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ABSTRACT

The composition of gut microbiota and its relationship to human health, particularly its links with obesity remain an ongoing challenge for scientists. The current gold standard for exploring human gut microbiota consists of using stool samples and only applying next generations sequencing techniques, which sometimes generate contradictory results.

Here, we comprehensively describe nutrient absorption, fat digestion, carbohydrate and protein absorption, demonstrating that absorption of these diverse nutrients occurs mainly in the stomach and small intestine. Indeed, bariatric surgery, including Roux-en-Y, removes part of the upper intestine, resulting in weight loss, while colonic surgery is associated with a stable weight. However, most studies only use stool samples rather than small intestine samples because of the easy with which this can be accessed. Metagenomics studies are associated with several biases such as extraction and primer biases and depth bias, including the more modern platforms. High-throughput culture-dependent techniques, such as culturomics, which uses rapid identification methods such as MALDI-TOF, remain time-consuming, but have demonstrated their complementarity with molecular techniques.

In conclusion, we believe that a comprehensive analysis of the relationships between obesity and gut microbiota requires large-scale studies coupling metagenomics and culture-dependent research, in order to analyse both small intestine and stool samples.

1. Introduction

Exploration of human gut microbiota was revolutionized at the beginning of the 21st century. The culture-based approach was initially used to describe human gut composition in the 1970s(1). Metagenomics, first used for exploring the complex ecosystems of the environment (such as sea composition), promised to discover the 'uncultivable' [2]. Explorations of human bacterial communities were initially applied to healthy patients [3,4] but they rapidly suggested links between gut microbiota and diseases. Indeed, this progress suggested relationships between the composition of the human gut microbiota and several human diseases, such as polyposis, colorectal cancer [5], irritable bowel syndrome [6], inflammatory bowel diseases such as Crohn's disease [7,8] in adults, and necrotizing enterocolitis in preterm infants [9]. Finally, most of the studies were published on the relationships between human gut microbiota and obesity [10–13]. Results that now seem naive initially associated obesity with phyla composition (*Firmicutes*/*Bacteroidetes* ratio) [13], while subsequent research suggested links between obesity and microbiota at the species and strain levels [14]. Nevertheless, all research on this topic compared gut microbiota composition from stool samples collected in obese and non-obese individuals. This approach does not take into account the place where nutrient absorption occurs [15]. Furthermore, we can question the fact that stools are rather a reflection of the relationship between microbiota and obesity pathophysiology or, in other words, a microbe dustbin.

Indeed, in this comprehensive review, we propose to briefly summarize the physiopathology of digestive nutrient absorption in the different sections of the human intestine. As one example of considering the actual role of each section of the human intestine in the potential relationship with weight gain, we also describe the consequences of digestive surgery (Fig. 1), which are totally different depending on whether the small intestine or the colon are involved. Finally, we describe the pros and cons of the different techniques available (including culture-based and culture-

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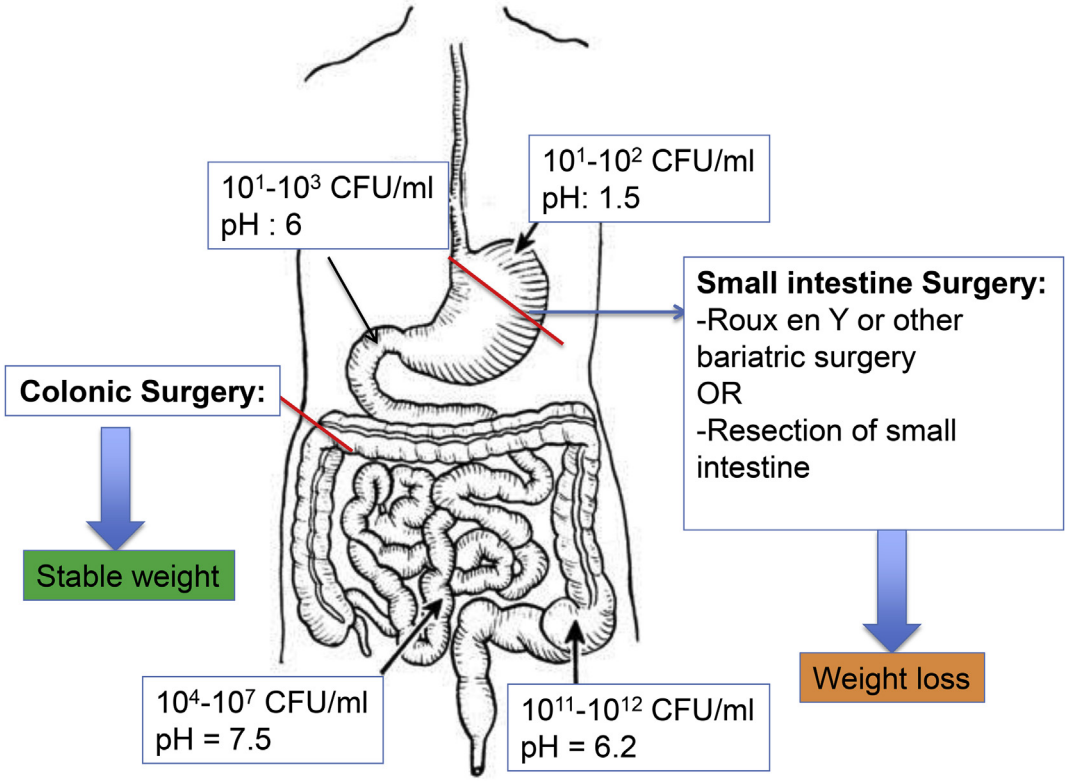

Fig. 1. Summarize of the weight variations consequences of intestinal surgery and of the gut composition.

independent approaches) to study gut microbiota.

2. Nutrient digestion and absorption in humans

2.1. Nutrient absorption (Fig. 2)

Diets, which are high in calories, particularly from refined sugars, saturated and trans-fats, and sedentary lifestyles all, contribute to the obesity epidemic. The typical Western diet is about 50% carbohydrates, 15% proteins, and 35% fats, which is over the dietary guidelines for the amount of fat (below 30%), below the guidelines for carbohydrate (above 55%), and at the upper end of the guidelines for the amount of protein (below 15%) recommended in the diet [16].

Digestion is the mechanical and chemical break down of food into small organic fragments [17]. Proteins, polysaccharides and lipids must be reduced to simpler particles before they can be absorbed by the digestive epithelial cells. Proteins are degraded into small peptides and amino acids before absorption. The mechanisms for controlling food intake involve an interplay between the gut, the brain, and adipose tissue, among the major organs [17]. Nutrients created by the digestion of food are assumed to activate G-protein-coupled receptors on the luminal side of enteroendocrine cells, e.g., the L-cells [18]. This stimulates the release of gut hormones. The hormones released from the gut and adipose tissue play an important role in the regulation of food intake and energy expenditure [18].

2.2. Fat digestion

Dietary fat is the most important source of energy, supplying 9 kcal/g, about double that contributed by either protein or carbohydrate, at 4 kcal/g [19]. Dietary lipids account for ~42% of the calories ingested in the Western diet, while nutritional recommendations are 20–35% fat for adults [20]. In the Western diet, ~95% of dietary lipids are triacylglycerols, mainly composed of long-chain fatty acids, and the remaining are phospholipids and cholesteryl esters [20]. The digestion of fat begins in the stomach where dietary constituents are mixed together with lingual and gastric enzymes, resulting in partial fat digestion by preduodenal lipases and emulsification by peristalsis [21]. The combined action of bile and pancreatic juice markedly alter the chemical composition of the lipid emulsion in the upper part of the small intestine. Pancreatic lipase is present only in the pancreatic juice [22]. Its high concentration in pancreatic secretions, together with its great catalytic efficiency, ensures complete digestion of dietary fat in the small intestine. Phospholipids are the second main source of fat in the intestine and they are also digested in the small intestine [22]. Upon entering the small intestine, dietary cholesterol is typically mixed in a lipid emulsion with triacylglycerols and phospholipids. The mixed micelles in the small intestinal lumen promote the absorption of fatty acids and cholesterol by facilitating transport of these lipids across the unstirred water layer adjacent to the surface of the apical membrane of enterocytes [22].

Fatty acids, cholesterol and bile acids that escape intestinal absorption are excreted as faecal fatty acids, as well as neutral and acidic sterols, respectively [23]. This constitutes the major route for sterol elimination from the body. In the fasting state or in fat-free diets, faecal sterol excretion in humans ranges from 0.7 to 1 g/day, emphasizing the fact that faecal sterols do not only consist of dietary constituents [24].

2.3. Carbohydrates

Carbohydrates in the form of mono-, oligo-, and especially polysaccharides, form the main energy supply. With a daily intake

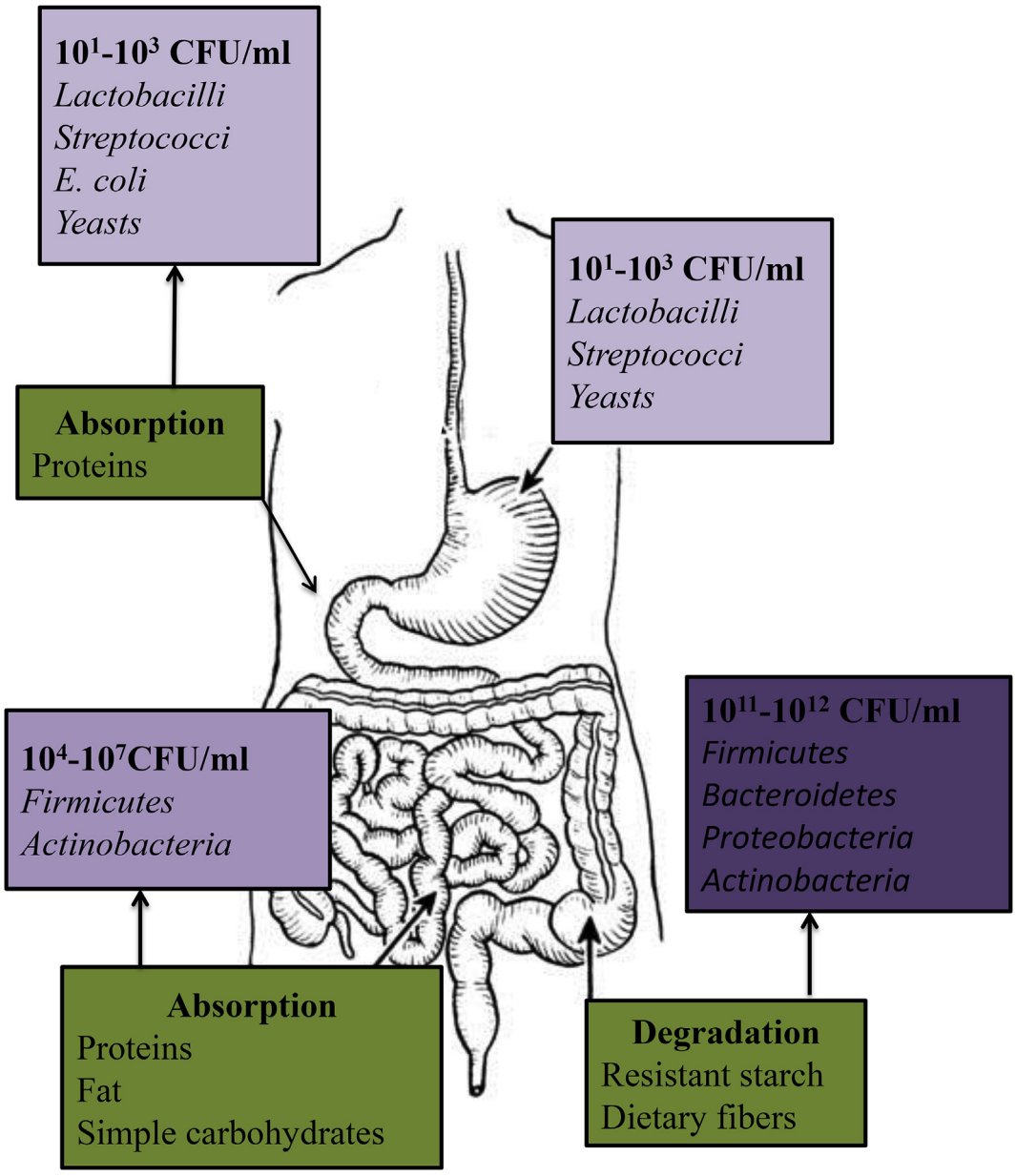

Fig. 2. Summarize of the nutrients absorption along human gut.

of 250–800 g, with the average being slightly over 300 g, dietary carbohydrates account for ≥50% of the daily calories in adults in Western industrialised countries [25]. Carbohydrates are ingested mostly in the form of starch from long chain glucose polymers with a small percentage in the form of disaccharides and a smaller amount in the form of monosaccharides. Carbohydrates are broken down by salivary and pancreatic amylases and then by saccharidases at the brush border membrane, forming monosaccharides which are the only forms by which carbohydrates can enter the cell. The oligosaccharides follow, with the disaccharide sucrose representing the quantitatively most significant carbohydrate in this case. Monosaccharides play only a subordinate role as dietary components with regard to carbohydrate supply in humans [25].

The digestion of carbohydrates begins in the mouth as amylase breaks down the food starches into maltose [25]. In the stomach, no significant digestion of carbohydrates takes place and the acidic environment stops the action of amylase. In the duodenum, pancreatic juice amylase continues to break down starch and glycogen into maltose and other disaccharides. These disaccharides are then broken down into monosaccharides by maltases, sucrases, and lactases. The monosaccharides which are produced are then absorbed. Enzymatic hydrolysis of starch proceeds very rapidly in the duodenum and was it was assumed that even complex starch-containing polysaccharides can be completely absorbed by the small intestine [25]. However, a certain amount of starch enters the large intestine in an undigested form in the same manner as dietary fibre. Within the colon, both unavailable starch and dietary fibre are then subject to bacterial break down and fermentation [26]. Carbohydrates that cannot be completely absorbed in the small intestine reach the colon, where they are subject to bacterial degradation [25]. Colonic microbiota is largely driven by the efficient degradation of complex indigestible carbohydrates, but that of the small intestine is shaped by its capacity to quickly import and convert relatively small carbohydrates, and its ability to rapidly

adapt to overall nutrient availability [27]. The fermentation process of carbohydrates in the colon is an important phenomenon, especially with respect to the salvage of energy [27]. Bacterial fermentation results in an energy salvage of 62% of the fermented carbohydrates and the colon can reabsorb short chain fatty acids very efficiently [27].

2.4. Proteins

Proteins are ingested in the form of peptides that are partly digested by gastric pepsin and then by pancreatic proteases and converted into amino acids and small peptides [28]. The denaturation of protein in the acidic stomach environment by various pepsins represents the first step in protein digestion. This process is of minor importance because only small amounts of amino acids are released, while the bulk of predominantly large polypeptides appears in the duodenum. The main event in terms of intraluminal digestion consists of the cleavage of polypeptides by pancreatic proteases including trypsin, chymotrypsin, elastase and caboxypeptidase. As a result, most proteins are absorbed in the upper jejunum. Digestion and absorption may well occur in the distal jejunum and the proximal ileum [29].

In conclusion, the small intestine is responsible for most nutrient digestion and absorption in humans and about 85% of carbohydrates, 66%–95% of proteins, and all fats are absorbed before entering the large intestine [15]. Short-chain fatty acids (SCFAs) absorbed in the colon contribute 6%–10%, while carbohydrates and proteins contribute 10%–30% of the entire energy requirements for humans [15].

3. The use of samples to comprehensively study the human gut and the interaction between microbiota and obesity

3.1. Surgery

The importance of the small intestine for nutrient absorption is also provided by extensive surgical data that can help us better understand digestive physiology. Gastric bypass procedures, including the Roux-en-Y gastric bypass, biliopancreatic diversion, and biliopancreatic diversion and duodenal switch induce weight loss through both restriction and malabsorption of food (Fig. 1). In these bypass procedures, a portion of the small intestine, including the duodenum, is excluded from the digestive tract. The most efficient bariatric surgery technique used to treat obesity is the Roux-en-Y gastric bypass and resections of the small intestine which leave less than 50 cm of the small intestine intact can lead to severe malnutrition [30]. It was characteristic of the first bariatric surgeries, in which the proximal jejunum was joined to the distal ileum, bypassing a large segment of the nutrient absorbing small intestine, that there was a significant malabsorption of carbohydrates, lipids, vitamins and proteins, resulting in serious long-term morbidities, including cirrhosis and liver failure [31].

Zhang et al. reported in 2009 the first study applying molecular techniques to investigate the microbiota after bariatric [32]. The authors demonstrated that obesity and gastric bypass strongly affected the composition of the intestinal microbiota with an increase of Gammaproteobacteria and a decrease of *Clostridia* in the post-bariatric surgery group. Additionally, Verrucomicrobia, a usually rare but sometimes predominant phyla [33], were abundant in obese but rarely detected in post-surgery individuals. In addition, Archaea were more abundant in obese individuals compared with patients after surgery. Furet et al., studied more individuals and detected after bariatric surgery, an increase number of *Bacteroides* and *Prevotella* genera that was lower in obese and an increase of *Escherichia coli* after surgery [34]. Kong et al. also described an increase of the diversity and specific changes in the microorganisms concentration detected after surgery. As example, the authors described an increase of *Bacteroides*, *Escherichia* and *Alistipes* but a decrease of *Bifidobacterium*, *Blautia*, *Dorea* and *Lactobacillus* in post-surgery [35]. Finally, a long-term stability of the changes observed has been recently reported by Tremaroli et al. [36]. In addition, in animal models, Zhang et al. have recently observed after duodenal-jejunal bypass in a diabetic rat model an increase of *Firmicutes* and *Proteobacteria* but a decrease of *Bacteroidetes* [37].

In contrast, colectomies or colostomies, which exclude the colon from its role in digestion, are not accompanied by significant weight loss in obese subjects or in patients with type 2 diabetes [38]. The major task of the large intestine is the re-absorption of water and salts. Indeed 1000–1500 ml of isotonic fluid passes the Bauhin valve daily and only 50–200 ml are excreted in the faeces. Moreover, in cases of a normal small intestine function, low levels of available carbohydrates and proteins are detected in the ileostomy effluent.

3.2. Duodenal versus stool microbiota

Duodenal bacterial composition is also very different compared to that of stools [39]. Indeed, the gastrointestinal tract harbours $>10^{14}$ microorganisms and different species and quantities of bacteria are presented at different parts along the digestive track depending upon major variations in the environmental niche [14]. The largest number of bacteria, approximately 10^{11-12} microorganisms per gram of content, reside in the large intestine and consist mainly of anaerobes [14]. In contrast, much lower bacterial concentrations, approximately 10^{1-4} microorganisms per mL of content, are present in the upper two-thirds of the small intestine [30] (Fig. 1).

3.2.1. Studies performed from stool samples

A metagenomic analysis of stools samples produced 698 phylotypes [40]. The most commonly represented bacterial phyla are *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* [40], and *Methanobrevibacter smithii* is the dominant methanogenic archaeal species in the gut [40]. The overall and individual microbiota structures are dominated by the *Bacteroidetes* and *Firmicutes* phyla [40]. In addition, three gut microbiota studies [41] assigned 98% of 16S rRNA sequences to only four bacterial phyla: *Firmicutes* (64%), *Bacteroidetes* (23%), *Proteobacteria* (8%) and *Actinobacteria* (3%). *Verrucomicrobia*, *Fusobacteria* and the *TM7* phylum together accounted for the remaining 2%. However, the ratio of the *Firmicutes*: *Bacteroidetes* is not the same in all individuals [42]. As a result, big differences in stool microbiota have been found between different populations tested [43,44]. If the differences detected through the first studies comparing obese and non-obese were at phylum level, currently the studies reports relationships between obesity and gut microbiota at the strain level [12].

3.2.2. Studies performed from small intestine samples

Based on a limited number of studies, we know that the small intestine contains a very different abundance and composition of bacteria, with much more variation in the time and depending of the section compared to the colon. *Lactobacillus* sp., *E. coli* and *Enterococci* have been found as the predominant species in the duodenum and jejunum [30,45] in healthy individuals. In a recent study performed from obese and non-obese samples, the predominant phyla of the duodenal microbiota were *Firmicutes* and *Actinobacteria*, while *Bacteroidetes* were almost absent [46]. Indeed, about 60% of the genera OTUs belonged to *Streptococcus*, *Actinomyces*, *Propionibacterium* and *Granulicatella* [46]. Moreover, the

phylogenetic mapping of the small intestinal metagenome of three different ileostomy effluent samples from a single individual indicated that *Streptococcus* sp., *E. coli*, and *Clostridium* sp. were most abundant in the small intestine [47]. In a culture-based study in infants, it was found that *Actinobacteria* and *Firmicutes* were the dominant phyla in the ileostomy samples, while *Bacteroidetes* were only detected following the reversal of the ileostomy in the final faecal sample [48]. In a recent article comparing duodenal microbiota in obese individuals to individuals of normal weight, it was found that the Acyl-CoA dehydrogenase (FAD), which targets the early enzymatic reaction of fatty acid β-oxidation, was enriched in the obese individuals [46]. This high occurrence of Acyl-CoA dehydrogenase in obese subjects might be associated with a higher β-oxidation capacity and energy mobilisation. Indeed, obese patients have an excessive intake of food and, particularly, fat and, as a result, their intestinal microbiota is adapted to high levels of dietary fats and free fatty acids released upon gastrointestinal lipolysis [49]. Free fatty acids could thus be used as a carbon and energy source for microbial growth. High fat loads are also associated with increased endotoxaemia, suggesting that fat and its lipolysis products have a deleterious effect on gut microbiota, leading to LPS release [50–52]. The harvest and degradation of fatty acids by bacteria might be viewed as an adaptive response to their antibacterial effects. In addition, obese individuals consume high levels of glucose, sucrose and readily digestible starch. It was also found [46] that the duodenal microbiota of the obese group showed a reduced abundance of genes encoding sucrose phosphorylase and 1,4-α-glucan branching enzyme, which catalyses the cleavage of sucrose to fructose and glucose-1-phosphate, and the transglycosylation reaction that creates α-1,6 branching points in bacterial glycogen, respectively. As a result, it is possible that the duodenal microbiota of obese individuals has been altered by a diet rich in readily digestible carbohydrates, which contributes to obesity in these individuals.

4. The use of techniques to comprehensively study the human gut microbiota

4.1. Culture

Culture techniques were the first method used to characterise the gut microbiota ecosystem [1,53]. Nevertheless, because culturing is a fastidious process, because of the lack of a rapid identification method, and because of the cost, culture techniques were rapidly abandoned after the advent of molecular techniques [54] (Table 1). In addition, culture techniques were associated with several biases, including the discrepancy between culture and microscopic counts, first described by Moore and Holdeman [55] in 1974, and later referred to as the 'great plate count anomaly' in 1985 by Staley and Konopka, were rapidly observed [56]. Considering that only a minority of bacteria can be easily grown *in vitro* [57], the majority of bacterial species easily cultured from stool samples consist of bacterial species that grow rapidly in the more usual culture conditions, neglecting minority populations [54]. Indeed, bacterial species usually cultured from the human gut belong to one of the four main phyla (*Actinobacteria*, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*) or, occasionally, to *Fusobacteria* [58] (Table 2).

In terms of the consequences of bypass surgery, culturing studies were primarily designed to elucidate the side effects of the surgical procedure [59,60]. In 1978, Corrodi et al., who were studying proximal jejunum and distal ileum samples, revealed that the small-bowel becomes colonised with colonic flora [60]. In 1987, Prakash et al., who were collecting biliopancreatic samples using catheters during surgery, evaluated bacterial composition before and after surgery, in order to explain the frequent diarrhoea observed in the post-operative period [59].

Zuo et al., in their culture-based study of the composition of gut microbiota from the stool samples of 52 obese and 52 non-obese individuals, detected significantly reduced levels of *Clostridium perfringens* and *Bacteroidetes* in obese individuals [61].

By multiplying culture conditions with a rapid and efficient identification method using MALDI-TOF [62], microbial culturomics explored the composition of gut microbiota using stool samples and demonstrated broad complementarity with metagenomics [63–67]. Indeed, in the proof of concept study, only 15% of the bacterial species were detected by both culture and pyrosequencing performed concomitantly on the three stool samples studied [64]. This complementarity was observed both at the genera level and at the species level [64]. In addition, culturomics was used to culture bacterial species from unusual phyla, including the first species of *Deinococcus–Thermus* isolated from humans and some species belonging to *Synergistetes* [58,64]. Indeed, many new bacterial species have been described, including their genome sequencing, and are available for the scientific community in strains collections [65,68,69]. Other reknowned teams proposed a new way of using culture, coupled with molecular techniques [70]. To date, microbial culturomics has never been applied to exploring the relationships between obesity and gut microbiota, but this concept was recently used to elucidate the relationships between *Clostridium butyricum* and necrotizing enterocolitis in preterm neonates [71]. This offers significant opportunities in addition to extending the human gut microbiota repertoire. In parallel, significant progresses on culture conditions for the culture of anaerobic bacteria will increase again the repertoire [72,73].

4.2. Molecular biology

Molecular biology revolutionized bacterial identification and allowed large studies to take place on the relationships between gut microbiota and obesity [14,65]. In addition, these techniques have dramatically increased the repertoire knowledge [74,75]. Of molecular techniques, qPCR [76–78], 16S clonal sequencing [13], flow cytometry coupled with fluorescence *in situ* hybridisation [79], pyrosequencing [32,80] and metagenomics [4,81,82] were successively used. Nevertheless, several biases inherent to these techniques limit their interpretation and explain the poor reproducibility of the results between different laboratories and sometimes even within the same team [83] (Table 1).

Overall, it appears that molecular techniques neglect a large part of gram negative prokaryotes. Hugon et al. explored 16 different stool samples concomitantly, using direct observations through

Table 1
Pros and cons of the main techniques used to explore gut microbiota.

	Advantages	Limitations
Culture-dependent techniques	- Detects viable bacterial populations - Allows genome sequencing of the isolates	- Time-consuming - No direct information on enzymatic abilities
Next generation sequencing	- Detection of 'not yet cultivable' microorganisms - Provides information on enzymatic abilities	- Technical biases (primers, extraction.....) - Neglects minority populations

Table 2
Pros and cons between small intestine and stool samples.

	Advantages	Limitations
Small-intestine samples	- Studying direct impact of antibiotics totally absorbed in small intestine - Studying direct impact of probiotics	- Difficulties to collect - Low diversity because of low bacterial concentration
Stool samples	- Easy to collect - Large diversity because of the bacteria concentration - Easy high throughput studies	- Very indirect picture of the real players with a role on obesity

transmission electron microscopy (TEM), quantitative real-time PCR (qPCR) of *Firmicutes* and *Bacteroidetes* phyla, and 16S rRNA pyrosequencing targeting the V6 region, and demonstrated that approximately 15% of the gram negative populations were overlooked by molecular techniques [84].

4.2.1. Inhibitors

DNA extraction methods cause bias in PCR amplification [85] because of inhibitors such as bile salts and complex polysaccharides which are very common in faecal specimens and cause bias in PCR amplification assays [86,87]. Complex glycans in faecal samples originating from vegetable consumption in the diet have been suggested as PCR inhibitors [88,89], and it has been proposed that polysaccharides may disturb the enzymatic process by mimicking the structure of nucleic acids [86]. Moreover, little attention has been paid to the potential biases introduced by biofilms [86]. In addition, the disruption and/or lysis of the bacterial membranes can be expected to be biased for specific bacterial taxa due to differences in cell wall structure and integrity. For example, DNA from gram-positive bacteria present in faeces is more efficiently extracted if a sample has been frozen [90]. Many bacteria produce an extracellular biofilm that contains glycoproteins and affects the sensitivity of molecular assays [91]. Indeed, exopolysaccharides produced by bacterial species which are present in faeces are extremely inhibitory to DNA restriction and modifying enzymes [92,93].

Various methods have been developed to remove or inactivate inhibitors in stools, and it is critical to optimise DNA extraction methods in order to obtain accurate results on the composition of gut microbiota [94]. Variations in DNA extraction methods, such as the use of different cell wall-degrading enzymes, chemical agents, bead sizes, bead-beating time, and DNA purification procedures may affect microbiota profiling. As a result, the need for internal controls to detect PCR inhibitors when gut microbiota samples are analysed can be critical.

4.2.2. Other biases

Next generation sequencing studies demonstrate various biases, depending on whether they are used in gut microbiota studies [95,96] or in environmental studies, such as for the exploration of activated sludge communities [97]. Of these biases, the extraction bias influencing phyla repartition has long been well-described [95] and the phylum distribution frequently depends on the primer choice [98,99]. For example, using 16S rDNA V4–V5 region primers preferentially detects *Bacteroidetes* phylum, despite using 16S rDNA V3–V4 preferentially detect *Firmicutes* phylum [100]. In previous pyrosequencing studies performed to explore gut microbiota, some bacterial genera were detected only by specific primers [96]. Finally, one of the main bias factors of metagenomics studies remains the depth bias. Indeed, only the most highly represented populations were detected by such studies, and the bacterial species composing the gut ecosystem at concentrations below a threshold of between 10^6 and 10^5 bacteria per gram of stools remain undetected [54,64].

The same limitations can be observed with more recent techniques, such as Illumina MiSeq platform. Jones et al. recently undertook a comprehensive exploration of the differences in taxonomy distribution among four different next-generation sequencing library preparations, using a DNA mock community and a cell control of known concentration [101]. The authors compared the data obtained by Illumina Nextera XT, Illumina TruSeq DNA PCR-free kits, KAPA Biosystems Hyper Prep PCR and PCR-free systems. In addition to the differences observed in taxonomy, the authors described error profiles, duplication rates and loss of reads with significant impact results [101].

5. Conclusion and perspectives

Human microbiota projects are being initiated throughout the world, with the goal of correlating human physiological phenotypes with the structures and functions of their indigenous microbial communities [54]. However, the nature of changes to the intestinal microbiota associated with obesity is a subject of controversy and one drawback of current metagenomic approaches is the presence of major discrepancies between different studies [14]. The development of experimental models to study the relationship between gut microbiota and obesity has mostly been based on the study of faeces [54]. However, we believe that faeces are not the optimal sample in order to examine the link between obesity and gut flora and believe that scientists should focus on the microbiota of the small intestine because this is where calories are absorbed. The low bacterial concentrations of the duodeno-jejunum microbiota compared to that of the distal microbiota make it difficult to evaluate its variation by testing stool samples. Probiotics have been consumed for centuries, either as natural components of food, or as fermented foods [102]. Probiotics in fermented food may contain as much as 10^9/ml living bacteria, mainly *Lactobacillus* and *Bifidobacterium* [102]. As a result, their influence on the duodenum where exist the same bacterial genera (but different species) but at much lower concentrations (10^5/ml) is considerable. Future work should focus on small intestine microbiota, even if this means greater difficulties in accessing samples than the use of stool specimens, because this is the place where the nutrients are digested [103]. Controversy around the best sample to be used to study efficiency shows that discussion has not yet been settled [83,104].

In conclusion, in order not to be blinded by the easiest technique, we believe that in order to be comprehensive, the study of the human gut microbiota and its relationships with obesity should combine different types of samples, including small intestine samples, and should combine culture-based studies and metagenomic studies. Finally, the future studies should use fresh samples or optimized transport media in order to explore the more wide diversity [72].

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