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The influence of bariatric surgery methods on the *Firmicutes/Bacteroidetes* ratio in obese patients

Bachelor's thesis

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ABSTRACT

The influence of bariatric surgery methods on the *Firmicutes/Bacteroidetes* ratio in obese patients

Obesity is a worldwide health problem that negatively affects the quality of life. Obese individuals have a high risk for a number of serious chronic diseases. The development of obesity in humans is influenced by proportions of the phyla *Firmicutes* and *Bacteroidetes* in the intestinal microbiota. Bariatric surgery is an invasive method that has been successfully used in obesity treatment. The present study was performed to compare the counts of phyla *Firmicutes* and *Bacteroidetes* in faecal samples of pre- and post- bariatric surgery (sleeve gastrectomy and roux-en-Y gastric bypass) patients. Obtained results indicated that both bariatric surgery methods had effect on body mass index (BMI) and the counts of the *Firmicutes* phylum. Patient gender also had an influence on the changes in gut bacterial composition after bariatric surgery.

Keywords: *Bacteroidetes*; *Firmicutes*; obesity; bariatric surgery; RT-PCR

CERCS: B726 Clinical biology; B230 Microbiology, bacteriology, virology, mycology; B570 Obstetrics, gynaecology, andrology, reproduction, sexuality

Bariaatrilise kirurgia meetodite mõju *Firmicutes/Bacteroidetes* suhtele rasvunud patsientidel

Rasvtõbi on ülemaailmne terviseprobleem, mis mõjutab negatiivselt elukvaliteeti. Rasvtõvega kaasneb kõrge risk haigestuda kroonilistesse haigustesse, näiteks diabeet, südamehaigused jne. Varasemad tööd on näidanud, et rasvtõve kulgu mõjutab hõimkondade *Firmicutes/Bacteroidetes* suhe seedetraktis. Antud töö eesmärgiks oli võrrelda hõimkondade *Firmicutes* ja *Bacteroidetes* kvantitatiivseid muutusi ja suhet enne- ja pärast bariaatrilist operatsiooni (vertikaalne maoreseksioon ja maost möödajuhtiv operatsioon) patsientide roojaproovidest. Saadud tulemused näitasid, et mõlemad kirurgilised meetodid mõjutavad kehamassiindeksi (KMI) ja *Firmicutes* hõimkonna arvukust. Peale bariaatrilist operatsiooni oli soolestiku bakterikoosluse koosseis sõltuv patsientide soost.

Märksõnad: *Bacteroidetes*; *Firmicutes*; rasvtõbi; bariaatriline kirurgia; RT-PCR

CERCS: B726 Kliiniline bioloogia; B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia; B570 Sünnitusabi, günekoloogia, androloogia, paljunemine, seksuaalsus.

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ABBREVIATIONS

| | |
|--------|-------------------------------------|
| BMI | Body Mass Index |
| CARB-R | Carbohydrate-Restricted |
| CFU | Colony Forming Unit |
| Ct | Thershold Cycle |
| FA | Fatty Acids |
| FAT-R | Fat-Restricted |
| GI | Gastrointestinal (tract) |
| GLP | Glucagon-Like Peptide |
| LD | Lipid Droplet |
| RT-PCR | Real-time Polymerase Chain Reaction |
| PYY | Peptide YY |
| RYGB | Roux-en-Y Gastric Bypass |
| SCFA | Short Chain Fatty Acid |
| SG | Sleeve Gastrectomy |
| T2D | Type II Diabetes |

GENERAL INTRODUCTION

The human intestine is a natural habitat for billions of archaea and bacteria. The human gut microbiota is a complex community, which has a mutualistic relationship with the host organism, having an influence on physiological processes. Characterization of the changes in microbial composition and diversity may help to understand the role of the gut microbiota in health and disease. Although, mechanisms of interactions and changes are not completely understood, it is important to know more about them for future diagnosing and treatment of different pathologies.

Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health (World Health Organization). The intestinal microbiota can be viewed as an 'organ' that may be involved in the development of obesity through metabolic activity and performs functions that host organism cannot perform on its own (Bäckhed et al., 2005). Two dominant phyla of bacteria in human gut microbiota are *Firmicutes* and *Bacteroidetes* (Mahowald et al., 2009). These phyla are shown in association with obesity (Ley et al., 2005).

Bariatric surgery is one of the modalities that is available today for obesity treatment. It is an invasive method used to change the capacity and shape of the stomach. Sleeve gastrectomy (SG) and roux-en-Y gastric bypass (RYGB) are most common currently used bariatric procedures, exerting different physiological changes in the gastrointestinal tract that possibly induce different changes in the gut microbiota and may contribute to different health outcomes such as weight loss and decrease of obesity related diseases. Quantitative characterization of the intestinal microbiota composition before and after bariatric procedures, especially two dominant phyla *Firmicutes* and *Bacteroidetes*, provide important information regarding potential associations between gut microbiota and obesity.

The purpose of this study is to expand our knowledge about the counts of the phyla *Firmicutes* and *Bacteroidetes* and their ratio in faecal samples of obese patients in pre- and post-bariatric surgery periods, as well as to assess the influence of sleeve gastrectomy and roux-en-Y gastric bypass bariatric surgery methods on these phyla and their ratio.

This study was performed at the Institute of Biomedicine and Translational Medicine, Chair of Medical Microbiology and the Institute of Molecular and Cell Biology, Tartu University.

1. LITERATURE OVERVIEW

1.1 Microbiota of human digestive tract

Digestive tract or gastrointestinal tract (GI) is an organ system responsible for extracting and absorbing energy and nutrients from the food. Human digestive tract (Figure 1) is divided into upper and lower tracts. Upper tract consists of oral cavity, esophagus, stomach and duodenum. Lower gastrointestinal tract includes the small intestine, which is subdivided into duodenum, jejunum and ileum, while the large intestine is subdivided into the cecum, colon, rectum and anal canal. (Treuting et al., 2012)

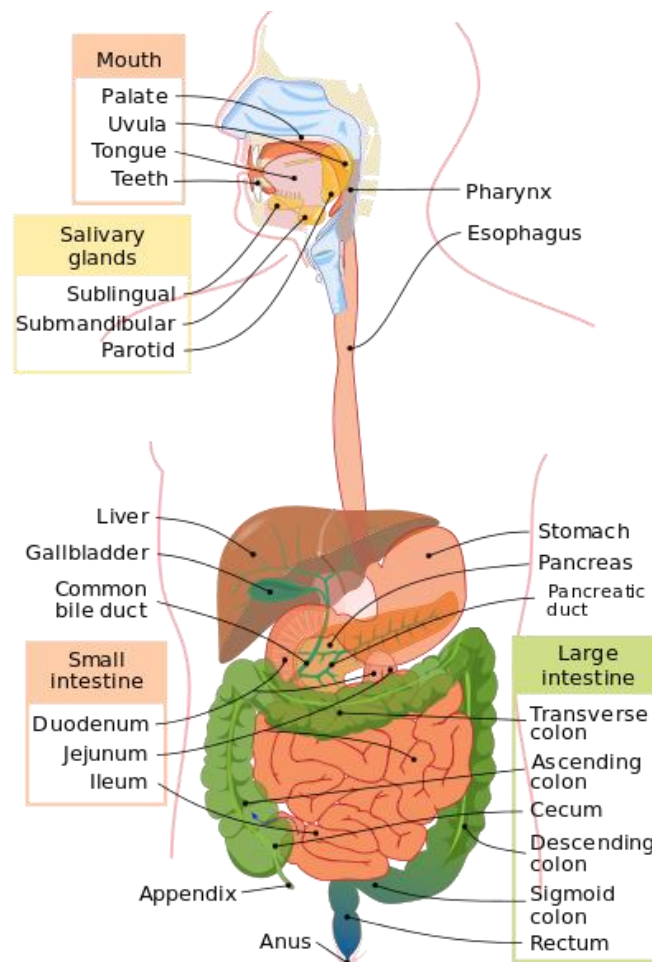


Figure 1. Anatomy of human gastrointestinal tract (Wikimedia Commons).

The human digestive tract is a natural habitat for different bacterial species. It contains a large variety of microorganisms, consisting of over 1000 different species (Huttenhower et al., 2012) and up to 10^{12} bacterial cells in the colon (Marteau et al., 2001). The total genome of human gut microbiota is 150 times larger than the human gene complement (Qin et al., 2010). The diversity of gut microbiota varies according to the location within the digestive tract

(Figure 2) and depends on oxygen availability, temperature and pH level. The most dominated bacteria are members of the *Firmicutes* (Gram-positive) and *Bacteroidetes* (Gram-negative) phyla (Stearns et al., 2011). Two major classes found in gastrointestinal tract from phylum *Firmicutes* are: *Clostridia* and *Bacilli*, however *Erysipelotrichi* and *Negativicutes* have been also determined. Within *Clostridia* class most commonly retrieved families are *Ruminococcaceae*, *Clostridiaceae* and *Lachnospiraceae*, within *Bacilli* class *Bacillales* and *Lactobacillales* orders were detected, nevertheless, it was not possible to identify bacterial families, common between different individuals. Within the *Bacteroidetes* phylum *Bacteroidaceae* family was most frequently recruited, though, *Porphyromonadaceae*, *Prevotellaceae* and *Rikenellaceae* families are also retrieved from almost all individuals. (Peris-Bondia et al., 2011) Composition of the microbiota of the gastrointestinal tract correlates with host's function, physiology and metabolism, including energy balance and storage (Bäckhed et al., 2005), maturation of the host immune system (Mazmanian et al., 2005), vulnerability to disease (Vijay-Kumar et al., 2010) and also social behavior (Bercik et al., 2011).

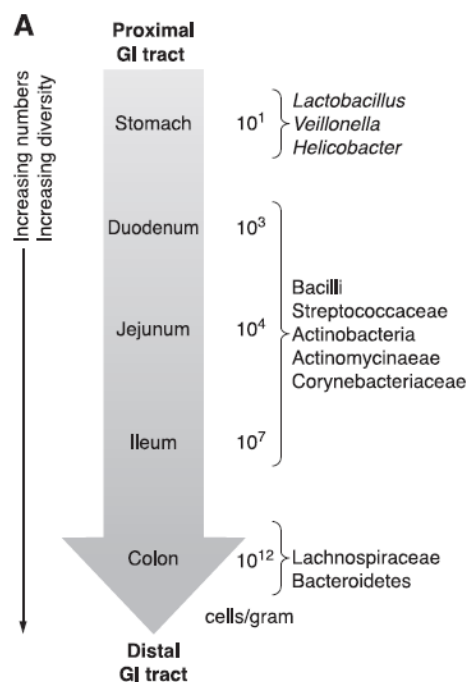


Figure 2. Bacterial biogeography in human gastrointestinal tract (GI) (Sekirov et al., 2010) (bacterial counts are expressed in CFU/g, colony forming units per gram of content).

Human oral cavity is characterized with the mixed microbiota with predominance of Gram-positive aerobic and anaerobic cocci. Average abundance is estimated to be 10^8 CFU (colony forming units) per ml of saliva, considering that organisms are derived from host surfaces, especially the tongue. (Marsh et al., 2016) Bacterial species are not only found in saliva, they

also accumulate on both soft and hard oral tissues to form biofilm. Mouth is mostly colonized by members of *Streptococcus* species (sp.), *Veillonella* sp., *Prevotella* sp., *Lactobacillus* sp., *Corynebacterium* sp., *Fusobacterium* sp. and *Staphylococcus* sp. that constitutes the major population of the upper part of the gastrointestinal tract (Zilberstein et al., 2007).

The epithelial layer of the esophagus is normally wet and has a similar pH to oral cavity (pH = 7) (Pilato et al., 2016). *Streptococcus* sp. and *Staphylococcus* sp. are dominant, but also *Peptococcus* sp. have been detected. (Zilberstein et al., 2007) Bacterial diversity of the esophagus microbiota is significantly smaller compared to the mouth microbiota (Manson et al., 2008). The flora in this segment is mostly transitional, *i.e.* it consists of microbes living on foods or in liquids, which human consumes.

Stomach and duodenum microbiota differs from esophagus and mouth microbiota. The main reasons for that are anaerobic environment and secretory activity, which predetermines low pH (pH = 2). There are five major phyla determined in the stomach: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria* and *Proteobacteria*. (Stearns et al., 2011) Duodenal microbiota predominantly consists of *Firmicutes* and *Actinobacteria*, whereas *Bacteroidetes* are almost completely absent (Angelakis et al., 2015). The abundance of microorganisms here is 10^1 - 10^3 CFU per gram of content (Hakansson et al., 2011) and predominant genera are *Clostridium* sp., *Lactobacillus* sp., *Veillonella* sp. and pathogen *Helicobacter pylori* (Zilberstein et al., 2007).

Microbiota of the lower digestive tract includes bacteria colonizing small (jejunum and ileum) and large intestines (colon, rectum, anal canal). Some of the bacterial genera in the small intestine are similar to upper tract, however microbiota of the large intestine has bigger variety of anaerobic bacteria (Figure 3), and overall diversity is also higher than in the upper tract. In the small intestine the abundance of bacteria is 10^4 - 10^7 CFU/g of content, while in the large intestine is up to 10^{12} CFU/g of content. (Marteau et al., 2001)

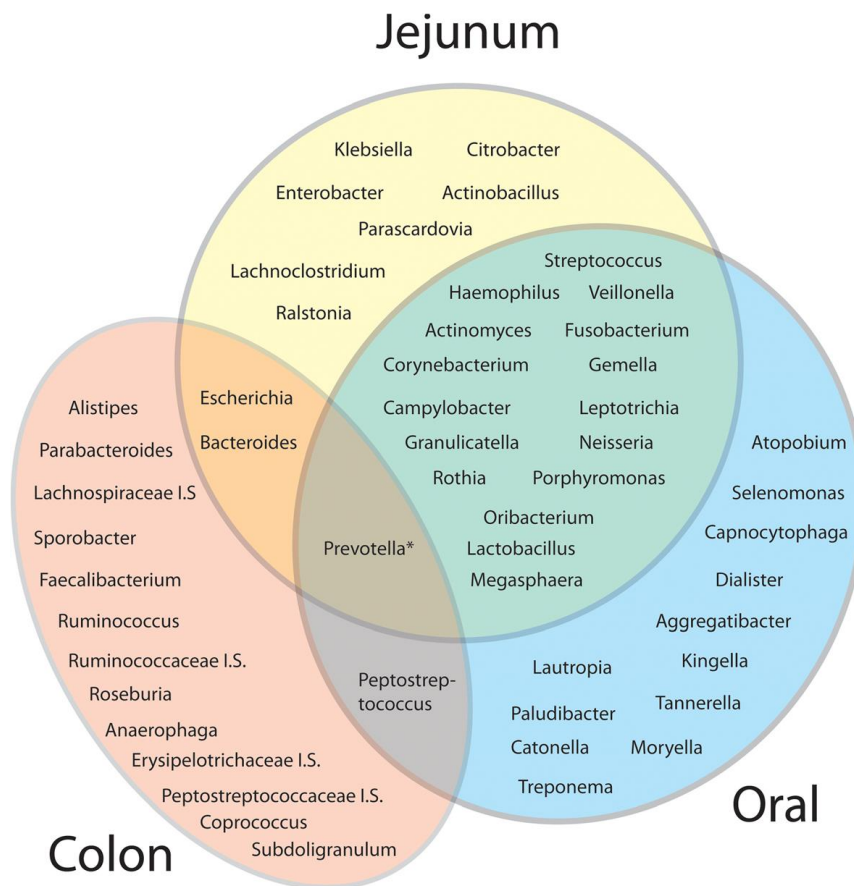


Figure 3. Venn diagram of major bacterial genera of colon, jejunum and mouth (Sundin et al., 2017).

Large surface area of the small intestine absorbs approximately 90% of the overall energy from the diet (Sekirov et al., 2010). The predominant species that inhabit the small-intestinal tract are facultative anaerobes and aerobes belonging to the genera *Lactobacillus*, *Escherichia*, *Enterobacter*, *Streptococcus* and *Klebsiella* (Hayashi et al., 2005). However, it was also reported that *Veillonella* sp. and genera such as *Clostridium* and *Turicibacter* are present in varying amounts per individual (El Aidy et al., 2015).

Microbiota in the large intestine is described by analyzing mucosal and faeces samples (Eckburg et al., 2005). In contrast with the small-intestinal microbiota, the large-intestinal microbiota is higher in number and predominant species are mainly anaerobic (Hayashi et al., 2005). Microbiota is presented mainly by members of the genus *Bacteroides*, *Bifidobacterium* and anaerobic cocci. It has been shown that facultative anaerobes and aerotolerant species from *Lactobacillus* genus also exist here but are less dominant. The dominant genera of colonic microbiota are *Coprococcus*, *Peptostreptococcus*, *Eubacterium* and *Ruminococcus*, but proportions usually vary between individuals. In lower numbers are reported the members of *Fusobacterium*, *Streptococcus*, *Lactobacillus*, *Enterococcus*, *Veillonella*, *Megasphaera*, *Propionibacterium* and *Enterobacteriaceae*. (Shigwedha et al., 2013)

1.2 The metabolic functions of the intestinal microbiota

Mammalian genomes lack of the ability to encode a great diversity of degradative enzymes, which are crucial for digestion (Flint et al., 2012). The metabolic activity of gut microbiota can regulate energy balance via mechanisms that affect energy harvest from the diet as well as modulate genes that affect energy storage (Nehra et al., 2016). Gut microbiota is a rich microbial community, which has predominantly saccharolytic and proteolytic activity. Substrates, which are not utilized in the small intestine move to the large intestine where they can be degraded by the microbiota alongside with endogenous polysaccharides. (Cummings et al., 1986) For example, carbohydrates, which reach the human colon, mainly consist of resistant starch, plant polysaccharides (plant cell wall and reserves) and some oligosaccharides as well as sugars such as inulin, gums, mucilages and fructooligosaccharides (Table 1) (Cummings et al., 1991).

Table 1. Fermentable components that reach the human colon (Lewandowska, 2010)

| Substrate | Component | Amount (g/day) |
|---------------|---|----------------|
| Carbohydrates | Resistant starch | 5-35 |
| | Non-digestible polysaccharides | 10-25 |
| | Oligosaccharides (FOS, GOS, inulin) | 2-8 |
| | Monosaccharides (sugar alcohols) | 2-5 |
| | Mucins | 3-5 |
| | Synthetic carbohydrates (lactulose, polydextrose, modified cellulose) | Variable |
| Proteins | Of dietary origin | 1-12 |
| | Of endogenous origin (pancreatic enzymes and other secretions) | 4-8 |
| | Desquamated epithelial cells | 30-50 |
| Others | Non-protein nitrogen (urea, nitrate) | ~0.5 |
| | Organic acids, lipids, bacterial recycling) | Unknown |

Gut microbiota is also capable of *de novo* synthesis of vitamins, which are essential for the host. B12 vitamin cannot be produced by animals, however, lactic acid bacteria can provide B12 to its host through metabolic reactions. (LeBlanc et al., 2013)

1.2.1 Digestion of polysaccharides

Carbohydrate fermentation reactions in the large intestine are affected by a variety of nutritional, host and dietary factors. The majority of carbohydrate reaching the large intestine are in the form of polysaccharides, which must be depolymerized, before they can be assimilated by the bacteria (Englyst et al., 1987). In this regard, metabolic activity of gastrointestinal tract microbiota is mainly dependent on substrate availability (Cummings et al., 1991). The analysis of gut bacterial community metagenome revealed the presence of high number and variety of genes involved in carbohydrate breakdown (Tasse et al., 2010). Metabolites produced during the fermentation are highly variable in composition and structure

- polysaccharides, oligosaccharides, proteins, peptides and glycoprotein precursors - are transformed into short-chain fatty acids (SCFAs) and gases. The formation of these products is the result of a complex interplay between diet and the gut microbiota within the gut lumen environment. Members of bacterial community compete for a given substrates and may also catalyze complementary reactions. This confers stability of the whole ecosystem. (Blaut, 2015)

The main gaseous byproducts of the fermentation are CO₂ and H₂, which are used by methanogens for CH₄ production (Bauchop et al., 1981), by acetogens and sulfate-reducing organisms for acetate and H₂S production (Gibson et al., 1988).

1.2.2 Digestion of proteins

Connective tissue proteins (elastin and collagen), serum albumins, plant proteins and proteins secreted by the bacteria or released during cell lysis are commonly digested by the colon microbiota (Cummings et al., 1991). It has been shown that the rate of the protein fermentations is increased if the concentration of carbohydrates is high (Birkett et al., 1996). Consequently, the activation of metabolic pathways and then the flow of generated metabolites are highly correlated with the dietary carbohydrate/protein ratio.

In the digestive tract proteins are hydrolyzed by proteases and peptidases, derived from both host and bacteria, into small compartments such as peptides and amino acids (Macfarlane et al., 1988). Some of these products act as energy substrates for the colonic mucosa or can be precursors for metabolic reactions for the synthesis of numerous products (Davila et al., 2013). For instance, *Fusobacteria* and *Clostridia* are able to ferment amino acids to SCFAs and gaseous byproducts such as H₂, NH₃, CO₂ in the absence of electron acceptors (Kim et al., 2004).

1.2.3 Short-chain fatty acids

Short-chain fatty acids (SCFAs) are volatile fatty acids with 1 to 6 straight or branched-chained carbon (C) atoms that are the end products of fermentation of dietary fibers by the anaerobic intestinal microbiota, usually absorbed in the colon of the host and regulating mammalian metabolism. The main SCFAs, formed during this process are acetic (C₂), propionic (C₃) and butyric (C₄) acid in a molar ratio approximately 60:20:20, respectively (Cummings et al., 1979). Other fermentation products such as lactate, ethanol and succinate are the intermediates in the global fermentation process and, to varying extents, are metabolized to SCFA by cross-feeding species in the ecosystem (Bernalier et al., 1999).

Different bacterial species use various fermentation mechanisms. For instance, members of the *Firmicutes* phylum are the main producers of butyric acid (Duncan et al., 2002). The second most abundant bacterial division in human gut is *Bacteroidetes*, which mostly produces propionic and acetic acid (Rey et al., 2010). One of the studies suggested that faecal SCFA concentrations are higher in lean than in obese individuals (Schwiertz et al., 2010). Consequently, the *Firmicutes/Bacteroidetes* ratio has an impact on SCFAs absorption, having and influence on energy harvesting in host.

SCFAs have distinct physiological effects: they contribute to shaping the gut environment, stimulating cell-proliferation, affecting mineral balance, regulating the metabolism of lipids and glucose (Nowak et al., 2008) and they also can be oxidized and used as energy sources by host tissues (Roediger, 1980).

Acetic acid is one of the main SCFAs that has an effect on host physiology. Acetate is easily determined from blood samples and therefore it is applicable for monitoring the health of the colon. Acetic acid is metabolized by peripheral tissues and is a substrate for the synthesis of cholesterol and long-chain fatty acids (Wolever et al., 1989). Acetate reduces appetite through central hypothalamic mechanism (Frost et al., 2014), stimulates proliferation of normal crypt cell, enhances ileum motility and increases the colonic blood flow (Scheppach, 1994). One of the studies showed that acetate may have a role in adipogenesis, being the ligand for G protein-coupled receptor (GPCR) GPCR43 on adipose tissue. GPCRs are transmembrane receptors, which activate cellular responses, regulating food intake, energy expenditure and body weight. (Hong et al., 2005)

Propionic acid has a potential role in lipogenesis, being the ligand for GPCR43 and affecting adipocyte differentiation (Hong et al., 2005). It has an ability to increase spontaneous contractions of the colonic smooth muscle, resulting in formation of faecal pellets (Ono et al., 2004). Propionate, being the ligand for GPCR41, increases the production of leptine - a satiety hormone in human (Xiong et al., 2004).

Butyric acid is the principal energy compound for intestinal epithelial cells, therefore it is believed to maintain a normal colonocyte population. Being the main fuel for these cells, butyrate plays a critical role in moderating cell growth and differentiation. (Topping et al., 2001) Butyrate is able to promote satiety similarly to propionate (Zhou et al., 2006).

1.2.4 Digestion of dietary fats

Dietary fats are digested in the form of triglycerides by lipases within the intestinal lumen. As the result of this process fatty acids (FA) are released and absorbed in the intestinal epithelium

by enterocytes (Karasov et al., 2007). Later they may be oxidized to generate energy, re-esterified into triglycerides and temporarily stored as cytoplasmic lipid droplets (LD), incorporated into chylomicrons (small lipoprotein particles) for secretion into the lymph (Hussain, 2000) or released into circulation as free fatty acids (Figure 4) (Iqbal et al., 2009).

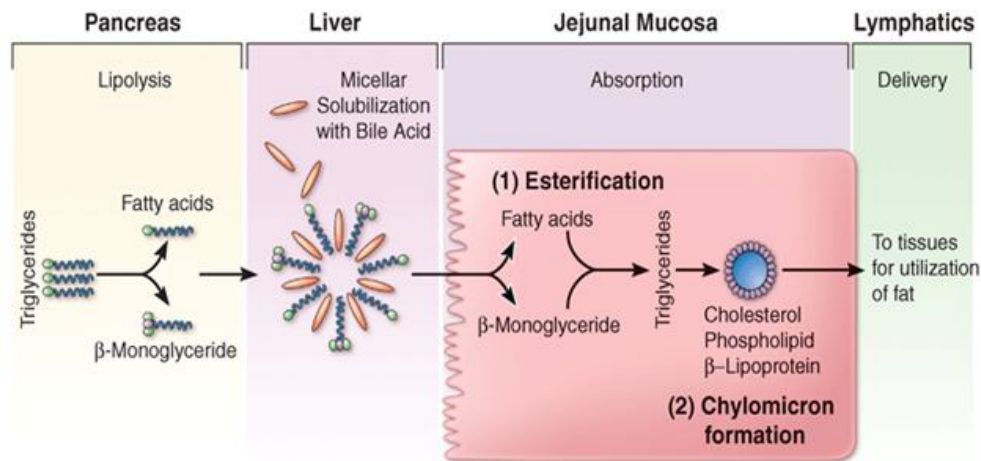


Figure 4. Lipid digestion and absorption by host (Chhabra, 2013).

Microorganisms contribute to lipid absorption by host tissues, influencing LD accumulation and FA absorption by enterocytes using four nonexclusive mechanisms. Microbes may increase biological availability of FAs, modifying the composition or production of bile salts. Bile acids are cholesterol derivatives, which act like detergent molecules, important for absorption of dietary fats and liposoluble vitamins in the small intestine and the maintenance of cholesterol homeostasis in the liver. Bile acids are also recognized as signaling molecules with systemic endocrine functions. (Swann et al., 2011) Furthermore, microbes are able to change the absorption of FA, contributing directly to luminal lipolytic activity (Ringø et al., 1995). Intestinal bacteria are capable of evoking physiologic responses in the intestinal epithelium to indirectly enhance its capability to absorb FAs. Microbiota promotes not only absorption, but also export of FAs to the liver LDs regardless of diet history. Finally, microbes in gut may reduce the oxidation rate of FAs, increasing their storage in LDs, affecting LD size regardless of diet history and increasing LD number. (Semova et al., 2012)

1.3 Human gut microbiota and obesity

Intestinal microbiota performs chemical reactions, which are important for regulation of metabolic activity of the organism. The proper symbiotic relationship between the host and bacteria provides appropriate development of the metabolic system. However, disturbances in this communication can result in dysregulation of the immune system and also contribute to mechanisms, which are important in the development of diseases such as obesity (Turnbaugh

et al., 2006). Obesity is a disease, described as an imbalance between energy consumption and expenditure, which course is influenced by different genetic (O’Rahilly et al., 2009), cultural, behavioral (Ogden et al., 2007), environmental (Mujico et al., 2013) and microbial factors (Turnbaugh et al., 2006).

Microbiological research continues to enrich the knowledge of the relationship between metabolic disorders and intestinal microbiota by providing additional ways to treat them. One of the most recent researches showed that *Lactobacillus plantarum* (LP) and *Lactobacillus fermentum* (LF) alone or in combination have weight-lowering effects in rats. The study found that food enriched with LP, LF or both has an influence on the size of adipose cells in fat tissue. (Li et al., 2018) Hou et al. (2017) observed that the amount of *Bifidobacterium* and *Lactobacillus* increases among the obese children during the process of weight loss, and offered that *Bifidobacterium* and *Lactobacillus* might be used as indicators of healthy conditions among obese patients, as well as a prebiotic and probiotic supplement in the diet for obesity treatment. (Hou et al., 2017)

The entire human gastrointestinal tract is covered by mucus layer. Its main functions are to cover epithelial cells and protect them from pathogens due to the inhibition of host-pathogen interaction. (Moal et al., 2006) Mucins are a family of glycoproteins and main organic components of mucus layer. Important commensal and probiotic bacteria use mucins as substrates for fermentation, degrading these proteins with specific enzymes. (Derrien et al., 2010) *Akkermansia muciniphila* is mucin-degrading bacteria, which is abundantly present in the human intestinal tract, making up to 1–4% of the bacterial population in the colon (Collado et al., 2007). Everald et al. (2013) demonstrated that the abundance of *A. muciniphila* was lower in obese and type II diabetic (T2D) mice than in lean mice gut microbiota. It was proposed that the metabolic products of *A. muciniphila* play an important role in maintaining and normalizing gut microbiota. (Everard et al., 2013)

Despite the fact that microbiota of gastrointestinal tract varies from host to host, *Bacteroidetes* and *Firmicutes* are two bacterial divisions, which maintain dominance in the human gut (Stearns et al., 2011). Changes in the *Firmicutes/Bacteroidetes* ratio have been associated with obesity development (Ley et al., 2005; Ley et al., 2006).

Human and mouse are quite similar in physiology and anatomical structures, in this regard mouse is a biomedical model, which is accepted for research purposes. Ley et al. (2005) analyzed 16S rRNA sequences of the distal intestinal (cecal) microbiota in genetically obese, lean and wild-type mice. The distal intestinal microbiota of obese mice had a statistically

significant 50% reduction in abundance of *Bacteroidetes*, relative to lean mice, and a significantly greater proportion of *Firmicutes*. These changes were not associated with specific bacterial subgroup, which means that they were division-wide. No subgroup was amplified or lost due to these divisions. (Ley et al., 2005)

Turbaugh et al. (2006) studied energy harvesting using cecal microbiota transplantation method from obese humans to germ-free mice. Recipients demonstrated a greater increase in body fat if their gastrointestinal tract was colonized with 'obese microbiota' rather than 'lean microbiota'. Researchers also speculated that these changes in microbiota correlate with energy harvest from diets and changes of body index within an individual, which in its turn contributes to the pathophysiology of obesity. (Turnbaugh et al., 2006)

1.4 Surgical treatment of obesity

One of the most effective and successful treatment methods of obesity and type 2 diabetes is bariatric or metabolic surgery (Dixon et al., 2011). Bariatric surgery stimulates weight loss by restriction (reduces the quantity of food intake) or malabsorption (reduces the absorption) or both (Karmali et al., 2010). The two most commonly performed bariatric surgery procedures are roux-en-Y gastric bypass (RYGB) and sleeve gastrectomy (SG) (Figure 5) (Buchwald et al., 2013).

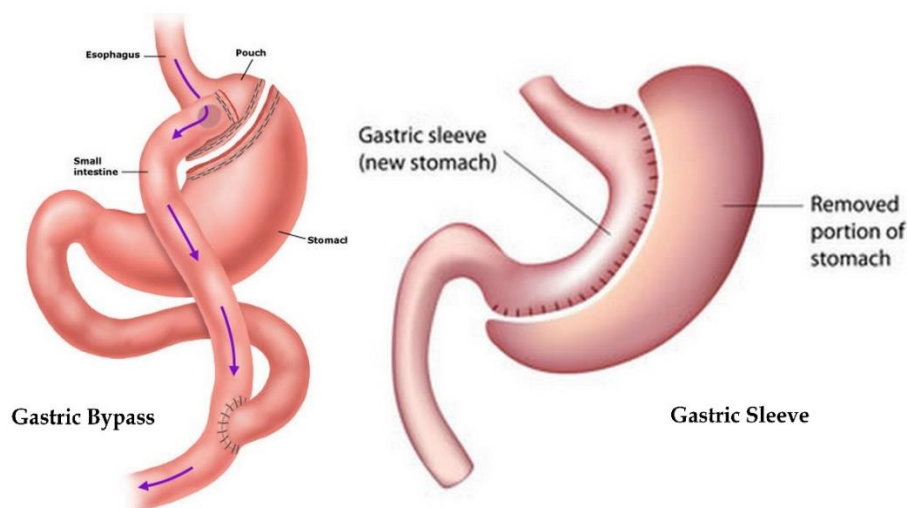


Figure 5. Bariatric surgery procedures. Left - roux-en-Y gastric bypass (RYGB), right - sleeve gastrectomy (SG) (Harley Street Bariatrics, 2014).

RYGB reduces the stomach capacity due to the creation a small gastric pouch while absorption is reduced by jejunojunostomy (dividing the small intestine into two parts, creating a bypass). The lower part of the divided jejunum (Figure 5, left) is integrated directly into the gastric pouch, while the other is surgically connected to the certain place down the

small intestine. (Schauer et al., 2003) As a result, the food 'bypasses' the upper part of the intestine. Therefore, gastric bypass is both restrictive and malabsorptive method. This procedure might lead to conditions that increase energy expenditure, nevertheless, it is considered to be technically complex and difficult. In addition, RYGB has the potential for long-term vitamin and mineral deficiencies. (American Society for Metabolic and Bariatric Surgery)

On the other hand, gastric sleeve is a purely restrictive modality. The stomach is transected, leaving a narrow stomach tube (Figure 5, right). All the curvatures, junctions and sizes must be calibrated, insuring proper intake and absence of leaks. (Darwish et al., 2017) Consequently, the capacity of the stomach is minimized and lesser curvature is preserved as the result of this procedure. This method does not require bypass or re-routing the food stream, which usually involves a relatively short hospital stay in contrast to RYGB. However, this procedure also can lead to long-term vitamin/mineral deficiencies, requiring lifelong supplementation and adherence to dietary recommendations. (American Society for Metabolic and Bariatric Surgery)

Gastric bypass surgery remodels gut microbiota, resulting in an increase in the relative abundance of *Gammaproteobacteria (Escherichia)*, which has not only pathogenic but also nonpathogenic commensal species, and *Verrucomicrobia (Akkermansia)*, which have a certain role in regulating host weight and adiposity. Additionally, it was reported that transfer of the gut microbiota from RYGB-treated mice to non-treated, germ-free mice promoted weight loss and decrease of the fat mass in the recipient animal. (Liou et al., 2013) One of the recent studies reported that unlike gastric bypass, gastric sleeve does not increase gut microbiota diversity, nevertheless it enhances the abundance of the *Bacteroidetes* phylum (Murphy et al., 2017).

Ghrelin is a hormone responsible for inducing appetite in rodents and humans. It has been shown that RYGB and SG are associated with suppressed ghrelin levels, which is believed to contribute to the weight-reducing effect of the whole procedure (Cummings et al., 2002; Tsoli et al., 2013). Hormones, glucagon-like peptide (GLP)-1 and peptide YY3–36 (PYY), are also important in appetite reduction, their levels increase after RYGB (Ochner et al., 2011). However, it was noticed that patients subjected to the gastric sleeve method had a greater appetite loss than RYGB group (Karamanakos et al., 2008). Bile acids are acknowledged as molecules with endocrine functions (Houten et al., 2006) and found to stimulate the release of hormones GLP-1 and PYY (Pournaras et al., 2012). It was discovered that bile acid concentrations are reduced in obese individuals, while RYGB has been reported to normalize

bile acid deviations (Ahmad et al., 2013). Both surgery procedures have an effect on weight loss resulting in an increased insulin sensitivity, improved β -cell functions and glucose tolerance (Bradley et al., 2014).

1.5 Real-time PCR in microbiological research

Real-time polymerase chain reaction (real-time PCR; RT-PCR) is widely used as a molecular approach, for quantification of specific bacterial groups in human microbiota. This method uses a chemiluminescent reaction to determine the kinetics of product accumulation during PCR amplification with specific primers for a specific group or species of bacteria. The product accumulation rate curves are usually used for calculation of number of original target molecules in a sample. (Espy et al., 2006)

There are two different approaches for RT-PCR: using DNA binding dyes such as SYBR-Green I and Taqman assay. Both methods are sensitive and rapid, however, principles of detection and optimization of these assays are different.

SYBR-Green I binds to double stranded PCR product increasing the level of fluorescence. The level of fluorescence detected is directly related to the amount of amplified target in each PCR cycle. (Wittwer et al., 2001) The primary disadvantage of the SYBR-Green I dye chemistry is that it may generate false positive signals; *i.e.* because the SYBR-Green I dye binds to any double-stranded DNA, it can also bind to nonspecific double-stranded DNA sequences. However, method is cheaper than Taqman assay because no additional probe is required. For additional support, the melting curve can be performed for SYBR-Green I assay, giving information about reaction quality.

Taqman assay is based on Taq polymerase 5' nuclease activity, which is able to cleave a non-extendible hybridization probe during the extension phase of PCR. Probes are labeled with two different fluorescent dyes: reporter and quenching dye. After denaturation stage Taqman probe and primers bind to its specific sequence of the template DNA. During the extension of the strand Taq polymerase then adds nucleotides and removes the probe from the template, removing quencher from the reporter and allowing the reporter to give off its energy, resulting in fluorescence. (Heid et al., 1996) The more denaturing and annealing occurs, the more Taqman probe binds and, in its turn, the higher is the level of fluorescence. Contrastingly to SYBR-Green I, Taqman assay provides a higher level of specificity and quantitation (Ponchel et al., 2003). However, this method still suffers from high sensitivity to contamination and requires the synthesis of probes, resulting in higher cost of the assay.

16S ribosomal RNA (rRNA) is the component of the 30S small subunit of a prokaryotic ribosome that binds to the Shine-Dalgarno sequence. The genes coding this component are referred to as 16S rRNA genes and are used in phylogeny, due to the slow evolutionary rate of this gene region. (Woese et al., 1977) Using these primers, it is possible to identify and quantify 16S rRNA genes, which allows measuring specific microbial population in samples.

Fluorescence data generated in real-time PCR assays are collected and analyzed with specific programs from PCR cycles that occur within the linear amplification portion of the reaction, where conditions are optimal and the fluorescence accumulates in proportion to the amplicon (Figure 6) (Mackay et al., 2002). Baseline shows early reaction cycles, in which little change in fluorescence signal is observed. Cycle threshold (Ct) is the fractional cycle number at which the fluorescent signal reaches the early exponential phase (Mackay, 2004). Every sample is characterized by its Ct value, which is proportional to the logarithm of the target copy numbers (Wilhelm et al., 2000).

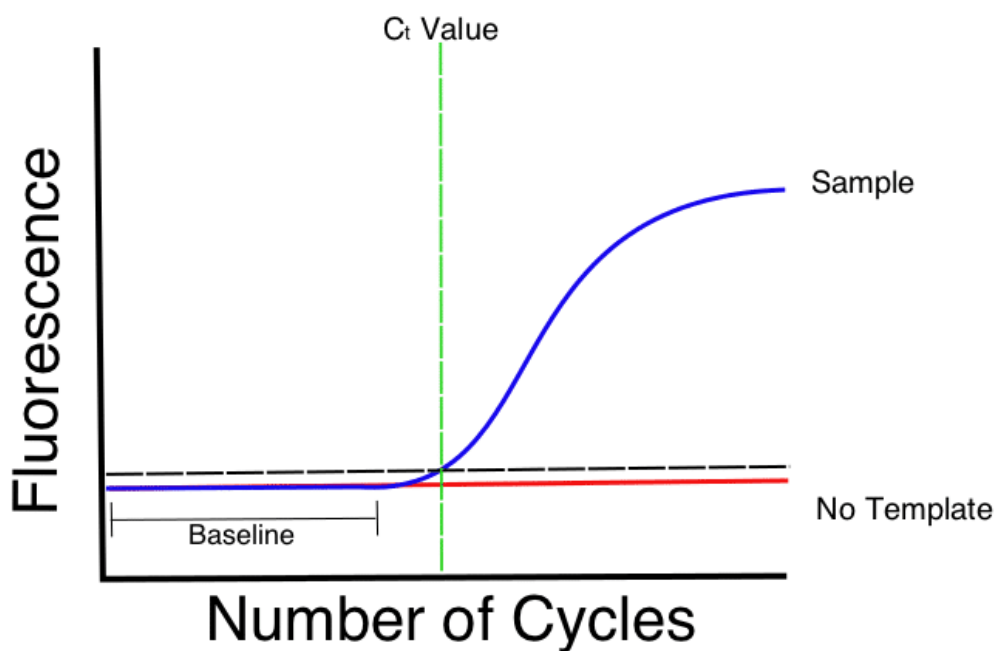


Figure 6. Amplification curve of real-time PCR, when plotted as fluorescence intensity (Porterfield, 2015).

It is necessary to arrange controls after the reaction. No template control should be arranged in order to verify amplification quality. Endogenous controls are used in order to exclude false-negative reactions. Performing this control usually includes amplifying household sequences (Niesters, 2001). The common practice for quantification is to use standard curve method, which is retrieved from the exogenous control (Mackay, 2004). This control is performed using a cloned amplicon, a portion of the target organism's genome, or simply

purified amplicon itself (Borson et al., 1998). Diluted amplicon series are created and used as templates in real-time PCR reaction, generating the standard curve. Later, the concentration of an unknown sample, amplified in the same reaction, but separate vessel, can be found from the curve. (Mackay, 2004)

Due to the fact that real-time PCR method is sensitive, relatively cheap and cross functional, it can be widely used in different microbiological research to quantify bacteria from various samples, including faeces (Requena et al., 2002; Malinen et al., 2003; Matsuki et al., 2004), dairy products and food (Kao et al., 2007). One of the studies used primers, which were specific for 16S rRNA genes of faecal bacteria, in order to estimate the total amount of faecal contamination in water (Layton et al., 2006). The use of multiple primer sets to amplify multiple templates within a single reaction has been investigated, but in some cases it has been considered to be problematic due to the limited quantity of fluorophores available for detection (Wittwer et al., 2001). Real-time PCR assay can also be performed for clinical pathogen diagnostics (Klein, 2002).

Real-time PCR was also used for defining the ratio of *Firmicutes* and *Bacteroidetes* in faecal samples, which are believed to be related to obesity syndrome. Guo et al. (2008) used specific bacterial primers to investigate the relative abundance of these two bacterial divisions. (Guo et al., 2008).

The current study is a part of the bigger research project, supported by Estonian Research Council (grant No. IUT34-19), Estonian Ministry of Education and Research (grant No. KOGU-HUMB) and Enterprise Estonia (grant No. EU48695) and was conducted at the Institute of Biomedicine and Translational Medicine. The aim of this study is to use the 16S rRNA gene specific primer pairs in real-time PCR method to quantify the number of bacteria belonging to the phyla *Firmicutes* and *Bacteroidetes* (more precisely genera *Bacteroides*, *Prevotella* and *Porphyromonas*, because these genera are the most abundant and found throughout the human intestine).

2. EXPERIMENTAL PART

2.1. Aims of the study

The purpose of the study was to compare the counts of the phyla *Firmicutes* and *Bacteroidetes* (more precisely genera *Bacteroides*, *Prevotella* and *Porphyromonas*) and their ratio in faecal samples of pre- and post-bariatric surgery patients as well as to assess the influence of bariatric surgery methods.

The present study set the following specific aims:

1. to assess the clinical data of pre- and post-bariatric surgery patients;
2. to quantify the phyla *Firmicutes*, *Bacteroidetes* and their ratio in faecal samples before and after the bariatric surgery;
3. to assess the influence of gender on the changes of studied bacterial phyla and their ratio after bariatric surgery.

2.2. Materials and methods

2.2.1. Study group

The study was carried out at Department of Microbiology, Institute of Biomedicine and Translational Medicine, University of Tartu in collaboration with Tartu University Hospital. The study group comprised 59 pre-bariatric (38 females and 21 males; age: 46.2±9.9 and 45.5±9.6 years) and 32 post-bariatric (23 females and 9 males; age: 47.5±9.7 and 47.2±9.6 years) attending the Surgery Clinic, Abdominal surgery department of Tartu University Hospital between March 2015 - December 2016. The experimental design applied to these cohorts was approved by the Ethics Committee of the Medical Faculty of the University of Tartu with approval No. 241T-13. The present study was conducted according to the guidelines laid down in the Declaration of Helsinki.

Subjects were exempted from acute or chronic inflammatory diseases, infectious diseases, viral infection, cancer, and/or known alcohol consumption. No antibiotics were taken before surgery or during the post-surgery follow-ups.

Participants' body weight was measured in light clothing to the nearest 0.1 kg using a calibrated scale. Height was measured without shoes to the nearest 0.1 cm using a vertical ruler. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m²).

2.2.2. Sample collection and preparation

Faecal samples were collected from patients 1 month before and 1-year after bariatric surgery. Fresh stool samples were placed into sterile containers. The samples collected at home were kept in a domestic refrigerator at 4°C for no more than 2 hours before transportation to the laboratory. Upon arrival, samples were mechanically homogenized with a sterile spatula, divided into aliquots and stored at -70°C until future molecular microbiological isolation.

2.2.3. Bacterial strains and culture conditions

Two culture collection strains *Bacteroides fragilis* ATCC 25285 and *Lactobacillus acidophilus* ATCC 4356 from the American Type Culture Collection (ATCC), were used to evaluate the specificity of PCR primer sets. *B. fragilis* was cultivated in an anaerobic glove chamber (Sheldon Manufacturing, Inc., Shel LAB, USA) with a gas mixture of CO₂/H₂/N₂:5/5/90% at 37°C on FAA (Fastidious Anaerobe Agar, LAB, UK) media and de Man-Rogosa-Sharpe agar (MRS; Oxoid) was used for cultivation of *L. acidophilus* in microaerophilic atmosphere (10% CO₂) at the same temperature.

2.2.4. DNA extraction from bacterial cultures and faeces

Bacterial DNA from faecal samples was extracted using a QIAamp DNA stool mini kit (QIAGEN, Hilden, Germany) with some modifications. 220 mg of faeces were resuspended in 200 µl of TE buffer (10 mM Tris, 10 mM EDTA pH=8, 20 mg/ml lysozyme, 200 u/ml mutanolysin) and incubated for 1 hour at 37°C (Štšepetova et al., 2011). The protocol was continued according to manufacturer instructions. Extracted DNA was quantified using NanoDrop™ 1000 Spectrophotometer 1.0 (NanoDrop Technologies, Inc., USA) at 260 nm.

2.2.5. Real-time PCR

In order to establish a quantitative assay, the 16S rRNA gene sequences of standard strains *L. acidophilus* and *B. fragilis* were amplified with specific primers: (Firm934F: 5'-GGAGYATGTGGTTTAATTTCGAAGCA-3' and Firm1060R: 5'-AGCTGACGACAACCATGCAC-3' (126 bp; Guo et al., 2008) and BAC-F: 5'-GGTGTCGGCTTAAGTGCCAT-3', BAC-R: 5'-CGGA(C/T)GTAAGGGCCGTGC-3' (140bp; Malinen et al., 2005), respectively. A reaction mixture (50µl) consisted of 25 µl Maxima HotStart PCR Master Mix (2X Hot Start PCR buffer, 400µM concentration of each deoxynucleoside triphosphate, 4 mM Mg²⁺), 1µM of each primer, 100 ng of bacterial DNA, HotStart Taq DNA polymerase (Fermentas, Lithuania) and water. The amplification program consisted of pre-denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. A cycle of 72°C for 10 min concluded the program. Amplification products were analyzed by agarose gel electrophoresis on 1.5% agarose gel in 1x TAE buffer with 100 bp DNA ladder (Fermentas, Lithuania), ethidium bromide staining (0.5 µg/ml) and UV transillumination. PCR products were purified with the QIAquick PCR purification kit (QIAGEN).

Purified PCR product was then cloned into the pGEM-T Easy vector system and recombinant vector were transformed into chemically competent *E. coli* JM109 cells (Promega, Madison, USA). Vectors were purified with NucleoSpin Plasmid QuickPure Kit according to manufacture instruction (Macherey-Nagel, Germany) and DNA concentration was quantified spectrophotometrically (NanoDrop ND-1000, USA). Obtained plasmids containing 16S rRNA gene sequences of *B. fragilis* or *L. acidophilus* were used as the standards for quantification of *Bacteroidetes* or *Firmicutes* assays, respectively. For standard curves serial tenfold dilution from 3·10⁵-3·10¹ plasmid copies per reaction of the previously quantified plasmid were used. Standard curves were generated by plotting the number of threshold cycles (Ct) of standard solutions against the logarithm of number of plasmid copies in the

standard solution. The functions describing the relationship between Ct (threshold number) and x (copy number) for both bacterial groups (Figure 7).

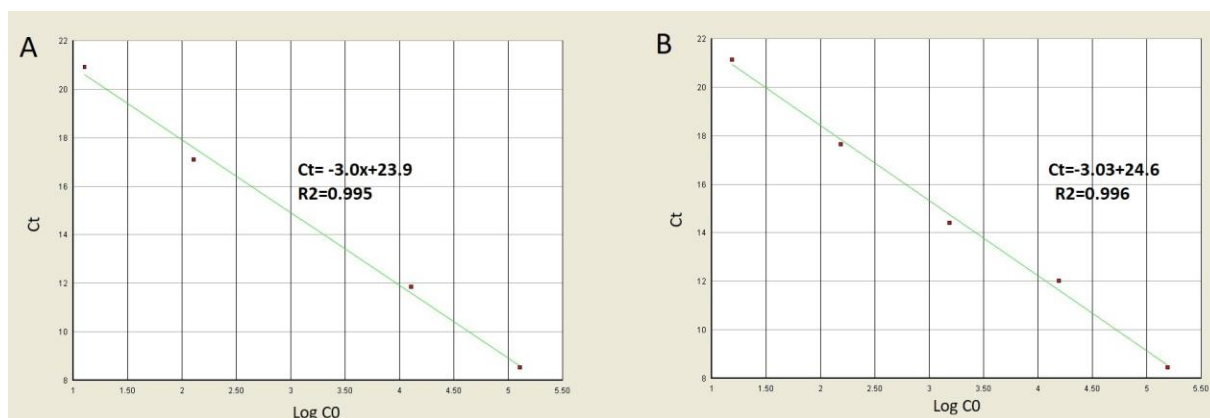


Figure 7. Standard curves for the *Firmicutes* phylum (A) and *Bacteroides-Prevotella-Porphyromonas* group (B) (Ct shows threshold number; Log C0 shows copy number; each point shows the value of copy number).

Amplification and detection of DNA by real-time PCR was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems Europe BV, Zug, Switzerland) using optical-grade 96-well plates. Triplicate sample analysis was routinely performed in a total volume of 25 μ l using SYBR Green PCR Master Mix (Applied Biosystems). Each reaction included 5 μ l of template DNA or water (no-template control), 12.5 μ l of SYBR Green Master mix (Applied Biosystems, USA), 4mM MgCl₂ and the appropriate primers for the *Firmicutes* phylum (Firm934F and Firm1060R, Guo et al., 2008) and for *Bacteroides-Prevotella-Porphyromonas* group (BAC-F and BAC-R, (Malinen et al., 2005), above mentioned, with concentration 150 nM and 300 nM, respectively. The real-time PCR conditions were set as follows: 2 min at 50°C; 10 min at 95°C; and 40 cycles of 15 s at 95°C and 1 min at 60°C.

A melting curve analysis was done after each amplification. Standard curves were routinely performed for each real-time PCR run using serial dilutions of control plasmid DNA. Data were analyzed using the Sequence Detection Software version 1.6.3 (Applied Biosystems, USA).

2.2.6. Statistical analysis

The statistical analysis was performed using SIGMASTAT 2.0 (Jandel Scientific Corporation, San Rafael, CA, USA). Clinical and microbiological data were expressed as mean \pm standard deviation (SD) (\log^{10} plasmid gene copies per gram of faeces) and compared by t-tests or the Mann-Whitney rank-sum test according to the distribution of data.

2.3. Results

2.3.1. Clinical data

In general, all patients after surgical interventions (32 of 59 patients) showed a significant reduction in BMI. The mean preoperative BMI in the study group was 45 ± 5.67 kg/m² vs 29.5 ± 3.7 kg/m² in post-bariatric group ($p=0.001$).

2.3.2. Quantitation of the phyla *Firmicutes*, *Bacteroidetes* and their ratio in faecal samples of pre- and post-bariatric surgery patients

In order to examine the counts of the *Firmicutes* phylum in faecal samples of pre- and post-bariatric surgery patients real-time PCR method was used. The counts of the *Firmicutes* phylum were higher in post-bariatric surgery patients in comparison to pre-bariatric patients (\log^{10} plasmid gene copies/g faeces 8.89 ± 0.62 vs 9.29 ± 0.54 ($p=0.004$)) (Figure 8).

The counts of the *Bacteroidetes* phylum in faecal samples of pre- and post-bariatric surgery patients assessed by real-time PCR method were slightly higher after bariatric surgery 8.54 ± 1.07 vs 8.96 ± 0.94 (\log^{10} plasmid gene copies/g faeces) (Figure 8), but the difference from that of pre-surgery value was not statistically significant ($p=0.070$).

The *Firmicutes/Bacteroidetes* ratio in faecal samples of patients after bariatric surgery was not statistically different (1.05 ± 0.1 vs 1.04 ± 0.06 , $p=0.957$).

The ratio was analyzed as the counts of the *Firmicutes* phylum of every patient divided by the counts of the *Bacteroidetes* phylum of the same patient and is shown as mean \pm SD.

The differences in counts of the phyla *Firmicutes*, *Bacteroidetes* and their ratio were also assessed in association with gender and surgical method, however, the results were not statistically significant ($p>0.05$, data not shown).

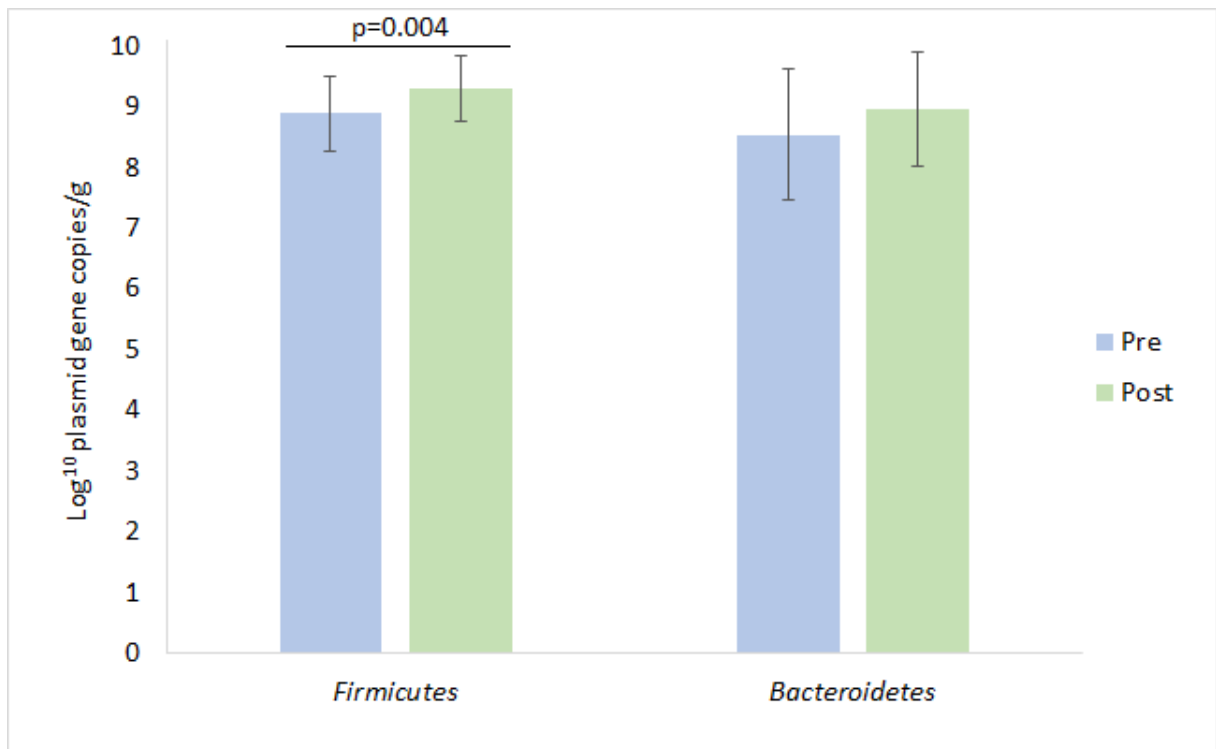


Figure 8. The counts of bacteria belonging to *Firmicutes* and *Bacteroidetes* phyla in faecal samples before (N=59) and after (N=32) the bariatric surgery (Charts indicate mean value \pm standard deviation (SD)).

2.3.3. Comparison of BMI, the counts of the phyla *Firmicutes*, *Bacteroidetes* and their ratio in faecal samples of patients having both pre- and post-surgery samples

Due to the fact that previously compared groups contained a significantly different number of patients (59 before vs 32 after), the analysis of data obtained from the faecal samples of 25 patients (17 females and 8 males) who gave both pre- and post-surgery samples was done. The mean BMI of this group before bariatric surgery was 43.9 ± 5.5 kg/m² and 29.7 ± 3.9 kg/m² in post-operative group ($p < 0.001$). SG method was performed in 14 patients (9 females and 5 males) and 11 patients (8 females and 3 males) were treated by RYGB method. We considered BMI changes in association with gender and surgery method, however, the differences were not found.

The counts of both phyla *Firmicutes* (8.75 ± 0.55 vs 9.3 ± 0.51 , $p < 0.001$) and *Bacteroidetes* (8.3 ± 0.83 vs 9.04 ± 0.91 , $p = 0.004$) increased after bariatric surgery (Figure 9). The difference in the *Firmicutes/Bacteroidetes* ratio was not found (1.059 ± 0.07 vs 1.034 ± 0.06 , $p = 0.342$) (data not shown).

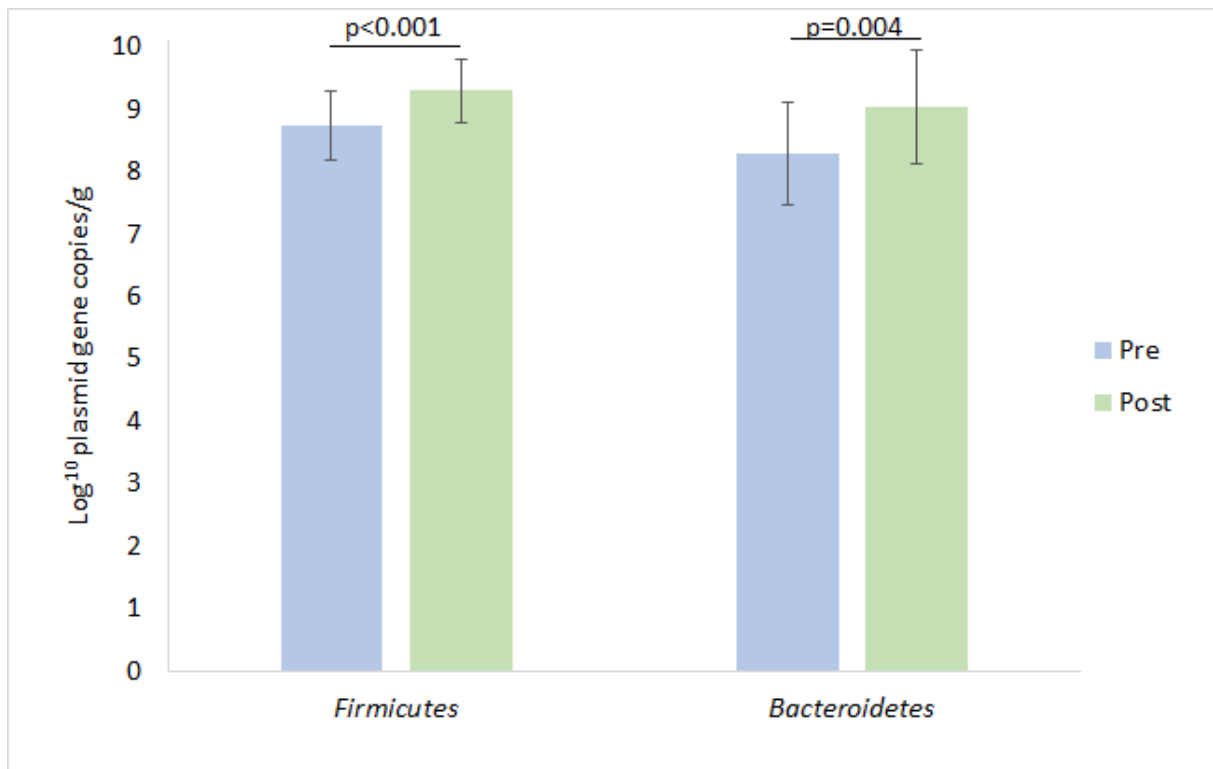


Figure 9. The counts of bacteria belonging to the phyla *Firmicutes* and *Bacteroidetes* before and after the bariatric surgery in faecal samples of the same patients (N=25) (Charts indicate mean value \pm standard deviation (SD)).

The counts of bacteria belonging to the *Firmicutes* phylum decreased (9.25 ± 0.57 vs 8.77 ± 0.48 , $p = 0.024$) after SG method (N=14), contrastingly, it increased (8.7 ± 0.65 vs 9.4 ± 0.4 , $p = 0.012$) after RYGB surgical intervention (Figure 10).

The abundance of *Bacteroidetes* increased after both surgical methods (8.2 ± 0.7 vs 8.89 ± 0.95 , $p = 0.041$ for SG; 8.4 ± 0.97 vs 9.3 ± 0.86 for RYGB, $p = 0.053$ (borderline of statistical significance)) (Figure 10).

The difference in the *Firmicutes/Bacteroidetes* ratio in pre- and post-operational period in association with surgical method (SG or RYGB) was also studied, but the change in this ratio was not found statistically significant ($p > 0.05$, data not shown).

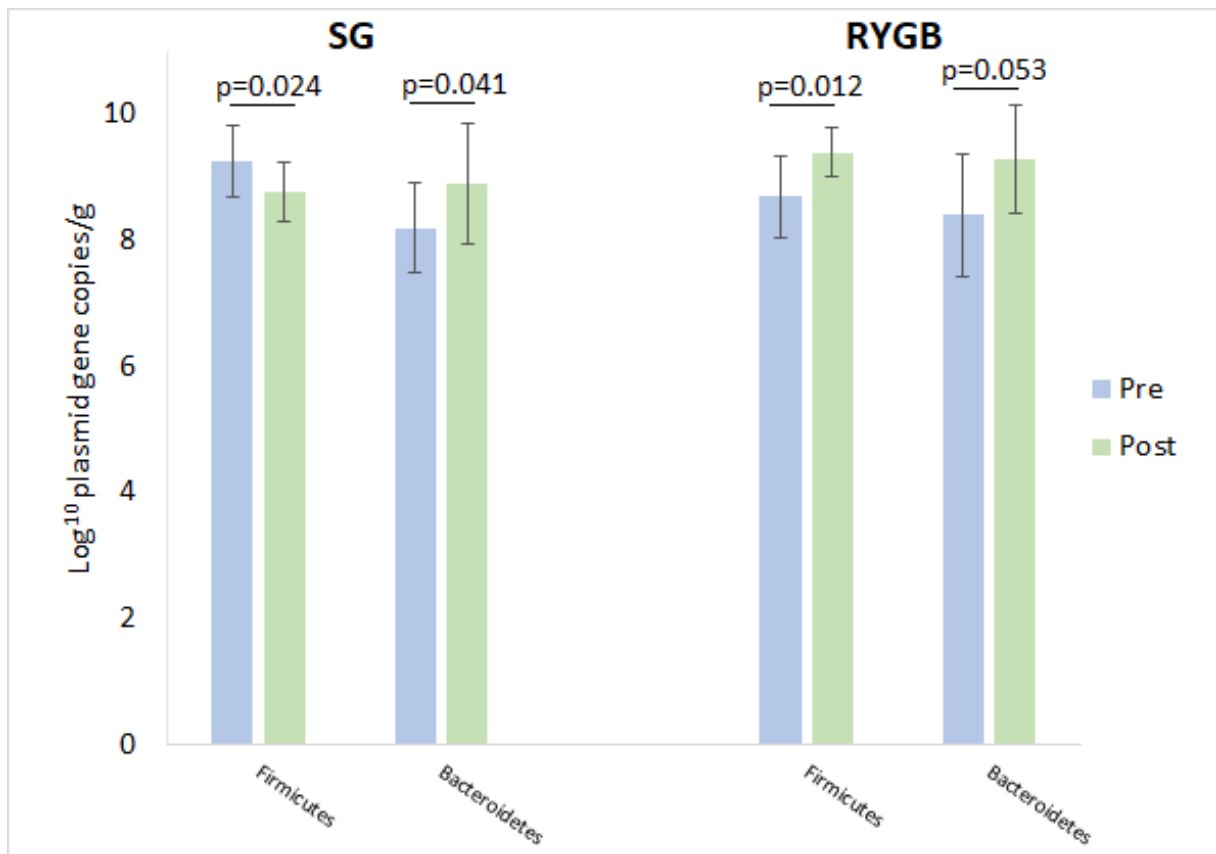


Figure 10. The counts of bacteria belonging to the phyla *Firmicutes* and *Bacteroidetes* before and after SG (N=14) and RYGB (N=11) methods in faecal samples of the same patients (Charts indicate mean value \pm standard deviation (SD)).

The change in counts of *Firmicutes* in faecal samples of patients (N=25, both pre and post-surgery samples) in association with gender was analyzed. The abundance of this phylum was increased for females (8.79 ± 0.5 vs 9.29 ± 0.5 , $p=0.01$) and decreased for males (9.36 ± 0.5 vs 8.68 ± 0.6 , $p=0.032$) (Figure 11).

The abundance of the *Bacteroidetes* phylum was higher in faecal samples of male group (7.9 ± 1.13 vs 9.2 ± 0.89 , $p=0.024$), while it was not statistically different for females 8.5 ± 0.59 vs 8.9 ± 0.9 , $p=0.084$) (Figure 11).

The difference in the *Firmicutes/Bacteroidetes* ratio in association with gender was not retrieved (data not shown).

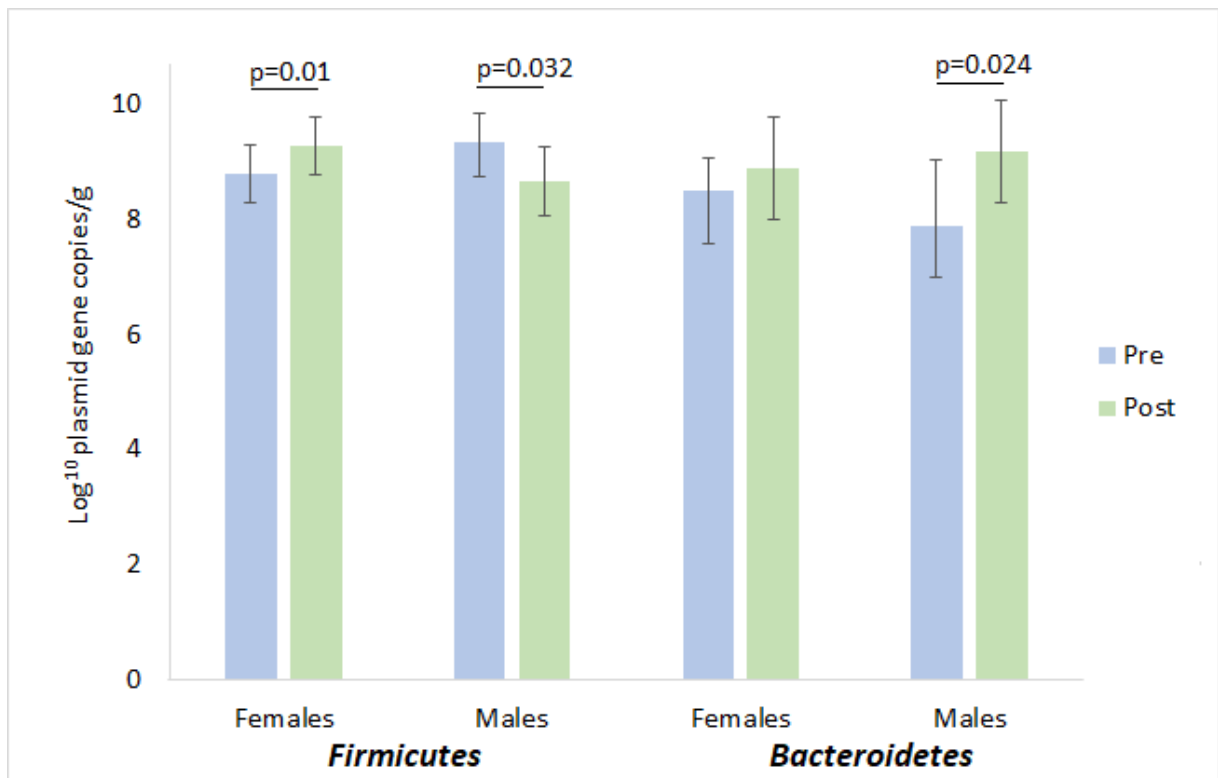


Figure 11. The counts of bacteria belonging to the phyla *Firmicutes* and *Bacteroidetes* before and after surgery in faecal samples of the same patients (N=25) but different gender (N females=17; N males=8) (Charts indicate mean value \pm standard deviation (SD)).

Our study also examined the possible influence of gender on the outcome in counts of these two bacterial phyla in association with different bariatric methods (SG or RYGB). The study found the significant increase in the *Firmicutes* phylum for females if they were treated with RYGB method (8.66 ± 0.59 vs 9.36 ± 0.43 , $p=0.018$). Remarkably, for sleeve gastrectomy method the counts of *Firmicutes* increased (8.5 ± 0.4 vs 9.3 ± 0.59 , $p=0.044$) and the counts of *Bacteroidetes* decreased for male group (8.83 ± 0.65 vs 7.7 ± 0.8 , $p=0.048$) (Figure 12).

The difference in the *Firmicutes/Bacteroidetes* ratio was not found (data not shown).

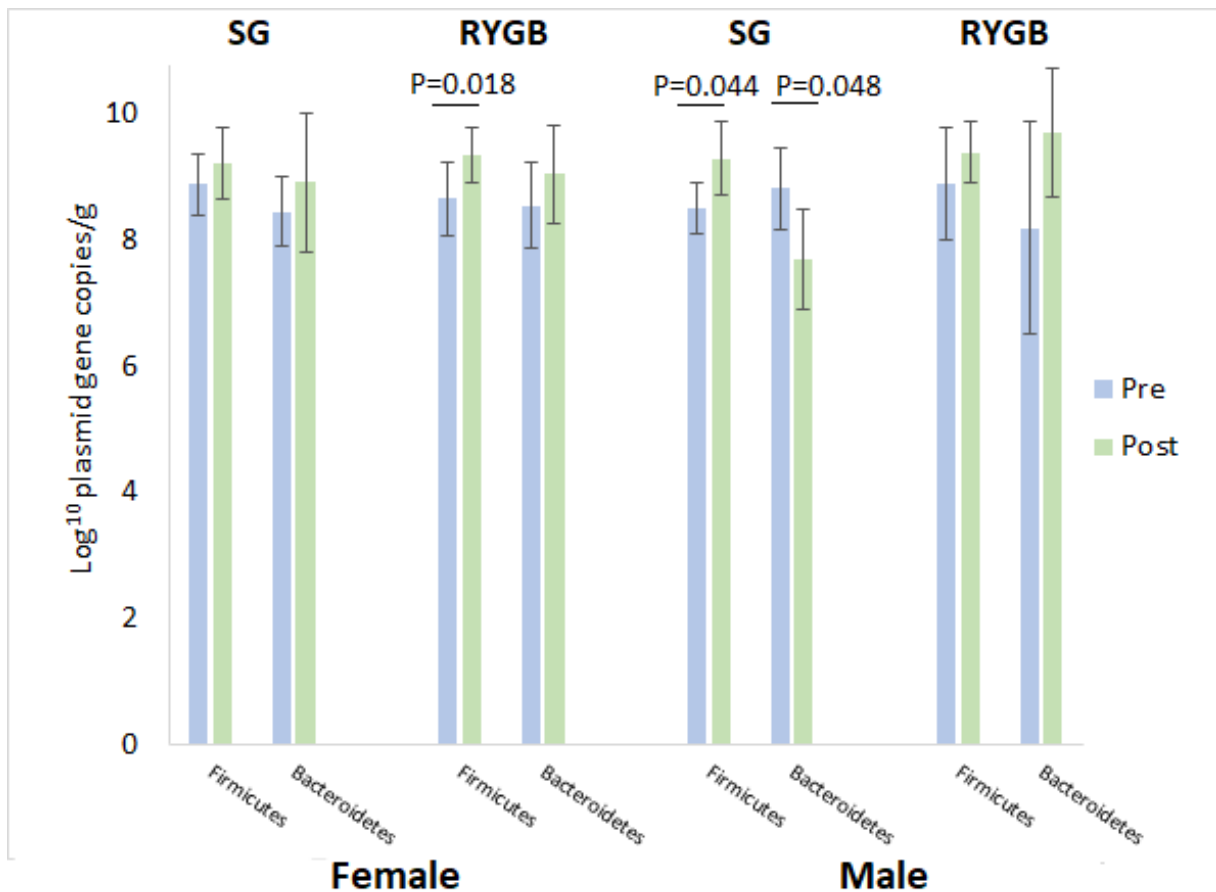


Figure 12. The counts of bacteria belonging to the phyla *Firmicutes* and *Bacteroidetes* in faecal samples before and after SG (N females=9; N males=5) or RYGB (N females=8; N males=3) method of the same patients but different gender (Charts indicate mean value \pm standard deviation (SD)).

2.4. Discussion

In this study, we compared the impact of two different surgical interventions (sleeve gastrectomy and roux-en-Y gastric bypass) on the two most predominant phyla and their ratio in the GI tract of obese patients. The influence of the patient's gender on the changes of bacterial composition was also assessed. Our study revealed decreased BMI and changes in the counts of the phyla *Firmicutes* and *Bacteroidetes* in the case of both bariatric surgery methods in obese patients. The difference in *Firmicutes/Bacteroidetes* ratio was independent of the used bariatric surgery method.

The *Firmicutes* and *Bacteroidetes* are the two most predominant phyla in the human colon and together comprise around 90% of the total gut microbiota (Guarner, 2015). The phylum *Firmicutes* includes Gram-positive, rod-shaped or circular bacteria that represent the largest part of the gut microbiome. Due to their negative influence on glucose and fat metabolism, they are commonly referred to as 'bad' gut microbes. Bacterial strains belong to the

Bacteroidetes phylum are rod-shaped, Gram-negative bacteria and commonly found in the human GI tract. Members of this phylum are among the so-called 'good' bacteria because they produce favorable metabolites, including SCFAs, which have been correlated with reducing inflammation (Vinolo et al., 2011). Increased ratios of *Firmicutes/Bacteroidetes* have been correlated with obesity and Type II diabetes (T2D) (Remely et al., 2016).

The first option for obesity treatment is diet. Ley et al. (2006) examined two groups of obese people who received a diet with limited fat (FAT-R group) and saccharides (CARB-R group). The study demonstrated that the proportion of *Bacteroidetes* increased and *Firmicutes* decreased with weight loss regardless of the type of a diet. However, the counts of *Bacteroidetes* decreased in patients only when patient lost at least 6% of body weight on the FAT-R diet and at least 2% on the CARB-R diet. The study suggested that *Firmicutes* and *Bacteroidetes* have a dynamic linkage with obesity and may have a minimized competition for resources due to their coexistence in the human gut. (Ley et al., 2006)

However, the bariatric surgery is more effective for the treatment of obesity and cardiovascular diseases than common dietary interventions (Santacruz et al., 2009; Aron-Wisnewsky et al., 2012).

Surgery methods allow to understand the molecular adaptations underlying the observed health benefits and the potential role of calorie restriction in changes in gut microbiota pattern (Furet et al., 2010). Both selected bariatric surgery methods induce important changes in the gastrointestinal tract and therefore they are expected to change the composition of the gut microbiota. The data obtained in the current study indicated that the abundance of the phyla *Firmicutes* and *Bacteroidetes* increased after RYGB surgical intervention. However, we showed decrease in the counts of the *Firmicutes* phylum and increase in *Bacteroidetes* for patients after SG method, as it was suggested by one of the previous studies (Damms-Machado et al., 2015). On the contrary, many of the other studies showed the increased counts of *Bulleidia* spp. and *Roseburia* spp. (both belong to the *Firmicutes* phylum) (Medina et al., 2017; Murphy et al., 2017) after SG bariatric surgery. Similarly, increase within the *Firmicutes* phylum was demonstrated for *Lactobacillales* and *Enterococcus* after bariatric surgery (Guo et al., 2018). However, it should be noted that present study did not examine the changes on genus or species level and it may be considered for future projects. Correspondingly with our results, Murphy et al. (2017) demonstrated the increased counts of *Firmicutes* and *Actinobacteria* for RYGB method. Interestingly, the abundance of the *Bacteroidetes* phylum in this study was decreased after RYGB and increased after SG (Murphy et al., 2017), that is not in agreement with our study. Evidence suggests that

Bacteroidetes communities can shift according to dietary modulation and weight change, whereas *Firmicutes* numbers are more dependent on host's genetic makeup (Goodrich et al., 2014; Voreades et al., 2014). Moreover, the results concerning changes in *Firmicutes* and *Bacteroidetes* in older adults vary due to nationality and age of the subjects (Biagi et al., 2013).

The current study implied that gender may have an influence on the counts of the phyla *Firmicutes* and *Bacteroidetes* after bariatric surgery. We demonstrated that the counts of the *Bacteroidetes* phylum trended to increase in men after surgical intervention but was not significantly different in women. Haro et al. (2016) described the association of BMI with the differences in gut microbiota of men and women at the bacterial phyla (*Firmicutes/Bacteroidetes* ratio), genus (*Bacteroides*, *Bilophila*, *Veillonella*, and *Methanobrevibacter*), and species (*Bacteroides plebeius*, *Bacteroides caccae*, *Coprococcus catus*) levels. According to this study, in women the counts of *Bacteroides* genus remained unchanged in the different ranges of BMI, while it was decreased in man with the increase of the BMI. (Haro et al., 2016) Due to the fact that this data is contrary to our results, in future studies it would be useful to take gender into consideration for examination the changes in bacterial genus and species after bariatric surgery.

Additionally, we have shown that gender affects the microbiological profiles of SG and RYGB, increasing the counts of the *Firmicutes* phylum in females after RYGB and increasing the abundance of *Firmicutes* but decreasing *Bacteroidetes* in males after SG method. Therefore, it is necessary to consider association between gender, different bariatric surgery interventions and the abundance of *Firmicutes* and *Bacteroidetes* phyla in further research.

In the present study the *Firmicutes/Bacteroidetes* ratio remained the same after both bariatric surgery methods. Similarly, one of the previous studies observed no significant differences in the *Firmicutes* to *Bacteroides-Prevotella* ratio between lean and obese individuals (Fernandes et al., 2014).

It is important to notice that the intestinal microbiota of patients after bariatric surgery may be influenced by a number of factors, including genetics, geography of population, socioeconomic factors, antibiotics used, age, diet and lifestyle (Zoetanadal et al., 2001; Yatsunenکو et al., 2012; Ladirat et al., 2013; Carmody et al., 2015; David et al., 2014). The metabolic activity of intestinal microbiota and host metabolism figure a tight interplay for development of host structure and physiology.

Real-time PCR is one of the most important assays in diagnostic microbiology. It is commonly used for quantification of the target bacterial divisions. This method has been found to be suitable for wide range laboratory research, including determination of the counts of *Firmicutes* and *Bacteroidetes*.

Considering that this project continues, it is necessary to intimately examine the changes in the proportions of different species, which belong to *Firmicutes* and *Bacteroidetes* phyla and increase the number of patients in pre- and post-bariatric surgery groups for better understanding of the association between these bacterial phyla ratio and obesity.

CONCLUSIONS

The intestinal microbiota has strong impact on host health and it is considered as a metabolic organ. The gut microbiota composition of obese people has been associated with a higher intestinal *Firmicutes/Bacteroidetes* ratio. This study was performed to compare the counts of the phyla *Firmicutes* and *Bacteroidetes* and their ratio in faecal samples of pre- and post-bariatric surgery patients and to assess the influence of gender on the changes of studied bacterial phyla and their ratio after bariatric surgery (sleeve gastrectomy (SG) or roux-en-Y gastric bypass (RYGB)).

We used faecal samples of pre- and post-bariatric surgery patients collected at the Chair of Medical Microbiology. Bacterial DNA was extracted from all faecal samples collected before and after surgery. Real-time PCR method with specific primers was applied for quantification of target bacterial groups.

The results of this study can be summarized as follows:

- the BMI of all patients was significantly decreased after both methods of bariatric surgery;
- in general, the abundance of the *Firmicutes* phylum increased in post-operational patients in comparison with pre-surgery individuals and the number of bacteria, which belong to the *Bacteroidetes* phylum did not change a year after bariatric surgery;
- in the specified group of patients both bariatric surgery methods influenced the abundance of the phyla *Firmicutes* and *Bacteroidetes*. However, there was also found the difference between SG and RYGB surgical interventions. After both methods the counts of the *Bacteroidetes* phylum increased, while the abundance of the *Firmicutes* phylum decreased after SG and increased after RYGB.
- patient gender had an influence on the counts of the phyla *Firmicutes* and *Bacteroidetes* after bariatric surgery. In particular, the counts of the phylum *Firmicutes* increased in females and decreased in males while the *Bacteroidetes* phylum increased in male group only;
- patient gender also affected the outcomes of different bariatric surgery methods for the counts of *Firmicutes* and *Bacteroidetes* phyla increasing the *Firmicutes* phylum in females after RYGB and increasing the abundance of *Firmicutes* but decreasing *Bacteroidetes* in males after SG method;
- the *Firmicutes/Bacteroidetes* ratio remained the same after both bariatric surgery methods.

In conclusion, our study has found that application of both bariatric surgery methods, SG and RYGB, is successful for obesity treatment due to decreasing of BMI. Our results show that

both bariatric surgery methods produce an effect on the counts of the *Firmicutes* phylum. The differences in the *Firmicutes/Bacteroidetes* ratio were not found. This study implies that different bariatric modalities may have different microbiological profiles in association with gender or by itself. In future studies our results may be used as a marker in surgical treatment of obesity and eventually to design microbiome-based bacto-therapies aimed at obesity treatment.

SUMMARY IN ESTONIAN

Bariaatrilise kirurgia meetodite mõju *Firmicutes/Bacteroidetes* suhtele rasvunud patsientidel

Olga Botšarova

Resümee

Inimese mikrobioota on kompleksne kooslus, mis on oma peremehega mutualistlikes suhetes. Mikrobioota mõjutab peremeesorganismis toimuvaid füsioloogilisi protsesse. Koosluse mitmekesisuse ja muutuste iseloomustamine aitab aru saada inimese seedetrakti mikrobioota rollist haigus- ja tervises seisundites. Kõige suurem osakaal selles koosluses on hõimkondadel *Bacteroidetes* ja *Firmicutes*.

Mõnede raskete krooniliste haiguste teke on tingitud inimese rasvumisest. Rasvtõve kujunemist seostatakse tasakaalustamata energia tulu ja kuluga. Inimese seedetrakti mikrobioota metaboolne aktiivsus on oluline toidu seedimiseks vajalike ensüümide tootmiseks ja energia tulu reguleerimiseks. Varasemad uurimistööd on näidanud, et hõimkonna *Firmicutes* esindajad mõjutavad negatiivselt seedetrakti metaboolseid protsesse. Samas bakterid, kes kuuluvad hõimkonda *Bacteroidetes*, mõjutavad nimetatud protsesse positiivselt, produtseerides elutegevuse käigus mitmesuguseid kasulikke ühendeid. Seega on nende bakterihõimkondade suhte jälgimisega võimalik hinnata üldise mikrobioota tasakaalu ja kasutada rasvtõve kujunemise indikaatorina.

Rasvumise invasiivseks ravimeetodiks on bariaatrilised operatsioonid, mille käigus vähendatakse mao mahtu. Käesoleva töö eesmärgiks oli võrrelda hõimkondade *Firmicutes* ja *Bacteroidetes* arvukust ja suhet rasvtõvega patsientidel enne ja pärast operatsiooni. Kasutati kahte bariaatrilise kirurgia meetodit, kas: vertikaalse maoreseksiooni (SG) või maost möödajuhtiva operatsiooni (RYGB). Bakteriaalne DNA eraldati patsientide roojaproovidest ning analüüsiti RT-PCR meetodil kasutades hõimkonna spetsiifilisi primereid.

Käesoleva bakalaureusetöö tulemused võib kokku võtta järgnevalt:

- kõikide patsientide kehamassindeks (KMI) vähenes märkimisväärselt pärast bariaatrilist operatsiooni;

- hõimkonda *Firmicutes* kuuluvate bakterite arvukus oli üldiselt kõrgem pärast operatsiooni kui enne operatsiooni võetud roojaproovides. Hõimkonda *Bacteroidetes* kuuluvate bakterite arvukus jäi aga samale tasemele;
- võrreldes omavahel vaid nende patsientide andmeid, kes andsid proovi nii enne kui pärast operatsiooni näidati, et bariaatriline kirurgia mõjutas seedetrakti mikrobiotas hõimkondade *Firmicutes* and *Bacteroidetes* arvukust. Samuti leiti erinevus kahe meetodi vahel (SG ja RYGB). Hõimkonna *Bacteroidetes* arvukus suurenes roojaproovides pärast mõlemat operatsioonilist sekkumist, aga hõimkonna *Firmicutes* arvukus vähenes pärast SG ja suurenes pärast RYGB;
- patsiendi sugu mõjutas hõimkondade *Firmicutes* ja *Bacteroidetes* arvukust - naistel oli peale operatsiooni hõimkonda *Firmicutes* kuuluvate bakterite arvukus kõrgem ja meestel madalam, samas hõimkonna *Bacteroidetes* arvukuse muutus oli oluline vaid meespatsientide korral;
- erinevast soost patsientidel oli sõltuvalt kasutatud kirurgilisest meetodist fekaalne mikrobiota erinev. Hõimkonna *Firmicutes* arvukus oli suurem naistel pärast RYGB operatsiooni. Meespatsientidel oli pärast SG operatsiooni *Firmicutes* arvukus vähenenud, samas *Bacteroidetes* hõimkonna arvukus aga suurenenud;
- *Firmicutes/Bacteroidetes* suhe ei muutunud peale operatsiooni sõltumata patsiendi soost või kasutatud kirurgilisest meetodist.

Kokkuvõttes leiti käesolevas töös, et mõlemad bariaatrilise kirurgia meetodid (SG ja RYGB) sobivad kasutamiseks rasvtõve invasiivseks raviks. Nii vertikaalsel maoreseksioonil kui ka maost möödajuhtival operatsioonil on mõju KMI-le ja *Firmicutes* hõimkonna arvukusele. Mõju *Firmicutes/Bacteroidetes* suhtele ei olnud statiliselt oluline. Antud töös näidati, et erinevatel bariaatrilise kirurgia meetoditel võib olla erinev mõju soolestiku mikrobiotale sõltuvalt patsiendi soost. Rasvtõve ja kahe bakterihõimkonna suhte vaheliste seoste täpsemaks selgitamiseks on vajalik suurendada patsientide arvu ja pöörata ka tähelepanu perekondade- ja liigisestele muutustele. Käesolev uurimistöö on aluseks ka *Firmicutes* ja *Bacteroidetes* arvukuste määramise kasutamiseks markerina rasvtõve ravi jälgimiseks ja mikrobioomipõhiliste bakteriaalsete teraapiate loomiseks.

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