to follow lifelong medical surveillance, failure of non-surgical treatments, aged 18 to 65 years, body mass index (BMI) ≥ 40 kg/m² or between 35 and 40 kg/m² with obesity-related comorbidities. Exclusion criteria were: patients affected from documented liver cirrhosis, patients consuming an amount of alcohol > 25 g/day, the presence of other liver diseases like: patients infected with the HBV, HCV, HIV and presence of neoplasia. Ten patients underwent laparoscopic gastric bypass (LGB) and ten laparoscopic sleeve gastrectomy (SG). Twenty normal weight (NW) subjects, with characteristics matching with obese patients, were recruited as control. Surgical interventions were performed at the General Surgery Clinic, Cattinara Hospital of Trieste. The microbiota characterization was performed on faecal samples by Ion Torrent next-generation high-throughput sequencing of the 16S rRNA gene, before surgery and 3 months later, at the Advanced Microbiological Laboratory, IRCCS-Burlo Garofolo, Trieste. All subjects provided informed consent before taking part in the study. The study was approved by the local ethical committee.

3.2. Ion Torrent sequencing

DNA was extracted from faeces with the NucliSENS® easyMAG® system (BioMérieux, Gorman, North Carolina, USA). A real-time EvaGreen® dye (Fisher Molecular Biology, Waltham, USA) PCR was performed with the degenerated primer 27FYM and subsequently with Bt338F in conjunction with the U534R primer targeting the V1-V3 region as previously described (50). Quantitative Insights Into Microbial Ecology (QIIME) 1.8.0.1. was used to process the sequence data. High quality (Q >25) sequences were de-

Table 1. Alpha diversity metrics

Author: Metric	NW	Before SG	After SG	Before LGB	After LGB
Chao1	623 ± 228	698 ± 169	722 ± 176	609 ± 51	812 ± 129
Observed Otus	329 ± 116	343 ± 63	375 ± 76	308 ± 26	418 ± 64
PD whole tree	17 ± 0.6.1	18 ± 3	18 ± 2	17 ± 0.6.	19 ± 2

Bacterial diversity values are given as mean ± standard deviation at a rarefaction depth of 10,000 sequences per sample. Alpha diversity was compared between groups by means of a non-parametric t-test using the `compare_alpha_diversity.py` script of QIIME. None of the comparisons was significant. Abbreviations: NW: normal weight; SG: laparoscopic sleeve gastrectomy; LGB: laparoscopic gastric bypass.

multiplexed and filtered by quality using `split_libraries_fastq.py` with default parameters, except for the length parameter (150 bp). Alpha diversity was assessed by the Chao1, observed otus and PD whole tree metrics. Differences in community composition between cohorts were investigated using the Kruskal-Wallis test. The sequences were aligned against the human intestinal microbiota 16S rRNA database (HITdb) (51).

4. RESULTS

4.1. Microbiota composition

It has been shown that obese people, compared to lean ones, have a dysbiosis in terms of quantity and variability of bacterial species (52), which is referred as to alpha diversity and represents the diversity of a habitat or a community of microorganisms and describes its richness and evenness of individuals. In our cohort, the three alpha diversity metrics were not significantly affected based on the intestinal dysbiosis among patients before and after surgery and compared to NW (Table 1). Nevertheless, in patients eligible for LGB, a slight decrease of the alpha diversity, compared to NW patients, was observed before surgery while an increase of this value was detected after both surgeries.

The bacterial phyla *Bacteroidetes* and *Firmicutes* were differentially represented in obese patients compared to healthy subjects. More precisely, the relative abundances of *Bacteroidetes* were similar between NW and obese patients (Figure 1 A, B and D) while differed between obese patients eligible for SG or LGB. After bariatric surgery, this phylum decreased in LGB group, in which also *Firmicutes* were significantly modulated, showing an increase (p-value < 0.05) (Figure 1 E). Nonetheless, looking into the variation of *Firmicutes* and *Bacteroidetes* by means of the *Firmicutes/Bacteroidetes* ratio, we observed that the ratio was affected by surgery (Table 2) but there were not significant changes. The trend that we observed, comparing to NW patients, showed that the ratio was not affected by the surgery in obese patients who were selected for SG. Conversely, the patients eligible for LGB showed an increased ratio.

We hypothesize that the reconfiguration of the *Firmicutes/Bacteroidetes* ratio may be influenced by the concomitant changes of other phyla present in the gut. Indeed, the surgical technique influenced the variation of the amount of *Proteobacteria* (p-value < 0.05). Based on the surgical technique, the amount of *Proteobacteria* decreased after SG while it showed a significant increase after LGB (p-value < 0.05) (Figure 1 C and E). The *Proteobacteria* were also responsible for the differences between obese patients eligible for SG with respect to the NW and to obese patients eligible for LGB (p-value < 0.05).

Based on recent evaluation documenting that the intestinal microbiota in adults is mainly clustered into two dietary enterotypes which are distinguished by a greater abundance of genera *Prevotella* and *Bacteroides*, which belong to *Bacteroidetes* phylum, we evaluated the predominant enterotypes in our series. The NW patients showed a doubled amount of *Bacteroides* comparing to *Prevotella* (Figure 2). Nevertheless, the ratios were not significantly different among groups. With regard to the most abundant species belonging to *Prevotella* and *Bacteroides* genera and involved in biofilm formation (Figure 3), in NW patients, *Bacteroides uniformis* (9%), *Bacteroides vulgatus* (6%) and *Prevotella copri* (8%) were predominant, alongside *Bacteroides stercoris* and *Hafnia alvei*, the latter belonging to *Proteobacteria*. In patients before SG, compared to NW and to obese patients eligible for LGB, a lower amount of *B. uniformis* (3%), which increased after SG toward the NW value, and a higher amount of *P. copri*, which decreased after SG, were observed. The relative amount of *B. uniformis* and *P. copri* antagonized, being the most influenced species by the surgical technique. Indeed, SG group switched from a *Prevotella* enterotype to a *Bacteroides* enterotype, as observed in NW, even if the same *Prevotella/Bacteroides* ratio before and after surgery was observed (Table 2). On the other hand, obese patients before LGB, comparing to NW, showed a lower level of *B. vulgatus* (3%) and a higher level of *B. uniformis* (15%) and *B. stercoris* (5%), this last specie found at very low percentages in NW patients. After LGB the percentages of *B. vulgatus* and *uniformis* regained values similar to NW (5% and 7% respectively) and a high presence of *H. alvei*

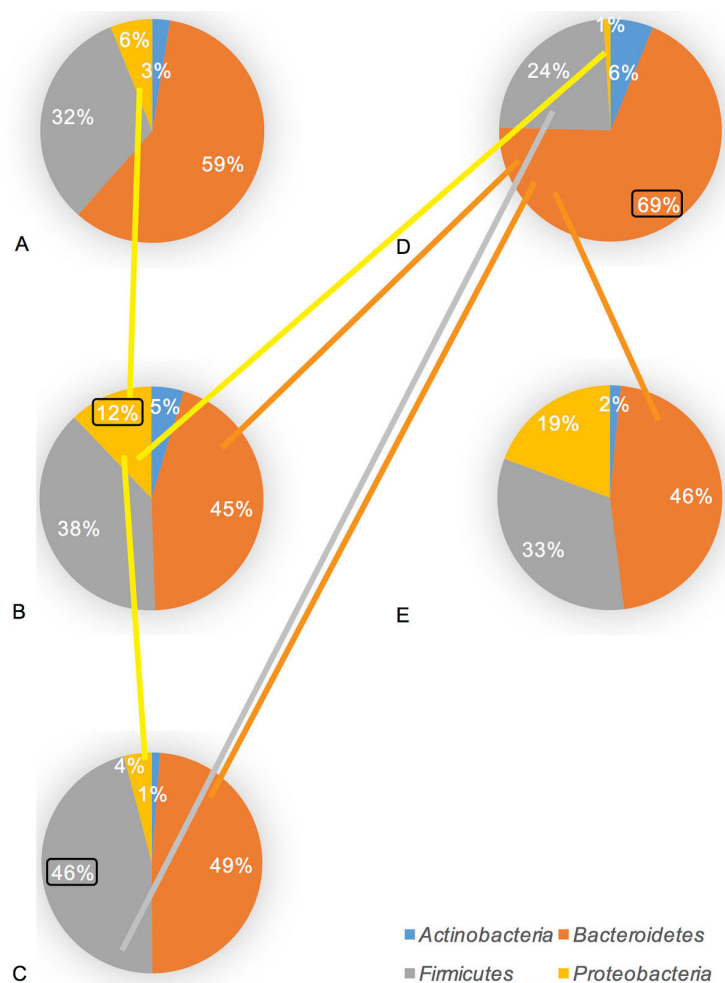


Figure 1. The intestinal bacterial communities from normal weight and obese patients. The output of plot_taxa_summary.py of QIIME showing the relative abundance of the predominant phyla. The coloured lines indicate the significant comparisons ($p < 0.05$) between the relative amounts of phyla, compared by means of the Kruskal-Wallis test. A) Normal weight. B) Before laparoscopic sleeve gastrectomy. C) After laparoscopic sleeve gastrectomy. D) Before laparoscopic gastric bypass. E) After laparoscopic gastric bypass.

Table 2. Firmicutes/Bacteroidetes and Prevotella/Bacteroides ratio

Ratio	NW	Before SG	After SG	Before LGB	After LGB
<i>Firmicutes/Bacteroidetes</i>	0.5.0	0.8.0	0.9.0	0.3.4	0.7.0
<i>Prevotella/Bacteroides</i>	0.4.4	0.2.4	0.2.0	0.0.9	0.3.7

The ratio between the relative abundances of the phyla *Firmicutes/Bacteroidetes* and the genera *Prevotella/Bacteroides*. The ratios were compared by means of the Kruskal-Wallis test. None of the comparisons was significant. Abbreviations: NW: normal weight; SG: laparoscopic sleeve gastrectomy; LGB: laparoscopic gastric bypass.

(6%) was detected. Regardless of the decrease of *B. uniformis* and *B. stercoris*, the enterotype remained dominated by *Bacteroides*.

5. DISCUSSION

Bariatric surgery has become a prominent therapeutic option to treat morbid obesity, with ben-

eficial consequences not only in terms of weight reduction but also in the improvement of the associated comorbidities such as type 2 diabetes mellitus (53, 54). In this study two surgical approaches have been used: the laparoscopic sleeve gastrectomy (SG) and the laparoscopic gastric bypass (LGB), which act on weight loss by restrictive and malabsorptive mechanisms.

Biofilm and bariatric surgery

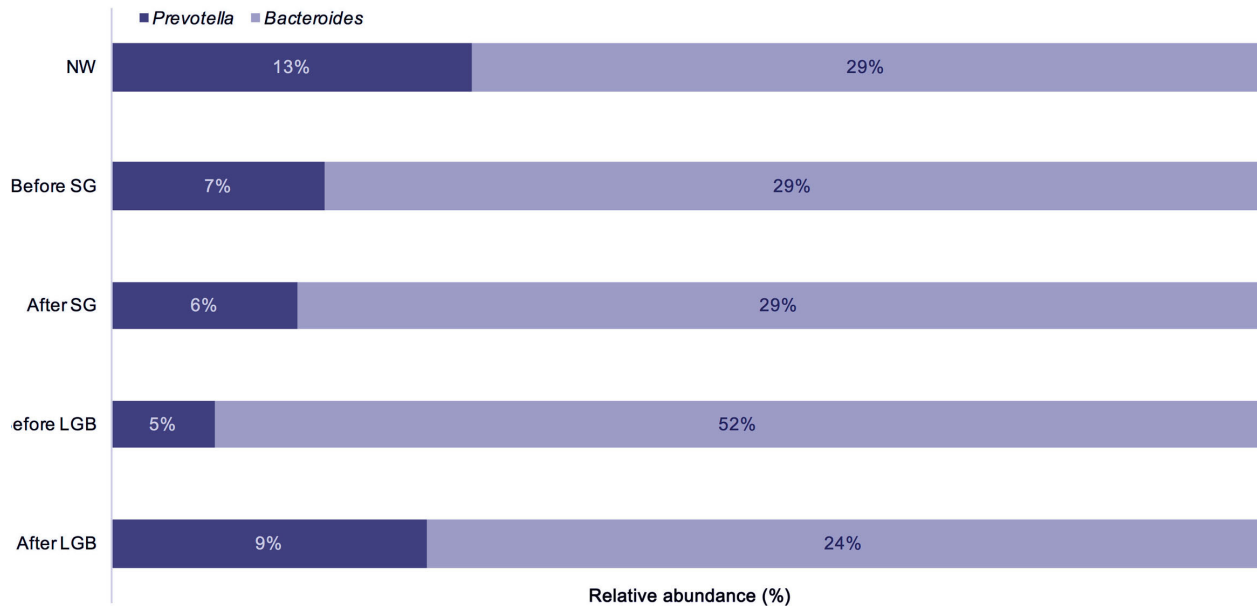


Figure 2. Relative abundance of *Prevotella* and *Bacteroides*. The output of `plot_taxa_summary.py` of QIIME showing the relative abundance of the genera *Prevotella* and *Bacteroides*. The ratios were compared by means of the Kruskal-Wallis test. None of the comparisons was significant. Abbreviations: NW: normal weight; SG: laparoscopic sleeve gastrectomy; LGB: laparoscopic gastric bypass.

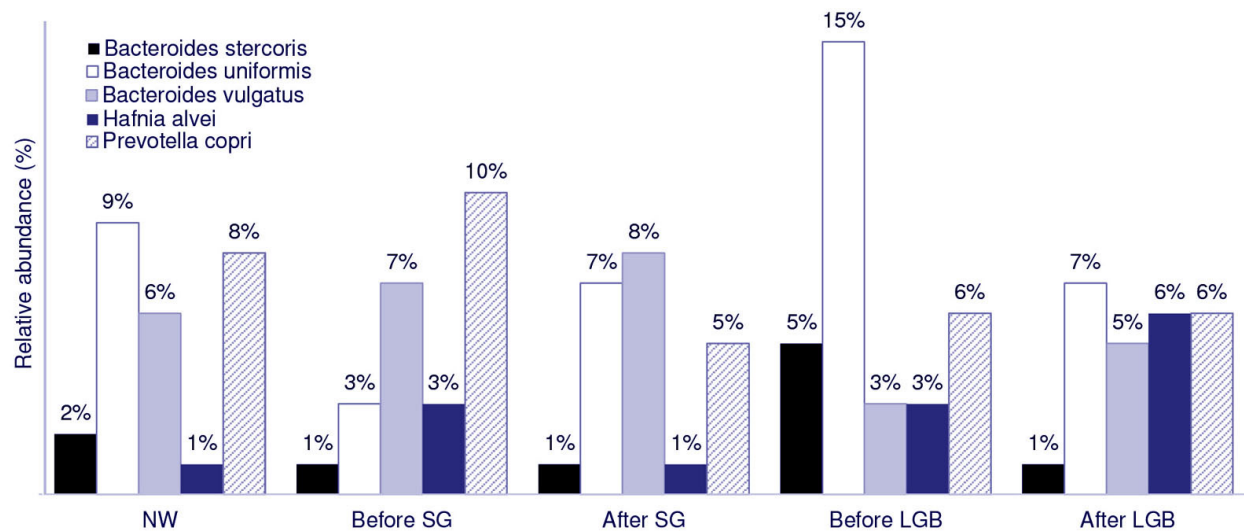


Figure 3. Species involved in biofilm formation. Abbreviations: NW: normal weight; SG: laparoscopic sleeve gastrectomy; LGB: laparoscopic gastric bypass.

We documented the influence of the type of surgical procedure on restoring the bacterial mucosal colonization which exerts an important impact on the clinical management of obesity and on the maintenance of good health. Basically, an increase of the bacterial alpha-diversity was observed in patients after three months from the surgery. According to our results, bacterial diversity seems to represent a more suitable marker of intestinal a condition of eubiosis/dysbiosis than the ratio between different phyla or

taxa, including the *Firmicutes/Bacteroidetes* ratio and the *Prevotella/Bacteroides* ratio, which have been for long time considered a surrogate marker of gut eubiosis/dysbiosis (31), with a physiologic status identified by a greater amount of *Bacteroidetes*. Indeed, even if a different relative amount of *Bacteroidetes* was demonstrated in patients eligible for SG with respect to LGB patients, the *Firmicutes/Bacteroidetes* ratios were not significantly changed. The increased relative amount of *Bacteroidetes* in LGB patients

before surgery is probably due to a more restrictive pre-operative diet/protocol followed by these patients. *Bacteroidetes* increase when a calorie-restricted diet is followed (55, 56). After surgery, all patients showed a similar relative amount of *Bacteroidetes*.

With regard to the enterotype, indicated by the ratio *Prevotella/Bacteroides*, it is maintained after surgery, with the predominance of *Bacteroides*. The predominance of *Bacteroides* may reflect their great ability to form microcolonies, conducive to a protective biofilm in which the cell mucus exerts a pivotal role against the invasions of pathogens (57). Moreover, thanks to its capability to modulate the surface polysaccharides, *Bacteroides* may avoid the host immune response and increase the formation of biofilms. This result suggests that the main role in gut recolonization after bariatric surgery may be absolved by the re-establishment of the correct equilibrium between taxa.

Among *Bacteroides*, we documented a similar proportion, after bariatric surgery, of the relative amount of *B. uniformis* and *B. vulgatus* species. Both species exert beneficial roles in the gut. In obese mice, *B. uniformis* has been seen to ameliorate high fat diet-induced metabolic and immune dysfunction, associated with intestinal dysbiosis and to reduce the production of some inflammatory cytokines (33). Moreover, in humans, this microorganism seems more efficient in digesting polysaccharides when part of biofilm colonies (47), contributing to the production of acetate as the principal fermentation product, which is a key player to inhibit enteropathogens (58). *B. vulgatus* is able to counteract the adhesion of *Escherichia coli* to the intestinal mucosa. The ability of *B. vulgatus* to adhere to host tissue and to form biofilms is crucial to affect the spatial distribution of *E. coli* in the gut (59).

Conversely, the relative amount of *Prevotella* remained constantly low and largely represented by *Prevotella copri*. The role of this specie is still debated. It seems that a single strain of *P. copri* can act in both beneficial or detrimental manner, basically due to its high genetic strain variability which adapts to several factors, including the diet, the species and/or strains of *Prevotella* present and other microbe–microbe interactions (60).

Interestingly, *Proteobacteria* showed a peculiar association with the type of surgery, showing an increased before SG and after LGB. The most abundant species identified in this phylum was *Hafnia alvei*, a facultative anaerobic bacterium, usually involved in a series of intestinal and inflammatory disorders (39). While in patients eligible for SG the colonization of this bacterium decreases after surgery, the patients who undergo LGB procedure show the drawback of promoting *H. alvei* expansion (61). One could speculate that this effect may be associated to

the LGB surgical protocol itself that, involving a GIT reconfiguration, expose the gut both to a transient aerobic condition and alteration of several metabolic functions that may additionally affect the abundance of *Proteobacteria* (62).

Although this study suggests that bariatric surgery restores a physiological colonization of the gut microbiota, with a plausible effect on the composition of gut biofilm, we suggest that the characterization of the gut microbial communities in obese subjects before surgery could help the clinical management by supporting the use of tailored microbiome modulators (e.g. antimicrobials, diet, prebiotics or probiotics). At this regard, promising results have been already obtained with the administration of a mucin degrading, *Akkermansia muciniphila*, to mice preventing the development of high-fat diet-induced obesity and ameliorating metabolic endotoxemia-induced inflammation through the restoration of the gut barrier (63, 64).

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Abbreviations: BMI, body mass index; CD, Crohn's disease; FDR, false discovery rate; GIT, gastrointestinal tract; IBD, inflammatory bowel disease; IgA, immunoglobulin A; LGB laparoscopic gastric bypass; NW normal weight; SG, sleeve gastrectomy; UC, ulcerative colitis

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