CHAPTER 1

The human microbiota: an historical perspective

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1.1 Introduction: the discovery of the human microbiota: why do we care?

The discovery by Antony van Leeuwenhoek in 1683 that we have a microbiota was very surprising and undoubtedly of great interest to 17th-century scientists. However, as modern-day researchers know only too well, this alone is not sufficient to ensure continued investigation of a subject. Further research into the microbes that inhabit humans proceeded at a very slow pace until it was realized that these microbes were able to cause disease and, much later, that they contribute to human health (i.e., in modern-day research parlance the research would be recognized as having "impact"). Our knowledge of those microbes with which we coexist has increased enormously during the last few years. An indication of the effort that has been devoted to determining the nature and function of the microbial communities inhabiting the various body sites of humans can be gleaned from the number of publications in this field listed in PubMed: in 2013 more than 2500 papers were published, nearly four times as many as in 2000.

What accounts for this recent huge growth of interest in the human microbiota? There appear to be two main driving forces: (a) increasing awareness of its importance in human disease, development, nutrition, behavior and wellbeing; (b) the development of technologies that enable us not only to identify which microbes are present but also to determine what these microbes are up to. In this chapter these two driving forces are described from a historical perspective.

1.2 The importance of the indigenous microbiota in health and disease

It has long been known that members of the indigenous microbiota of humans are responsible for a variety of infections, but only relatively recently has it been recognized that these microbes play an important role in maintaining human health and wellbeing.

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1.2.1 The indigenous microbiota and human disease

In the late 19th and early 20th centuries many members of what we now recognize as the indigenous microbiota of humans were found to be the causative agents of a number of human infections (Table 1). However, at that time there was little understanding of what constituted the indigenous microbiota and therefore it was not realized that these newly recognized, disease-causing microbes were in fact regularly present on some, if not all, healthy humans and that, for the most part, they lived in harmony with their host (Table 1).

Subsequently, as knowledge of the indigenous microbiota improved, the involvement of members of these communities in disease processes became of great interest and was the subject of more intense research. Other members of the indigenous microbiota now known to cause human disease are shown in Table 2. More recently, it has become apparent not only that individual members of the microbiota are able to cause disease, but that shifts in the overall composition of the microbiota at a site can result in disease (Table 3). Such "dysbioses" are discussed in greater detail in subsequent chapters of this book. Recognition of the disease-inducing potential of the indigenous microbiota became an important stimulus to research into the characterization of the microbial communities associated with humans.

1.2.2 The indigenous microbiota and human health

Towards the end of the 19th century it became evident to many researchers that the intestinal microbiota was important in intestinal physiology, and Pasteur in 1885 went even further by suggesting that animal life would not be possible in the absence of the indigenous microbiota[19]. In the second half of the 20th century it became evident that the indigenous microbiota not only contributed to mammalian health and wellbeing in a number of ways but that it also played an important

Table 1 Early discoveries of the involvement of members of the indigenous microbiota in human infections.

Year	Researcher	Organism	Disease	Reference
1881	Alexander Ogston	staphylococci	abscesses	1
1884	Friedrich Rosenbach	*Strep. pyogenes*	Wound infections	2
1884	Friedrich Rosenbach	*Staphylococcus aureus*	Wound infections	2
1884	Friedrich Rosenbach	*Staphylococcus albus (i.e. Staph. epidermidis)*	Wound infections	2
1884	Albert Fraenkel	*Diplococcus pneumoniae (i.e. Strep. pneumoniae)*	Lobar pneumonia	3
1890s	Theodor Escherich	*Bacterium coli commune* (i.e. *Escherichia coli*)	Colicystitis (i.e. urinary tract infection)	—
1892	George Nuttall and William Welch	*Bacillus aerogenes capsulatus* (i.e. *Clostridium perfringens)*	gangrene	4
1898	Veillon and Zuber	A variety of anaerobic species including *Bacteroides fragilis*, *Fusobacterium nucleatum*	gangrene	5
1906	Thomas Horder	*Strep. salivarius*	infective endocarditis	6
1891	Albert Fraenkel	*Bacillus coli communis (i.e. Escherichia coli)*	peritonitis	7

Table 2 Diseases caused by members of the indigenous microbiota (in addition to those listed in Table 1).

Organism	Disease
Enterococcus faecalis	Urinary tract infections, endocarditis, meningitis, wound infections
Moraxella catarrhalis	Bronchopneumonia, sinusitis, otitis media
Haemophilus influenzae	Meningitis, pneumonia, sinusitis, otitis media, epiglottitis
Proteus mirabilis	Urinary tract infections
Helicobacter pylori	Gastritis, ulcers, carcinoma
Streptococcus mutans	Dental caries, endocarditis
Porphyromonas gingivalis	periodontitis
Actinomyces israelii	actinomycosis
Staphylococcus saprophyticus	Urinary tract infections
Neisseria meningitidis	meningitis
Malassezia spp.	Atopic dermatitis, seborrhoeic dermatitis, folliculitis
Gardnerella vaginalis	Bacterial vaginosis
Corynebacterium minutissimum	erythrasma

Table 3 Diseases resulting from dysbiosis.

Disease	Microbiota involved	Reference
obesity	intestinal tract	8
rhinosinusitis	nasal cavity	9
chronic obstructive pulmonary disorder	lungs	10
asthma	lungs	11
autism	intestinal tract	12
inflammatory bowel diseases	intestinal tract	13
multiple sclerosis	intestinal tract	14
arthritis	intestinal tract	15
periodontitis	oral cavity	16
colorectal cancer	intestinal tract	17
type II diabetes	intestinal tract	18

role in mammalian development (Table 4). While many of these discoveries were made in animals such as mice and rats, in some cases these effects have also been demonstrated in humans.

That the indigenous microbiota exerted a protective effect by preventing colonization of exogenous pathogens was demonstrated in 1962 when it was found that mice were 100,000-fold more susceptible to infection with *Salmonella enteritidis* following the administration of a single dose of streptomycin[20]. This was attributed to the disruptive effect of the antibiotic on the composition of the intestinal microbiota, thereby destroying its barrier function. This protective effect was termed "colonization resistance".

Most information regarding the role of microbes in mammalian development has been obtained by comparative studies involving germ-free animals, animals with a normal microbiota and those colonized with particular microbial species. Such studies became possible following the successful breeding of germ-free animal colonies (mainly rats and mice) in the 1950s and were well underway by the 1970s. The absence of an indigenous microbiota can have dramatic effects on

Table 4 Beneficial effects of the human microbiota.

Role of microbiota	Body site involved
colonization resistance (i.e. exclusion of exogenous pathogens)	all
development of immune functions	all
tissue and organ differentiation and development	intestinal tract
development of nutritional capabilities	intestinal tract
provision of nutrients	intestinal tract
provision of vitamins	intestinal tract
detoxification of harmful dietary constituents	intestinal tract
prevention of bowel cancer	intestinal tract

Table 5 Attributes of germ-free animals compared to their counterparts with an indigenous microbiota[22].

decreased mass of heart, lung and liver
increased size of cecum (may be eight times larger)
decreased water absorption by large intestine
increased redox potential of large intestine
decreased concentration of deconjugated bile salts
increased pH of stomach
altered surface epithelial mucins
decreased mass of small intestine
absence of immune cells in lamina propria
decreased intestinal peristalsis
prolonged intestinal epithelial cell cycle time
shorter, more slender villi in intestinal epithelial cells
increased length of microvilli
decreased surface area of intestinal mucosal
decreased number of lymphocytes in lamina propria
decreased size of Peyer's patches, mesenteric lymph nodes, spleen and thymus
decreased macrophage chemotaxis and phagocytic activity
decrease in plasma cells in lamina propria and Peyer's patches
decreased production of IgG and IgA
decreased number of TCR$\alpha\beta$+ intra-epithelial lymphocytes

the anatomy and physiology of an animal and examples of these are listed in Table 5; these are often termed "germ-free animal characteristics" (GACs)[21]. Those aspects of the host's anatomy, immunology, physiology or biochemistry that are influenced by the indigenous microbiota have been termed "microbiota-associated characteristics" (MACs) and, as is evident from Table 5, these are many and varied. Many of the abnormalities observed in germ-free animals can be reversed by inoculation with the indigenous microbiota or constituents of the microbiota.

A number of studies in the early 2000s involving the gut symbiont *Bacteroides thetaiotaomicron* and germ-free animals revealed the multiple contributions of the gut microbiota to host development[22,23]. Colonization of germ-free mice with *B. thetaiotaomicron* results in changes in the expression of several host genes involved in the processing and absorption of carbohydrates, lipids and micronutrients and thereby contributes to the development of the host's nutritional capabilities. Germ-free adult mice have a greatly reduced capillary network in their

intestinal villi compared with conventional mice and it has been shown that in conventional mice the development of the capillary network coincides with the establishment of a complex intestinal microbiota. Inoculation of germ-free mice with *B. thetaiotaomicron* induces the formation of a normal capillary network, thereby greatly increasing the host's ability to absorb nutrients.

One of the major roles of the intestinal microbiota is in stimulating the growth and differentiation of intestinal epithelial cells[22,24]. Germ-free rodents have fewer crypt cells than conventional animals and the rate of production of such cells is reduced. Hence, in conventional rodents the rate of enterocyte turnover is almost twice that found in germ-free animals. The microbially induced proliferation and differentiation of epithelial cells is mediated by the short chain fatty acids (SCFAs) produced by fermentation of carbohydrates and amino acids. Although all three major SCFAs (i.e. butyrate, acetate and propionate) are able to induce this trophic effect, butyrate is the most potent in this respect and has been shown to alter the expression of a number of genes in epithelial cells *in vitro*. Butyrate can also inhibit DNA synthesis in, and proliferation of, neoplastic cells, and it has been suggested that this may account for the protective effect that dietary fibre exhibits against bowel cancer.

It is well established that the indigenous microbiota plays a key role in the development of a competent immune system[22,25]. Because the gut-associated lymphoid tissue (GALT) contains the largest collection of immunocompetent cells in the human body, most studies have involved the gastrointestinal tract and its microbiota. As shown in Table 5, the immune system of germ-free animals has a number of structural and functional abnormalities including low densities of lymphoid cells in the gut mucosa, low concentrations of circulating antibodies, specialized follicle structures are small, etc. However, exposure of the gut mucosa to the indigenous microbiota has a dramatic effect on the GALT. Hence, the number of intraepithelial lymphocytes expands greatly, germinal centres with antibody-producing cells appear in follicles and in the lamina propria, the levels of circulating antibodies increase and increased quantities of IgA are secreted into the gut lumen. Many studies have shown that the indigenous microbiota stimulates the secretory IgA system and B lymphocyte function in general[22].

The colonic microbiota functions as an effective scavenger of dietary constituents that the stomach and small intestine are unable to digest (mainly complex carbohydrates), have failed to digest (carbohydrates, proteins, peptides), or have failed to absorb (amino acids and monosaccharides). The colonic microbiota degrades these materials to assimilable molecules that can serve as nutrients for the host as well as for resident microbes[22,26]. Although a variety of microbes can digest the complex carbohydrates reaching the colon, the most effective species are those belonging to the genera *Bacteroides* and *Bifidobacterium*. In addition to carbohydrates, the colon also receives proteins and peptides from the diet, exfoliated epithelial cells and pancreatic enzymes. These are rapidly degraded by microbial proteases and peptidases and these may be of significant nutritional value to the host. Furthermore, many colonic microbes can ferment these amino acids to generate a range of products, including SCFAs, that are of great nutritional value to the host, providing up to 9% of the host's energy requirements[22]. Colonocytes can utilize each of the three SCFAs as an energy source, with butyrate being the most important and acetate the least important in this respect. It has been estimated

that the colonic epithelium derives up to 70% of its energy from these SCFAs[22]. The acids are also used as precursors for the synthesis of mucosal lipids. Apart from acting as a major energy source and its involvement in lipid synthesis, butyrate has a number of effects on the colonic epithelium. Hence, it can stimulate cell growth and proliferation, induce differentiation, alter gene expression, induce apoptosis, stimulate tight junctions, increase mucus production and reduce inflammation[27]. SCFAs, therefore, appear to play a key role in maintaining gut integrity. There is also some evidence to suggest that the butyrate produced by colonic microbes exerts a protective effect against large bowel cancer.

The colonic microbiota, therefore, constitutes a means by which the host can achieve maximum recovery of the nutrients present in its diet without it having to elaborate the vast range of enzymes that would be needed to degrade a wide range of dietary constituents. It also plays a major role in energy harvest, storage and expenditure, and consequently is an important factor in human obesity[8].

A number of vitamins are present in the colon and many are derived from the colonic microbiota — particularly *Bifidobacterium* spp., *Bacteroides* spp., *Clostridium* spp. and enterobacteria. Vitamins produced by colonic bacteria include biotin, vitamin K, nicotinic acid, folate, riboflavin, pyridoxine, vitamin B12 and thiamine[22,28]. A number of studies have shown that the tremendous metabolic capabilities of the colonic microbiota can achieve detoxification of potentially harmful dietary constituents[22,29]. Heterocyclic aromatic amines (HAAs) are pyrolysis products of amino acids found in cooked meat and fish products and may have a role in the etiology of colon cancer. *Lactobacillus* spp., *Clostridium* spp. and *Bifidobacterium* spp. are able to reduce the mutagenicity of HAAs by binding to them and/or by altering their structure. Studies in humans have shown that the consumption of *Lactobacillus casei* or *Lactobacillus acidophilus* results in a greatly reduced urinary and fecal mutagenicity following the ingestion of meat.

More recently (i.e. during the early years of the 21st century), it has become increasingly evident that the gut microbiota is able to communicate with the central nervous system (via neural, hormonal, immunological and metabolic pathways) and influence brain function and behavior[30] (see chapter 27). Hence, there is considerable evidence (from both animal and human studies) that the composition of the gut microbiota can play a role in regulating memory, cognition, anxiety, sleep, mood and pain[31]. It is becoming clear, therefore, that the influence of the microbiota on human wellbeing extends far beyond what was recognized in the latter years of the 20th century (summarised in Table 4).

1.3 The development of technologies for characterising the indigenous microbiota

The above section has outlined how our knowledge of the role of the indigenous microbiota in health and disease has progressed. The establishment of the indigenous microbiota as a reservoir of disease-causing microbes, the finding that changes in its overall composition (i.e. dysbiosis) can result in a range of chronic diseases, the realization that it has a profound role in the development and health of humans and, finally, the recent recognition of its ability to influence brain function and behavior have all provoked an enormous drive to characterize the microbial communities that live on us. The above-mentioned revelations would

not have been possible without the development and application of a range of new techniques for identifying what organisms are present in these complex communities and for establishing exactly what they are doing there. In this section, the historical emergence of these techniques will be outlined.

1.3.1 Light microscopy

The discovery of the microbial world and, indeed, the realization that we are colonized by microbes arose from the use of a simple light microscope. Not only was Antony van Leeuwenhoek the first person to report that he had seen microbes (in stored rainwater in 1676) but he was the first to report (in 1683) the presence of microbes in humans. On 17th September 1683, he wrote a letter to the Royal Society in which he reported

> Tho my teeth are kept usually very clean, nevertheless when I view them in a magnifying glass, I find growing between them a little white matter as thick as wetted flower: in this substance tho I could not see any motion I judged there might probably be living Creatures. I therfore took some of this flower and mixt it either with pure rain water wherein there were no Animals: or else with some of my Spittle (having no air bubbles to cause a motion in it) and then to my great surprize perceived that the aforesaid matter contained very many small living Animals which moved themselves very extravagantly. Their motion was strong and nimble, and they darted themselves thro the water or spittle as a Jack or Pike does thro water.

In a letter to Robert Hooke in 1719, he also reported the presence of "animalcules" in his feces.

Light microscopy continues to be an important tool for detecting and enumerating the microbes that colonize humans, although a number of developments since 1683 have greatly increased its usefulness and versatility. These include:

- the production of more powerful microscopes (increasing the magnification from X300 to more than X1000)
- dark-field microscopy
- phase-contrast microscopy
- the use of simple and differential staining techniques (e.g. Gram stain)
- the use of fluorescent probes
- the use of stains that distinguish between live and dead microbes
- confocal microscopy

These various techniques have not necessarily been used to investigate all of the microbial communities inhabiting humans. Some have proved to be more useful for studying particular body sites than others. For example, dark field and phase-contrast microscopy have been used extensively to investigate the oral microbiota whereas their use in studying microbial communities at other body sites has been more limited. A brief overview of these techniques is provided below:

- Light microscopy is one of the simplest and most direct approaches used to study microbial communities. One of its advantages is that it can reveal details of the physical structure of a community and the spatial arrangement of the constituent organisms. It also serves as a "gold standard" with respect to the total number of microbes that are present within a sample; this is often used as a yardstick for assessing the ability of other, less direct techniques to detect all

of the organisms present in a community. Hence, it has been shown that culture-based analysis of feces may detect as few as 20% of the organisms that can be observed microscopically. Differential counts of the various morphotypes in a sample give an indication of the diversity of the microbiota, and this has proved useful to ascertain whether the composition of the vaginal and subgingival microbiotas in an individual are indicative of health or disease.

- Dark field microscopy (invented in 1830) is useful for examining unstained, living microbial communities. The resolution (0.02 μm) is approximately 10X higher than that obtained with a traditional bright field microscope, which means that thin and fragile microbes (e.g. spirochetes) can be visualized. It has been used to provide information on the main bacterial morphotypes present in biofilms found in the gingival crevice of healthy adults[32]. The relative proportions of these morphotypes (cocci, straight rods, curved rods, filaments, fusiforms and spirochetes) were shown to be similar in biofilms present in healthy adults. Shifts in their relative proportions (e.g. a decrease in cocci accompanied by an increase in rods and spirochetes) were found to be indicative of disease (gingivitis or periodontitis).
- Phase-contrast microscopy, first described in 1934, depends on differences in refractive indices between microbes and their surroundings and, like dark field microscopy, enables microbes to be examined in their living state. It has been used to study the various bacterial morphotypes present in biofilms from the fissures on teeth in adults[33].
- Fluorescent probes have long proved useful in determining which organisms are present in a microbial community. Initially, antibodies were used to identify the organisms present, but nowadays oligonucleotide-based probes are increasingly being used in a technique known as fluorescent *in situ* hybridization (FISH). This involves the use of fluorescent-labeled oligonucleotide probes to target specific regions of bacterial DNA. Most of the probes currently used are those that recognize genes encoding 16S ribosomal RNA (16S rRNA). The gene encoding 16S rRNA in a bacterium consists of both constant and variable regions. Within the molecule there are regions that are highly specific for a particular bacterial species as well as regions that are found in all bacteria, in only one bacterial genus, or in closely-related groups of bacteria. Probes, therefore, can be designed to identify an individual species, a particular genus, certain related microbial groups, or even all bacteria. Oligonucleotide probes can, of course, also be used to detect microbes other than bacteria. An important advantage of this approach is that it can be automated and the resulting data can be processed using computerized image analysis software. One of the earliest studies to use fluorescent antibodies to investigate the indigenous microbiota was that of Ritz[34], who used this technique to detect *Nocardia* spp. in dental plaque. FISH has also been used to detect *Bifidobacterium* spp. in fecal samples[35]. Since then, it has been used to analyse microbial communities at other sites including the vagina[36], the oral cavity[37] and the skin[38].
- Staining procedures that can distinguish between live and dead cells can provide useful information about the physiological status of members of microbial communities. One such procedure involves treating the specimen with a mixture of two DNA-binding dyes — propidium iodide (which fluoresces red) only enters cells with a damaged cytoplasmic membrane, while SYTO9 (fluoresces green) enters all cells. Live cells (strictly speaking, those with an intact membrane)

appear green, while dead cells (strictly speaking, those with a damaged membrane) appear red. This has been employed to study biofilms in the oral cavity[39] but is more frequently used in conjunction with a confocal laser scanning microscope (CLSM) as described below.

- CLSMs, which became available in the early 1990s, produce a series of very thin optical sections through the object under examination. These sections can be built up to produce a 3D image of the object. A CLSM can be used to investigate communities in their living, hydrated state and so provides valuable information concerning the true spatial organization of the constituent cells as well as the overall shape and dimensions of the community. It is a technique that has revolutionized our understanding of the structures of biofilm communities. Additional information can be obtained by using vital stains, fluorescent-labeled antibodies and labeled oligonucleotide probes. Furthermore, information about the nature of the environment within the biofilm (e.g. pH, Eh, etc.) can be obtained using appropriate probes. It is also possible to monitor gene expression within biofilms using reporter genes such as green fluorescent protein. Because it is so useful for studying biofilms, CLSM has been widely used by researchers investigating the various types of dental plaque[40–43]. It has also been used to study biofilms formed on particulate matter in the human colon[44] and to study the microbial communities associated with the intestinal mucosa[45].

1.3.2 Electron microscopy

The electron microscope is capable of much higher magnifications (up to several million times) and has a greater resolving power than a light microscope, allowing it to see much smaller objects in finer detail. Consequently it can provide information that is not obtainable by ordinary light microscopy, and the high magnifications that are possible can be used to reveal details of microbial adhesins and adhesive structures. The organisms that are present can be identified using antibodies conjugated to electron-dense markers (e.g. gold or ferritin). However, a major disadvantage of electron microscopy is that specimen processing and the accompanying dehydration alters the structure of the sample. There are two basic types of electron microscope: the transmission electron microscope (TEM) and the scanning electron microscope (SEM).

The TEM became commercially available in 1939 and has been used to study microbial communities inhabiting most body sites. In 1969 the location of microbes within epidermal samples taken from various body sites was investigated by TEM[46]. The TEM has also been used to demonstrate the presence of viruses in feces[47], the development of biofilms on the tooth surface[48], bacterial attachment to the oral mucosa[49], the distribution of bacteria on the vaginal mucosa[50], the formation of bacterial microcolonies on the tonsillar epithelium[51] and the attachment of bacteria to the urethral epithelium[52]. The SEM, while capable of only lower magnification than the TEM, has the great advantage of being able to produce three-dimensional images. Hence, the overall shape and structure of microbial communities can be visualized. It became commercially available in 1965 and has been used to demonstrate the formation of dental plaque[53], the attachment of bacteria to the pharyngeal epithelium[54], the presence of bacteria in the urethra[55], the association of bacteria with the colonic mucosa[56], bacteria adhering to the oral mucosa[57] and bacterial microcolonies on skin[58].

1.3.3 Culture-based approaches to microbial community analysis

1.3.3.1 Techniques

In 1881 Robert Koch demonstrated the use of solid culture media (with gelatin as the solidifying agent) on glass plates to isolate pure cultures of bacteria. This was a major breakthrough in practical microbiology and forms the basis of all subsequent culture-based methods of isolating, purifying and identifying microbes from the mixed communities that inhabit humans. Shortly afterwards, other members of Koch's laboratory improved on this approach by replacing gelatin with agar (in 1882) and by introducing petri dishes in place of glass plates (in 1887). In the early 1900s, selective media were developed for the isolation of specific groups of microbes from mixed communities. One of the first of these (1905) was MacConkey agar, which incorporated bile salts to inhibit the growth of all but lactose-fermenting bacteria from faecal samples[59]. Since then a huge variety of media have been developed including elective, diagnostic and chromogenic media in addition to selective media — in 1930 a total of 2,543 different culture media formulations were recognized[60]. As well as nutrients, microbes also require appropriate environmental conditions for growth. Until 1861, it was thought that all living creatures needed oxygen for growth but in 1861 Pasteur discovered microbes that could grow in the absence of air — these he called "anaerobies". He discovered the anaerobe *Clostridium butyricum* and showed that it produced butyric acid under anaerobic conditions. In 1878, he recognized that microbes could be divided into three groups depending on their relationship to air: "either exclusively aerobic, at once aerobic and anaerobic, or exclusively anaerobic". Pasteur removed oxygen from his culture media by boiling, but subsequently many techniques were developed to enable the cultivation of anaerobic microbes[61]. Between 1888 and 1918 over 300 different methods for producing anaerobic conditions were described in the literature[62]. Subsequent developments led (in the 1960s) to the anaerobic cabinets widely used today for the isolation and cultivation of anaerobic microbes. The ability to grow and identify anaerobic microbes represented a huge step forward in characterising the indigenous microbiota, as these organisms comprise significant and, indeed, dominant proportions of the communities inhabiting many body sites.

Most of our knowledge of the composition of indigenous microbial communities has come from using quantitative culture techniques. This involves some form of sample dispersion, plating out the sample (and usually dilutions of it) on various media, incubation, subculture of isolated colonies and then identification of each isolate. Given the complexity of the communities at most body sites, this is a very labor-intensive and painstaking task. There are also a number of problems with the technique. First of all, if a non-selective medium is to be used, then one must be chosen that can support the growth of all of the species likely to be present; this is virtually impossible given the disparate (and often very exacting) nutritional requirements of the members of such communities. Furthermore, it is difficult to provide the optimum environmental conditions (e.g. pH, oxygen content, CO_2 content, etc.) necessary to enable the growth of all the physiological types of microbes present. Problems arise as a result of some organisms growing faster than others, resulting in overgrowth of plates and failure to isolate slow-growing organisms. In samples taken from sites with a very dense microbiota (e.g. the colon, vagina, dental plaque), it is essential to use dilutions of the sample to

obtain isolated colonies for subsequent identification. This means that organisms present in low proportions are "diluted out" and so are rarely isolated. Many studies have used selective media instead of, or in addition to, non-selective media. These can be useful, but analysis of a complex microbiota requires the use of a number of media selective for the various groups of organisms present. However, no medium can be relied upon to be truly selective and the inhibitory constituents may also have some adverse effect on the organisms for which the medium is supposedly selective. These problems all contribute to a greater workload, which inevitably results in an increase in the number of errors, a decrease in the number of samples that can be processed and hence a decrease in the statistical reliability of the data obtained[63].

Comparison of samples analysed by culture and by microscopy has revealed that even the best culture methods seriously underestimate the number of organisms present in the microbiotas of certain body sites — particularly those from the gastrointestinal tract (GIT) and oral cavity. The reasons for this are many and include: (i) the failure to satisfy the nutritional and/or environmental requirements for some of the organisms present, (ii) the presence in the community of organisms in a "viable but not cultivable" state, (iii) the failure to disrupt chains or clusters of organisms prior to plating out — this results in the production of only one "colony forming unit" from a cluster or chain consisting of many viable bacteria, (iv) the death of viable cells during transportation and processing of the sample[63]. Collectively, these difficulties have resulted in a serious underestimate of the number and variety of organisms in a sample taken from any environment, and it has been estimated that we are able to culture in the laboratory no more than 1-2% of the microbial species present on planet Earth (of which there are thought to be at least 10^{12}). Once individual isolates have been obtained, the next task is to identify each one. Traditionally this has involved the use of a battery of morphological, physiological and metabolic tests that is very labor-intensive and often not very discriminatory. The use of commercially available kits for this purpose has made the process less technically demanding. Other phenotypic tests that have been used for identification purposes include cell wall protein analysis, serology and fatty acid methyl ester analysis.

During the last few years, there has been an increasing trend to use molecular techniques for identifying the organisms isolated and one of these is based on the sequencing of genes encoding 16S rRNA[63]. The gene is amplified by PCR and the sequence of the resulting DNA determined and then compared with the sequences of the 16S rRNA genes of organisms that have been deposited in databases. If the sequence is >98% similar to that of one already in the database, then it is assumed that the gene is from the same species and hence the identity of an unknown organism can be established. The procedure is much simpler to perform than a battery of phenotypic identification tests and has the great advantage that it enables phylogenetic comparisons of the isolated organisms. However, some taxa are recalcitrant to PCR and some (e.g. many viridans streptococci) are so closely related that they cannot be differentiated using this approach. Alternative gene targets for speciation have been used including *recA, rpoB, tuf, gyrA, gyrB* and *cpn60* family proteins.

In fungi, the rRNA gene complex consists of four ribosomal genes, 18S (small subunit), 5.8S, 28S (large subunit) and 5S genes. Within this region, the internal transcribed spacer (ITS) and an approximately 600 bp D1/D2 region of the 28S

subunit are the most phylogenetically variable regions and have been widely used for fungal taxonomy and identification.

A variety of other techniques are used for the identification and further characterization of isolated colonies. These include pulsed field gel electrophoresis (PFGE), ribotyping, multiplex PCR, arbitrary-primed PCR, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), Raman spectroscopy and Fourier transform-infrared (FT-IR) spectroscopy.

1.3.3.2 Outcomes

Early culture-based studies of the oral cavity revealed the presence of a number of species and what was known of the indigenous microbiota of the mouth in 1875 was summarised in an essay by Peirce[64]. In this review, reference is made to the presence of bacteria, yeasts and protozoa including *Oidium albicans* (*Candida albicans*), *Cryptococcus cerevisiae, Leptothrix buccalis* (*Leptotrichia buccalis*), *Leptomitus oculi*, vibrios and paramecia. Williams (1899)[65] isolated a variety of microbes from the mouth including diphtheroids, actinomyces, *Staphylococcus pyogenes albus, Staphylococcus pyogenes aureus (Staphylococcus aureus), Sarcina lutea* and *Bacillus buccalis maximus* (*Leptotrichia buccalis*).

In 1884, Theodor Escherich isolated a number of bacteria from the feces of infants including *Bacterium coli commune* (*Escherichia coli*), *Proteus vulgaris, Streptococcus coli gracilis, Bacillus subtilis, Bacterium lactis aerogenes (Klebsiella pneumoniae)* and *Micrococcus ovalis*. He also cultured four different yeasts including *Monilia candida* (*Candida albicans*) and a *Torula* species. In 1886, he published his work on the intestinal microbiota as a monograph entitled "The Intestinal Bacteria of the Infant and Their Relation to the Physiology of Digestion" (this was republished in English in 1988[66]). In 1896, Harris isolated *Proteus vulgaris* and *Bacterium coli commune* from the human duodenum[67] and Strauss isolated the Boas-Oppler bacillus (i.e. *Lactobacillus acidophilus*) from the stomach of healthy individuals[68].

In the late 1880s and early 1890s a number of investigators, including Maggiora Vergano (1889), Preindlsberger (1891) and Welch (1892), isolated and identified large numbers of different types of bacteria from the skin of healthy individuals[69–71]. The predominant organism was found to be a staphylococcus of low pathogenicity, which was named *Staphylococcus epidermis albus* (i.e. *Staphylococcus epidermidis*). *Staphylococcus pyogenes aureus* was isolated from the skin by Bockhart in 1887[72]. Bordoni-Uffreduzzi cultured five different species of micrococci and two bacilli from the skin including the foul-smelling *Bacterium graveolens,* which was found between the toes[73].

In a book published in 1892, Sternberg reviewed those microbes that had been cultivated from various body sites[74] and these are shown in Table 6. However, at this stage, investigators were concerned exclusively with determining the identities of those microbes present at a body site. No quantitative studies were undertaken and therefore there was no attempt to define the relative proportions of the various microbes present in a particular community. Culture-based approaches to analysing the indigenous microbiota of humans reached their zenith in the early 2000s, but since then they are increasingly being replaced by culture-independent methods. The "state-of-play" of our knowledge of the cultivable microbes present in these communities prior to the large-scale use of culture-independent techniques will now be summarised.

Table 6 List of microbes that had been cultivated from various body sites as reviewed by Sternberg in 1892[74].

Body site	Organisms cultivated from the site
Skin	*Diplococcus albicans tardus, Diplococcus citreus liquefaciens, Diplococcus flavus liquefaciens tardus, Staphylococcus viridis flavescens, Bacillus graveolens, Bacillus epidermidis, Ascobacillus citreus, Bacillus fluorescens liquefaciens minutissimus, Bacillus aureus, Bacillus ovatus minutissimus, Bacillus albicans pateriformis, Bacillus spiniferus, Micrococcus tetragenus versatilis, Bacillus Havaniensis liquefaciens*
Feces	*Streptococcus coli gracilis*, *Hicrococcus ahogenes*, *Micrococcus ovalis*, *Porzellanococcus*, *Bacillus subtilis*, *Bacillus aerogenes*, *Bacterium aerogenes*, *Bacterium lactis erythrogenes, Clostridium foetidum*, *Bacillus muscoides*, *Bacillus putrificus coli*, *Bacillus subtilis similis, Bacillus zopfii*, *Bacillus liquefaciens communis*, *Bacillus intestinus liquefaciens*, *Bacillus intestinus motilis, Bacillus fluorescens liquefaciens*, *Bacillus mesentericus vulgatus*, *Staphylococcus pyogenes aureus*, *Bacillus septicaemise haemorrhagicae, Bacillus enteritidis, Bacillus pseudomurisepticus, Bacillus coli communis, Bacillus lactis aerogenes, Bacillus cavicida, Bacillus coprogenes foetidus, Bacillus leporis lethalis, Bacillus acidiformans, Bacillus cuniculicida, Bacillus cadaveris, Bacillus cavicida Havaniensis, Proteus vulgaris, Helicobacterium aerogenes*
Conjunctiva	various micrococci and occasional bacilli
Nose	*Micrococcus nasalis, Diplococcus coryzae, Micrococcus albus liquefaciens, Micrococcus cumulates tenuis, Micrococcus tetragenus subflavus, Diplococcus fluorescens foetidus, Micrococcus totidus, Vibrio nasalis, Bacillus striatus flavus, Bacillus striatus albus, Staphylococcus pyogenes aureus, Staphylococcus pyogenes albus, Streptococcus pyogenes,* Bacillus Of Friedlander*, Bacillus foetidus ozaenae, Bacillus mallei, Bacillus smaragdinus foetidus, Diplococcus fluorescens foetidus, Micrococcus albus liquefaciens, Vibrio nasalis*
Mouth	*Micrococcus roseus, Sarcina pulmonum, Sarcina lutea, Micrococcus candicaiis, Bacillus virescens, Vibrio rugula, Vibrio lingualis, Pseudo-diphtheria bacillus, Bacillus mesentericus vulgatus, Bacillus subtilis, Bacillus subtilis similis, Bacillus radiciformis, Bacillus luteus, Bacillus fluorescens non-liquefaciens, Bacillus ruber, Bacillus viridiflavus, Proteus zenkeri, Vibrio viridans, Micrococcus nexifer, Iodococcus magnus, Ascococcus buccalis, Bacillus fuscans, Staphylococcus pyogenes albus, Staphylococcus pyogenes aureus, Staphylococcus salivarius septicus, Streptococcus pyogenes, Micrococcus salivarius septicus, Micrococcus tetragenus, Micrococcus gingivae pyogenes, Streptococcus septo-pysemicus, Streptococcus articulorum, Micrococcus pneumoniae crouposae, Bacillus of Friedlander, Bacillus bronchitidse putridae, Bacillus septicaemias haemorrhagicae, Bacillus gingivae pyogenes, Bacillus pulpse pyogenes, Bacillus dentalis viridans, Bacillus crassus sputigenus, Bacillus saprogenes, Bacillus pneumoniae agilis, Bacillus pneumoniae of Klein, Bacillus pneumosepticus, Spirillum sputigenum, Spirillum dentium, Vibrio rugula, Vibrio lingualis*
Stomach	*Sarcina ventriculi, Bacillus pyocyaneus, Bacillus lactis aerogenes, Bacillus subtilis, Bacillus mycoides, Bacillus amylobacter, Vibrio rugula*, *Micrococcus tetragenus mobilis ventriculi*
Vagina	*Diplococcus albicans amplus, Diplococcus albicans tardissimus*
Urethra	*Streptococcus giganteus urethrae, Bacillus nodosus parvus*

1.3.3.2.1 The skin

Gram-positive species (belonging to one or more of the genera *Propionibacterium, Staphylococcus* and *Corynebacterium*) are usually the numerically dominant organisms at any skin site[75]. As can be seen in Figure 1, in general, propionibacteria are the predominant organisms of sebum-rich regions (e.g. scalp, forehead).

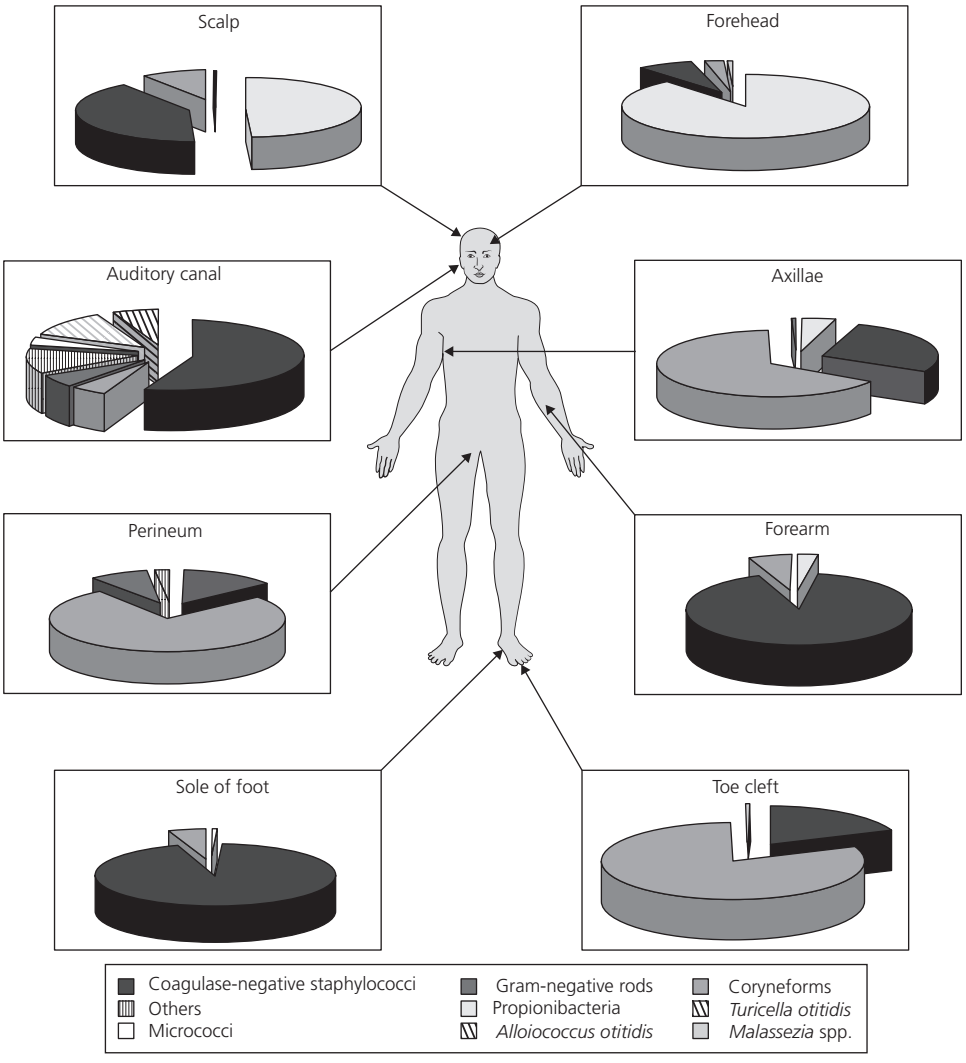

Figure 1 Relative proportions of the various organisms comprising the cultivable microbiota of a number of skin sites (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective*. Oxford: Wiley-Blackwell, 2008). (*see color plate section for color details*).

Staphylococci dominate in dry regions (e.g. arms, legs), while corynebacteria comprise the highest proportions of microbes in communities inhabiting moist regions (e.g. axillae, perineum). Apart from *Acinetobacter* spp., few Gram-negative species are present on the skin surface. As well as bacteria, fungi (Malassezia spp.) are found at many sites. Transients are often present on the skin surface, and these are derived from the environment and from other body sites that have openings onto the skin surface, e.g. the rectum, vagina, etc.

1.3.3.2.2 The conjunctiva

The conjunctival surfaces of a large proportion of individuals appear to be free of cultivable microbes and, when a microbial community is found, it tends to have a low population density and a simple composition — usually no more

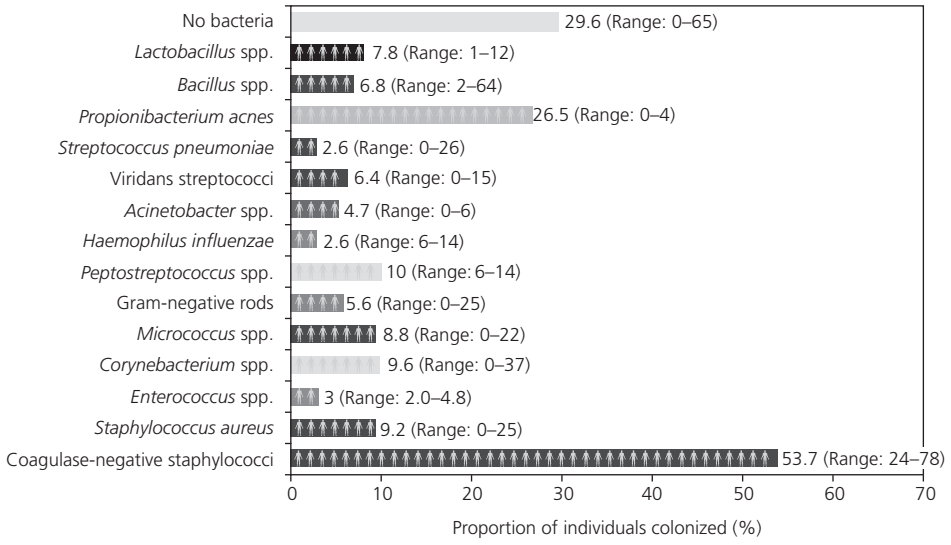

Figure 2 Frequency of detection of various microbes on the conjunctivae of healthy adults. The data shown are mean values (and ranges) derived from the results of 17 culture-based studies involving 4623 individuals from a number of countries (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective.* Oxford: Wiley-Blackwell, 2008).

than two species[76]. Coagulase-negative staphylococci (CNS) and, to a lesser extent, *Propionibacterium acnes* are the most frequently isolated organisms (Figure 2). Other organisms occasionally found include *Staph. aureus* and species belonging to the genera *Corynebacterium, Streptococcus, Lactobacillus, Peptostreptococcus, Bacillus* and *Micrococcus*. Gram-negative species are infrequently isolated. The eyelid margins have a similar cultivable microbiota to that found on the conjunctiva, but the population density tends to be higher.

1.3.3.2.3 The respiratory tract

Only the upper regions of the respiratory tract (the nose and pharynx) have resident microbial communities; the lower regions appear to be largely devoid of cultivable microbes[77]. Although the dominant cultivable organisms in the microbial communities at each site within the tract are known, the exact composition of each community is complex and poorly defined. This is not only because of the complexity of these communities, but is also attributable to the fact that the various regions of the respiratory tract are carriage sites of several very important human pathogens (*Streptococcus pyogenes, Neisseria meningitidis, Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis* and *Staph. aureus*) and, consequently, most bacteriological studies have focused on the detection of these organisms. Other more numerous members of the microbial communities have received little attention. In the nasopharynx (Figure 3) and oropharynx (Figure 4), the most frequently detected organisms include species belonging to the genera *Streptococcus* (mainly viridans streptococci), *Haemophilus, Neisseria, Staphylococcus* (mainly CNS), *Corynebacterium, Prevotella, Propionibacterium, Bacteroides, Porphyromonas* and *Veillonella*. Mollicutes are frequently present, but little is known regarding their identity or their exact prevalence.

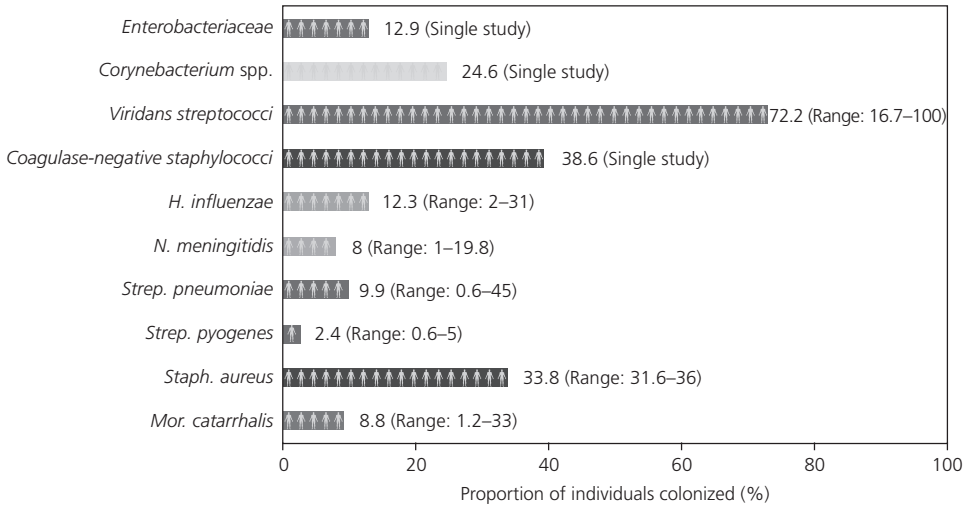

Figure 3 Organisms most frequently detected in the nasopharynx of adults (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective.* Oxford: Wiley-Blackwell, 2008).

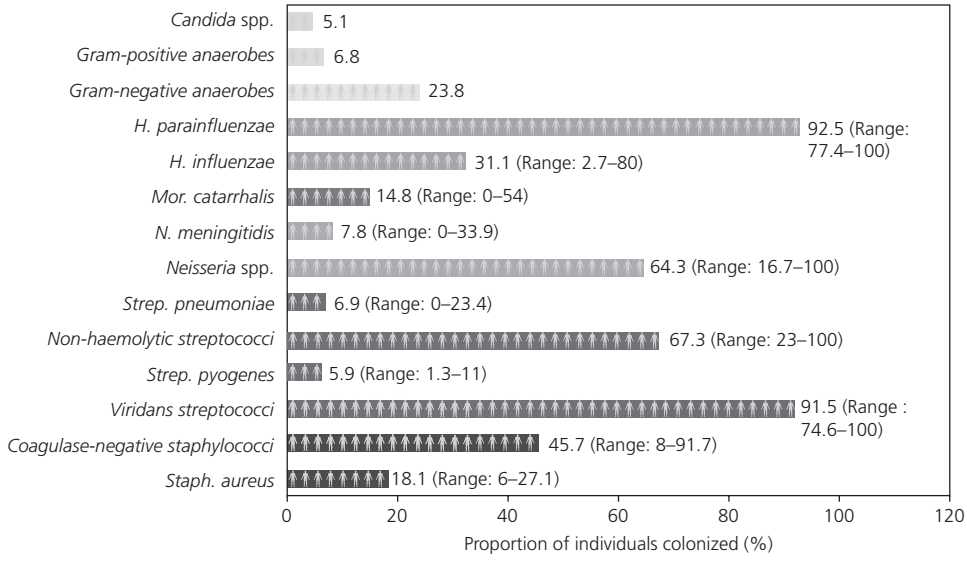

Figure 4 Organisms most frequently detected in the oropharynx (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective.* Oxford: Wiley-Blackwell, 2008).

The microbiotas of the nasal vestibule (Figure 5) and cavity (Figure 6) differ from those of the pharyngeal regions and the most frequently detected organisms are *Corynebacterium* spp., CNS and *Propionibacterium* spp.

1.3.3.2.4 The urinary tract of females

Only the urethra of the female urinary tract has a resident microbiota and, as this is relatively short, microbes can be detected along its entire length[78]. Remarkably few studies have been directed at ascertaining the composition of the urethral

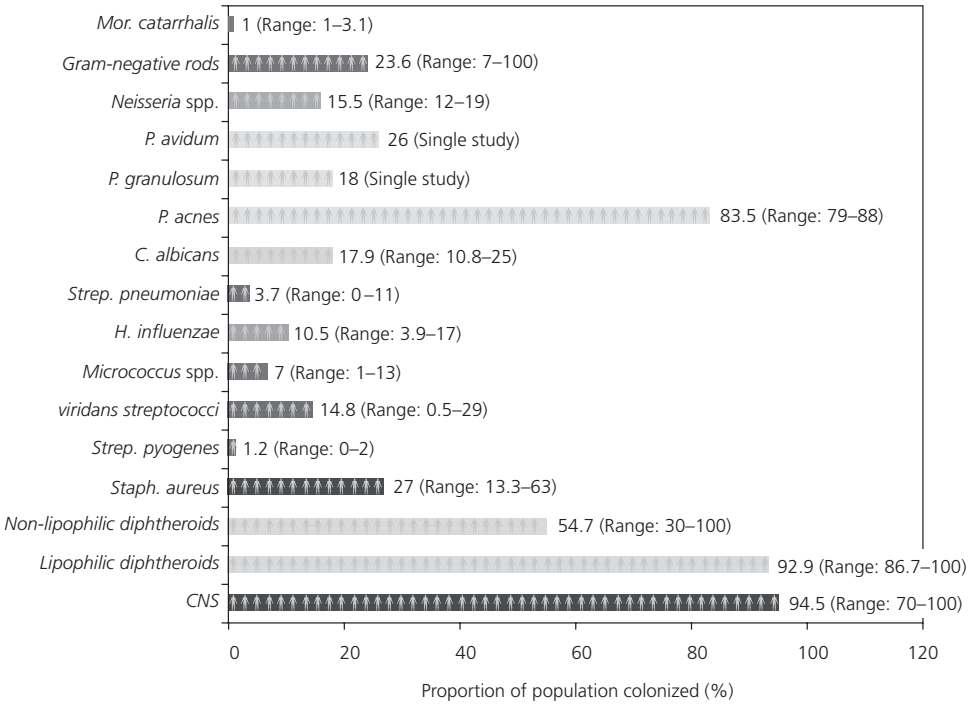

Figure 5 Frequency of detection (mean value and range) of various microbes in the nasal vestibule (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective*. Oxford: Wiley-Blackwell, 2008).

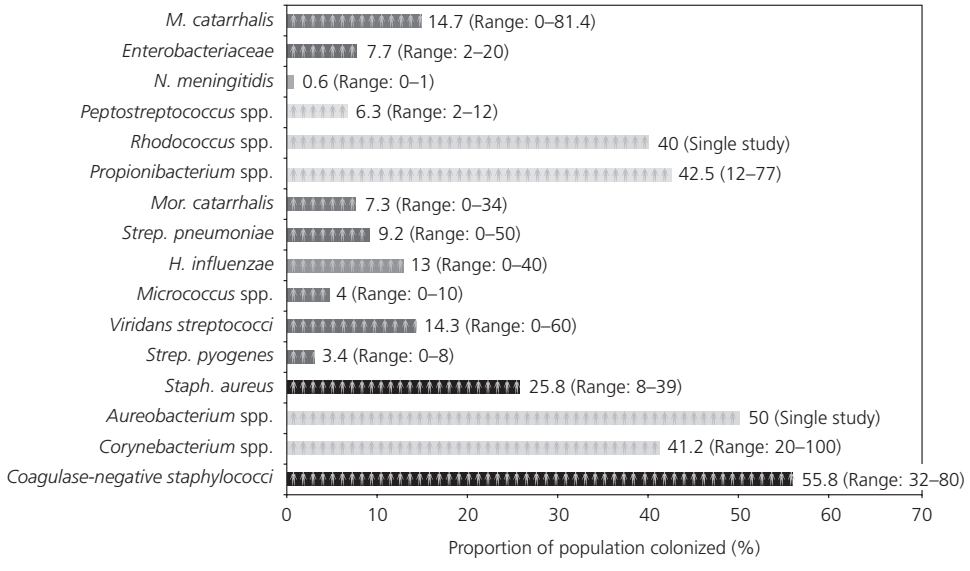

Figure 6 Frequency of detection (mean value and range) of various microbes in the nasal cavity (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective*. Oxford: Wiley-Blackwell, 2008).

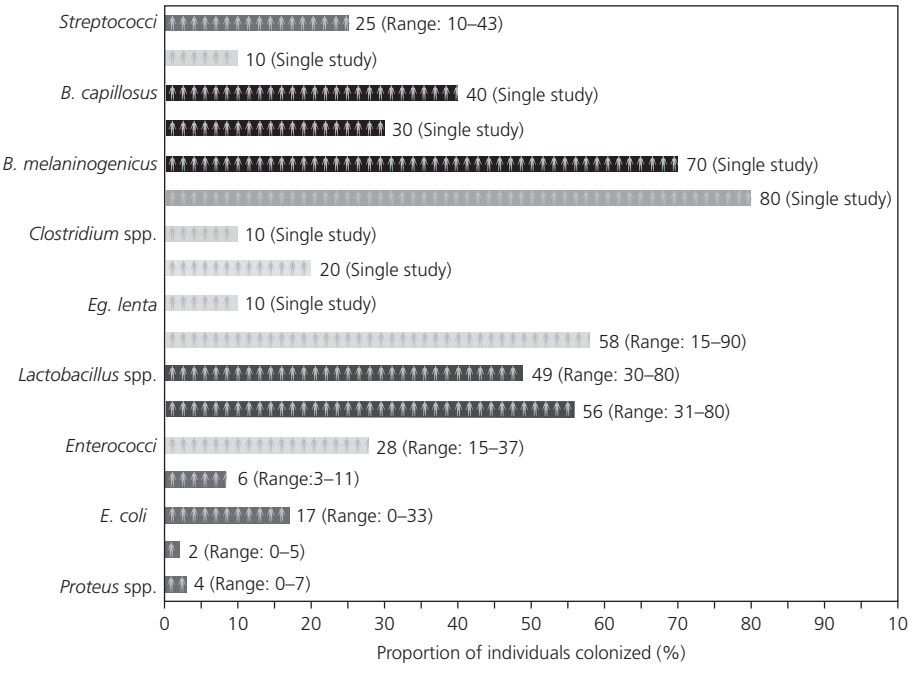

Figure 7 Frequency of isolation of bacteria from the urethra of healthy females. The data shown are the means (and ranges) based on the results of six studies involving 219 pre-menopausal females (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective*. Oxford: Wiley-Blackwell, 2008).

microbiota. The organisms most frequently isolated include *Corynebacterium* spp., Gram-positive anaerobic cocci (GPAC), *Bacteroides* spp., CNS and lactobacilli (Figure 7). However, the sexual maturity of the individual has a profound effect on microbial community composition. *Corynebacterium* spp., CNS, streptococci, and lactobacilli dominate the microbiota of pre-menarcheal girls and pre-menopausal women, whereas Gram-negative anaerobic bacilli and lactobacilli dominate that of post-menopausal individuals.

1.3.3.2.5 The reproductive system of females

Regions of the female reproductive system that are colonized by microbes are the vulva, vagina, and cervix[79]. In addition to being affected by the usual inter-individual variations (e.g. age, socioeconomic factors, etc.), the composition of the microbial communities at these sites is also profoundly influenced by the sexual maturity of the individual and, in females of reproductive age, the menstrual cycle. Although a wide variety of species have been detected within each of these communities in the population as a whole, in an individual female, each microbial community is generally dominated by a limited number of species. The species most frequently isolated from the vagina (Figure 8) and cervix (Figure 9) in females of reproductive age include lactobacilli, CNS, GPAC, Gram-negative anaerobic bacilli (GNAB), coryneforms and Mollicutes. Lactobacilli are generally the numerically dominant organisms in both the vaginal and cervical microbiotas — but this is not the case in pre-menarcheal girls and postmenopausal women who

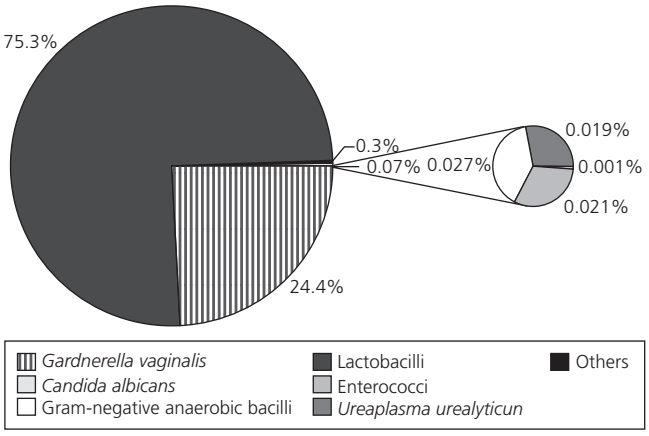

Figure 8 Relative proportions of the predominant organisms constituting the vaginal microbiota of 21 postmenarcheal/pre-menopausal, healthy, non-pregnant females (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective.* Oxford: Wiley-Blackwell, 2008).

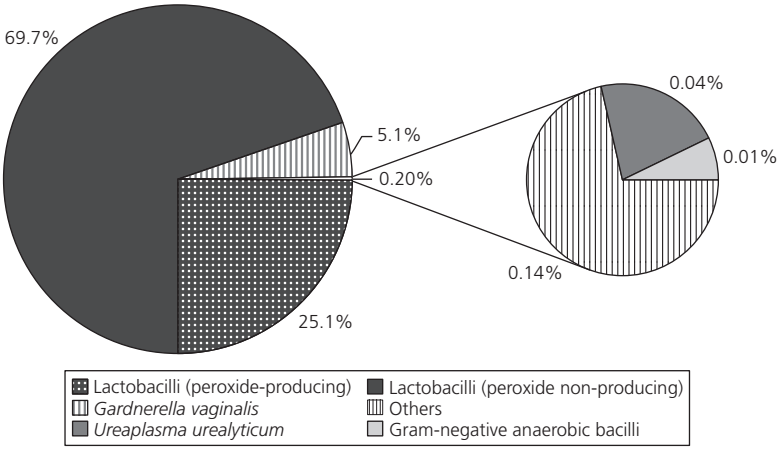

Figure 9 Relative proportions of species comprising the cultivable cervical microbiota of 21 healthy, pre-menopausal females (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective.* Oxford: Wiley-Blackwell, 2008).

are not on hormone replacement therapy. The microbiota of the labia minora is similar to that of the vagina, whereas that of the labia majora consists of both vaginal and cutaneous species (Figure 10).

1.3.3.2.6 The reproductive and urinary systems of males

Of the various regions of the urinary and reproductive tracts in males, only the distal portion of the urethra and the glans penis appear to be colonized by microbes[80]. Unlike in females, where microbes are found along the whole length of the urethra, microbes can be detected only in the distal 6 cm of the male urethra. CNS, viridans streptococci, *Corynebacterium* spp., GPAC and GNAB are the most frequently encountered organisms (Figure 11), and the composition of the microbiota varies along the urethra. Engaging in sexual activity has a dramatic effect on

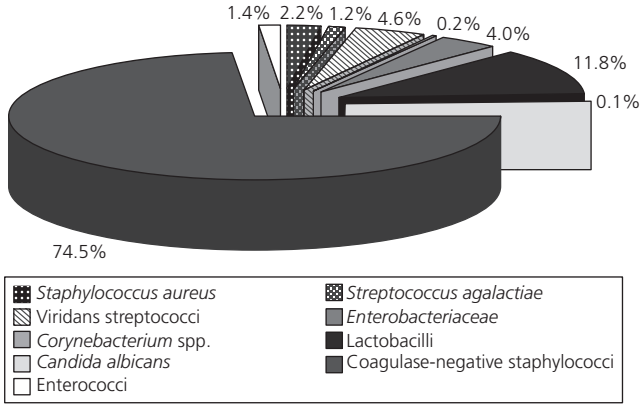

Figure 10 Relative proportions of the various microbes that comprise the cultivable microbiota of the labia majora of post-menarcheal/pre-menopausal females. Data represent the mean values obtained in a study involving 102 individuals (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective.* Oxford: Wiley-Blackwell, 2008). (*see color plate section for color details*).

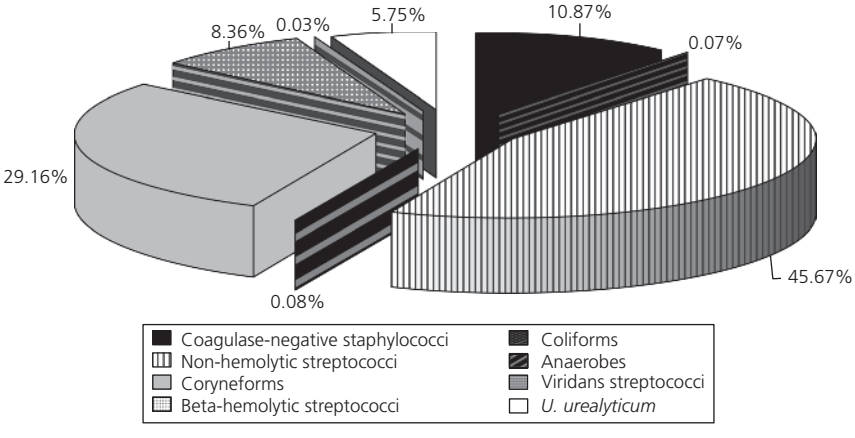

Figure 11 Relative proportions of the various organisms comprising the cultivable urethral microbiota of adult males. Data are derived from an analysis of 60 adult males (reproduced with permission Wilson M. *Bacteriology of humans: an ecological perspective.* Oxford: Wiley-Blackwell, 2008).

the urethral microbiota — it becomes more complex and contains organisms derived from the vagina. The microbiota of the glans penis differs substantially between circumcised and uncircumcised individuals, the population density and species diversity being greater in the latter. *Malassezia* spp., anaerobes, and facultative Gram-negative bacilli are frequently encountered in uncircumcised individuals, whereas CNS, *Propionibacterium* spp., and *Corynebacterium* spp. dominate in circumcised individuals.

1.3.3.2.7 The oral cavity

Because of its complex anatomy, the oral cavity has a large variety of habitats available for microbial colonization[81]. Uniquely, it also has non-shedding surfaces, the teeth, that make possible the formation of substantial and complex biofilms. The oral cavity harbors a variety of microbial communities, most of which have a high species diversity. As many as 700 phylotypes have been detected in the oral cavity, and approximately 50% of these have not yet been cultivated. Although mucosal surfaces comprise 80% of the total surface area of the oral cavity, most of the microbes present in the mouth are found on tooth surfaces in biofilms known as dental plaques. The microbial composition of these plaques is complex and is dependent on their anatomical location. In supragingival plaques, viridans streptococci and *Actinomyces* spp. are usually the dominant organisms, but anaerobes such as *Veillonella* spp. and *Fusobacterium* spp. are also invariably present (Figure 12). The microbial composition of plaque alters with time and is also affected by the host's diet. The microbiota of the plaque found in the gingival crevice is more diverse than that of supragingival plaques and, although streptococci are usually the dominant organisms, the proportion of anaerobes is greater than in supragingival plaques (Figure 13). Anaerobic organisms frequently detected include *Veillonella* spp., Gram-positive anaerobic cocci, *Prevotella* spp., *Fusobacterium* spp., *Selenomonas* spp., *Eubacterium* spp. and spirochetes.

The tongue is densely colonized by microbes, and the composition of the resident communities varies with the anatomical location (Figure 14). Streptococci, again, are generally the dominant organisms, and a variety of anaerobes are frequently present, including species belonging to the genera *Prevotella, Veillonella, Eubacterium* and *Fusobacterium*. Other mucosal surfaces are relatively sparsely populated compared with the tongue. The community composition varies with the anatomical location, but facultative anaerobes and capnophiles are usually the dominant organisms, e.g. streptococci, *Gemella* spp., *Neisseria* spp., *Haemophilus* spp. and *Capnocytophaga* spp. However, anaerobes such as *Fusobacterium* spp., *Veillonella* spp. and *Prevotella* spp. are also often present.

1.3.3.2.8 The gastro-intestinal tract

The gastro-intestinal tract (GIT) has a number of distinct regions, each harboring a characteristic microbial community or communities[82]. In the upper GIT (oral cavity, pharynx and esophagus), the resident microbiota is associated with surfaces, and because material (food, secretions, etc.) passes rapidly through these regions, microbial communities cannot become established in their lumen. As the passage of material becomes slower in the lower regions of the GIT, there is an opportunity for communities to develop within the lumen as well as on the mucosal surface — in the distal ileum, cecum, colon and rectum such communities are substantial. Very few studies have investigated the oesophageal microbiota, but these few have shown that it is dominated by staphylococci, lactobacilli and *Corynebacterium* spp. (Figure 15).

Because of its low pH, the stomach is a hostile environment for a wide range of organisms. Organisms detected in the lumen are mainly acid-tolerant species of streptococci and lactobacilli together with staphylococci, *Neisseria* spp., and various anaerobes. These organisms are also present in the mucosa-associated community, which in addition often contains the important pathogen *Helicobacter pylori* (Figure 16).

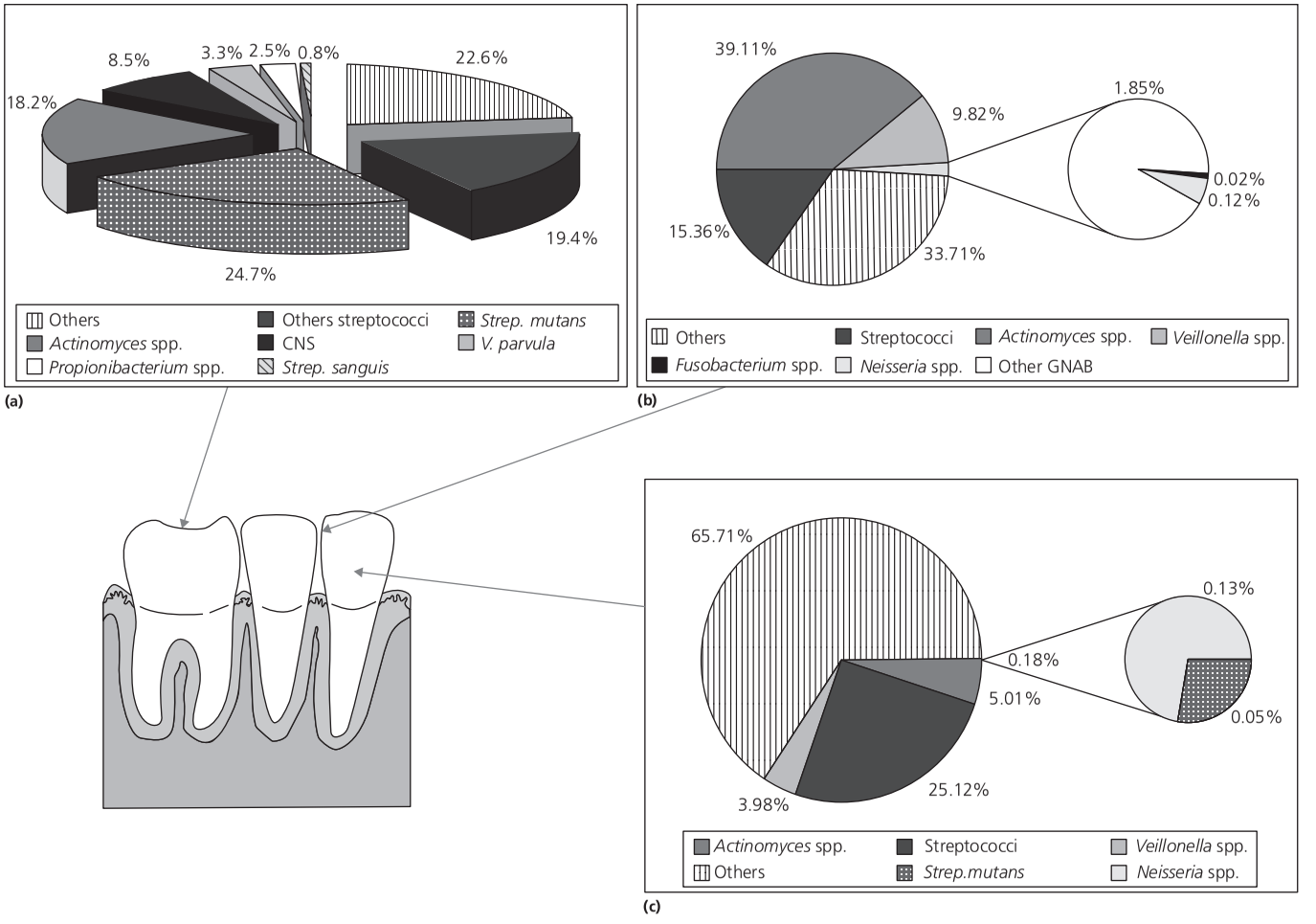

Figure 12 The predominant cultivable microbiota of the three main types of supragingival plaque: (a) fissure, (b) approximal, and (c) smooth surface. Data are derived from three studies involving a total of 40 healthy adults (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective*. Oxford: Wiley-Blackwell, 2008).

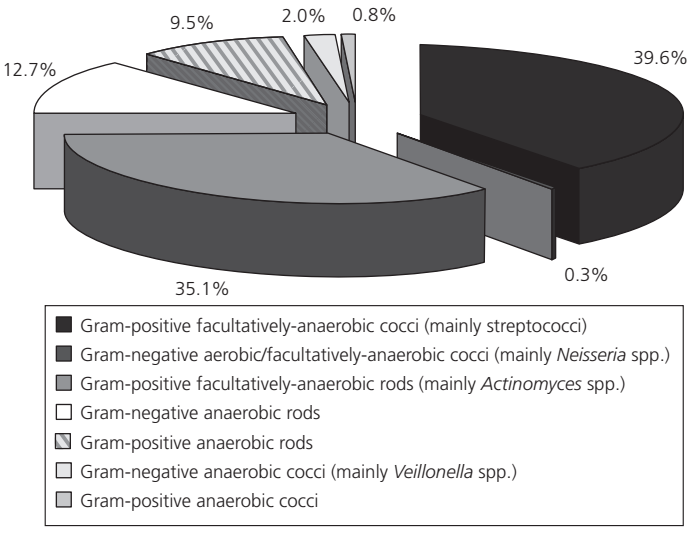

Figure 13 Relative proportions of organisms comprising the cultivable microbiota of the gingival crevice. Data are derived from a study involving seven healthy adults (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective*. Oxford: Wiley-Blackwell, 2008). (*see color plate section for color details*).

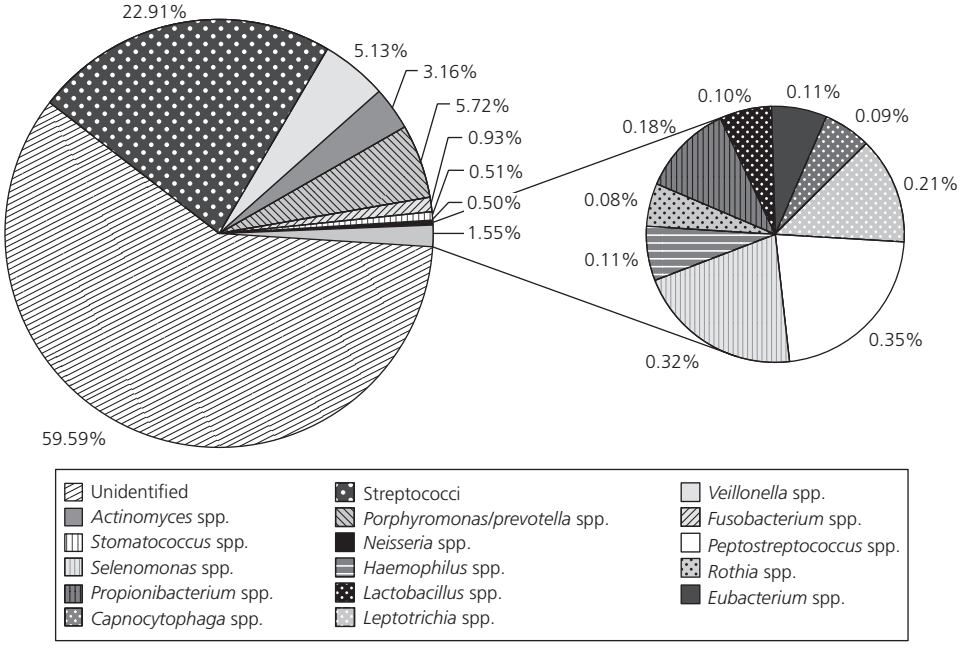

Figure 14 Relative proportions of the various organisms comprising the cultivable microbiota of the tongue. Data are derived from a study involving 17 healthy adults (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective*. Oxford: Wiley-Blackwell, 2008).

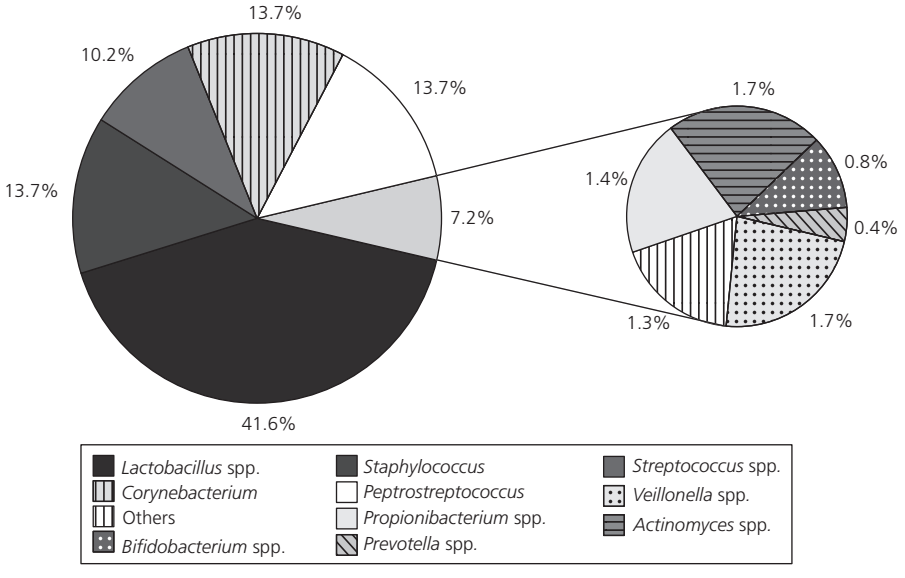

Figure 15 Culture-dependent study of the oesophageal microbiota. Relative proportions of the organisms present. Data are mean values derived from the results of two studies involving 17 healthy adults (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective.* Oxford: Wiley-Blackwell, 2008).

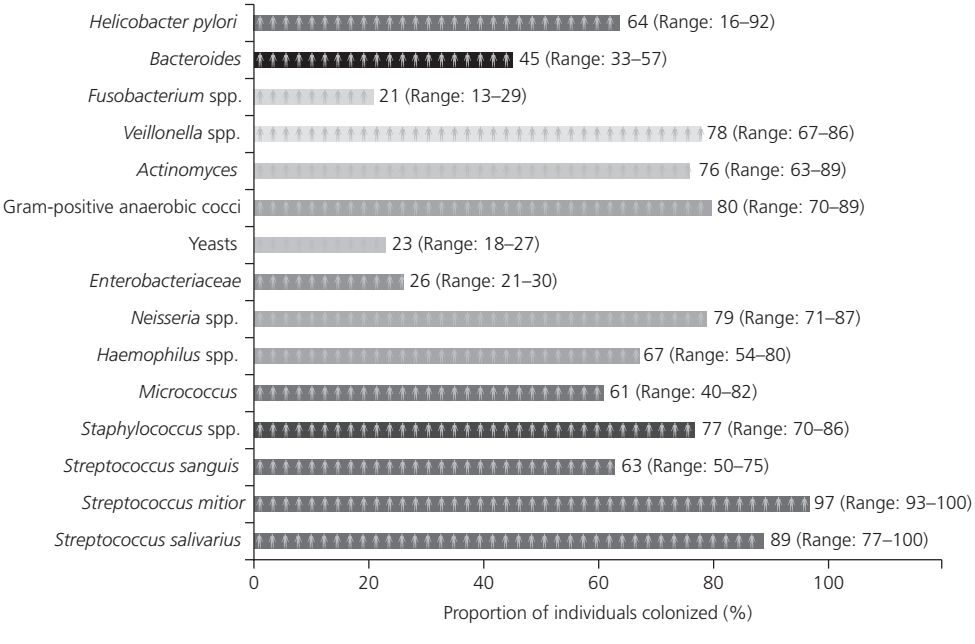

Figure 16 Frequency of isolation of microbes from the gastric mucosa. The data shown are the means (and ranges) based on the results of two studies involving 58 adults (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective.* Oxford: Wiley-Blackwell, 2008).

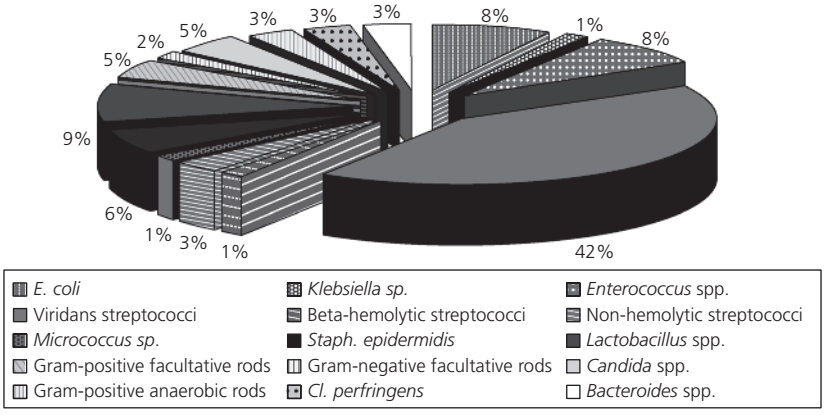

Figure 17 Relative proportions of organisms comprising the cultivable microbiota of the duodenal mucosa of 26 healthy adults (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective*. Oxford: Wiley-Blackwell, 2008). (*see color plate section for color details*).

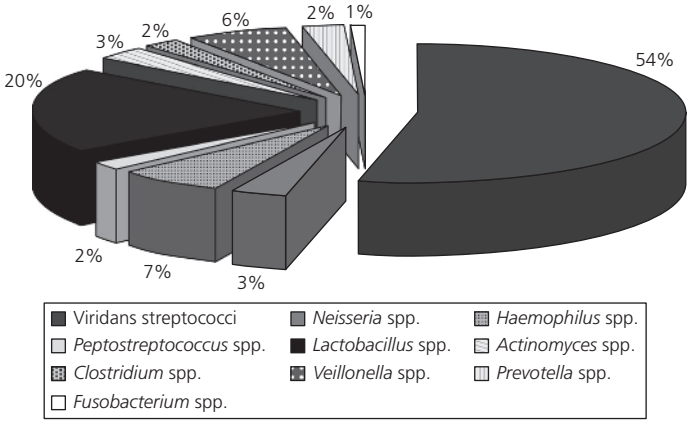

Figure 18 Relative proportions of organisms comprising the cultivable microbiota of the jejunal mucosa of 20 healthy adults (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective*. Oxford: Wiley-Blackwell, 2008).

The environments within the duodenum and jejunum are also largely inimical to many microbes because of the low pH, the presence of bile (and other antimicrobial compounds), and the rapid transit of material. Consequently, the mucosa and the lumen of both of these regions have sparse microbiotas consisting mainly of acid-tolerant streptococci and lactobacilli (Figures, 17 and 18).

In the ileum, especially the terminal region, conditions are less hostile to microbes, and the microbiotas within the lumen and on the mucosa are more substantial. Streptococci, enterococci and coliforms are the dominant organisms in the lumen, but the microbiota of the mucosa is very different and consists of high proportions of anaerobes including *Bacteroides* spp., *Clostridium* spp., GPAC and *Bifidobacterium* spp. The cecum has a lower pH and a higher content of easily fermentable compounds than the more distal regions of the GIT, and consequently

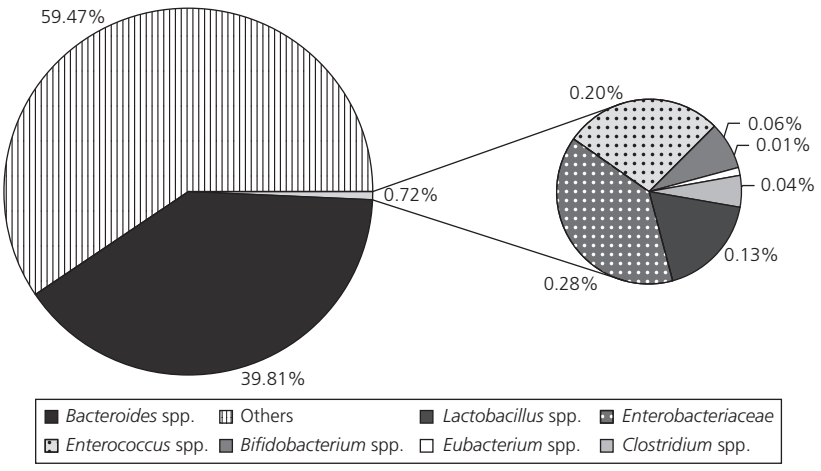

Figure 19 Relative proportions of organisms comprising the cultivable microbiota of the caecal mucosa in 19 healthy adults (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective*. Oxford: Wiley-Blackwell, 2008).

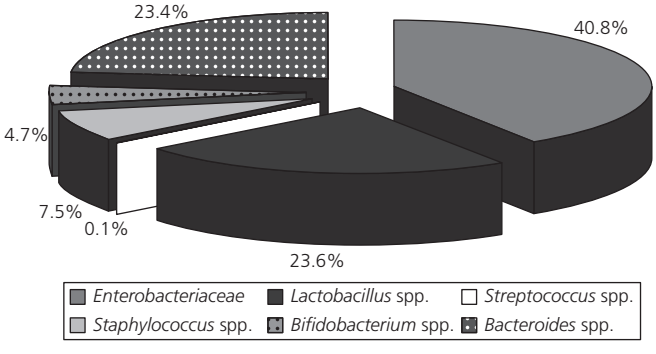

Figure 20 Relative proportions of organisms comprising the cultivable microbiota of the contents of the cecum in 21 healthy adults (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective*. Oxford: Wiley-Blackwell, 2008).

it harbors microbial communities that are very different from those in the rest of the large intestine. The lumen is dominated by facultative organisms (mainly Enterobacteriaceae and lactobacilli), although substantial proportions of anaerobes (*Bacteroides* spp. and *Clostridium* spp.) are also present (Figure 19). The mucosal microbiota appears to be dominated by *Bacteroides* spp. (Figure 20).

The colon is colonized by a very large and diverse microbial population. Up to 80% of the organisms present have not yet been grown in the laboratory, and many of these are novel phylotypes. Most of our knowledge of the colonic microbiota has come from analysis of feces in which obligate anaerobes are 1000-fold greater in number than facultative organisms — the predominant genera being *Bacteroides*, *Eubacterium* (and related genera), *Bifidobacterium* and *Clostridium* (Figure 21). The microbiota of the colonic mucosa is dominated by *Bacteroides* spp. (Figure 22).

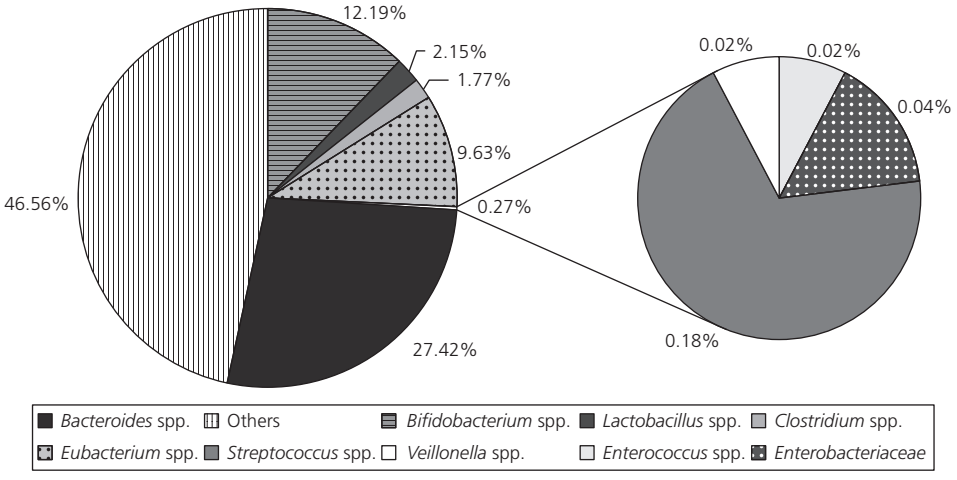

Figure 21 Culture-based analysis of the composition of the faecal microbiota. The figures represent mean values for the relative proportions of the various genera —— these have been derived from the results of ten studies involving 212 healthy adults from several countries (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective*. Oxford: Wiley-Blackwell, 2008).

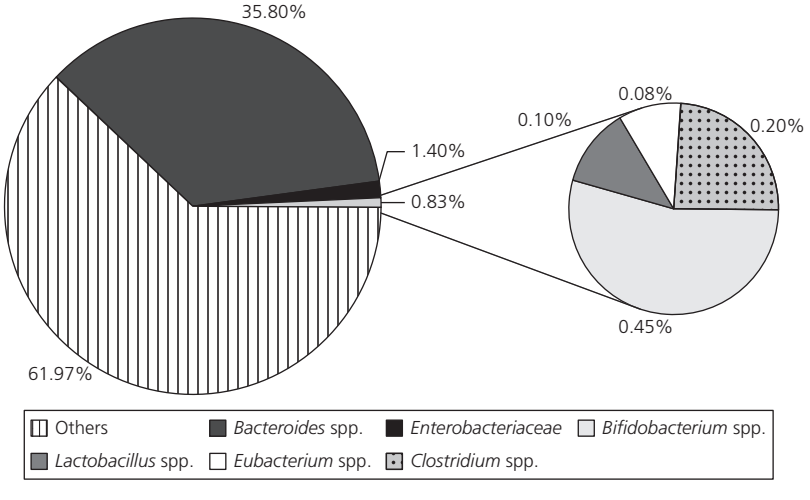

Figure 22 Cultivable microbiota of the colonic mucosa. Figures denote mean values for the relative proportions of the various organisms isolated, and are derived from the results of three studies involving 61 healthy adults (reproduced with permission from Wilson M. *Bacteriology of humans: an ecological perspective*. Oxford: Wiley-Blackwell, 2008).

1.4 Culture-independent approaches to microbial community analysis

Many of the problems inherent in culture-based approaches to analysing microbial communities can be circumvented by the use of molecular techniques, the main advantage being that organisms that have not yet been cultivated in the laboratory can be identified in these communities. However, it must be pointed out that such

approaches are not without their own problems[63]. The first stage in the analysis of a microbial community by a molecular technique is to isolate either DNA or RNA from the sample. Extraction of nucleic acids from microbes requires that the cells are lysed and the ease of lysis varies significantly among different organisms. Numerous protocols for the lysis of microbes present in samples have been devised and include the use of enzymes, chemicals and mechanical methods. Care has to be taken that, once lysed, the nucleic acids do not undergo shearing and are not degraded by nucleases. The extracted nucleic acids can then be used in a variety of ways to reveal the identity of the microbes originally present in the sample and/or to produce a "profile" or "fingerprint" of the microbial community.

Universal primers can be used to amplify all of the 16S rRNA genes present in the DNA extracted from the sample and the amplified sequences are then cloned and sequenced. The sequences of the clones are then determined and compared to sequences in databases. If a sequence is >98% similar to one already in the database, then it is regarded as identical and so the corresponding organism can be assumed to have been present in the sample[83,84]. In this way the sequences of the 16S rRNA genes of all organisms present in the community can be determined and, if these sequences match those of known organisms in databases, then the identities of all the organisms present will be revealed. However, not all of the sequences of the 16S rRNA genes obtained correspond to sequences in databases. A significant limitation of 16S rRNA sequencing is the introduction of biases by PCR primer design, which may select for or against particular groups of organisms. Furthermore, bacterial contamination of reagents may occur, and therefore extensive controls are required. The 16S rRNA operon is also present in between one and fifteen copies in bacterial genomes and this can influence the apparent relative abundance of an organism.

Another useful approach is to separate the amplicons produced by either temperature-gradient gel electrophoresis or denaturing-gradient gel electrophoresis[85]. Staining of the DNA in the resulting gel reveals a banding pattern or "fingerprint" that is characteristic of that particular community. The individual bands can be cut out and each amplicon eluted, re-amplified, sequenced and identified using databases as described above. Alternatively, the fingerprints produced from samples from the same individual obtained on different occasions can be compared and analysed for differences. Hence, bands appearing or disappearing with time can be sequenced to determine the gain or loss of an organism from the community. The method is also useful for comparing the microbiotas present at the same body site in different individuals and this is facilitated by computer and statistical analysis of the banding patterns obtained.

Microarrays offer yet another possible approach. A number of different phylogenetic microarrays, consisting of multiple probes designed to discriminate specific organisms or subgroups of organisms, have been developed[86]. However, the probes and the organisms targeted must be preselected, which limits their usefulness in detecting novel organisms in the community under investigation. A microarray known as the Human Intestinal Tract Chip (HITChip) has been developed to detect target 1140 different phylotypes in the human intestinal microbiota[87].

Quantitative PCR can be used to quantify species, or groups of organisms, in a community[88]. However, this is technically demanding and time-consuming.

Metagenomics, the analysis of all the genomes present in an ecosystem, was first described in 1998 and has become an extremely powerful tool for analysing microbial communities both in terms of what species are present as well as the activities of which they are capable[89]. New sequencing technologies (e.g. pyrosequencing, Illumina) have made possible shotgun sequencing of the metagenomic DNA of a microbial community inhabiting a body site in a rapid and cost-effective manner. For example, in a study of the faecal microbiota of 124 subjects, metagenomic analysis revealed the presence of approximately 1150 bacterial species of which about 160 species were associated with each individual[90]. 57 species were common to more than 90% of individuals.

The above approaches let microbial community structure be determined in terms of species richness, community evenness and diversity. Such measures can reveal a great deal about the dynamics and selection pressures experienced by the system. Increased richness, evenness and diversity can be associated with stable, longer-established or less-active ecosystems[91]. Furthermore, association of these measures with environmental and clinical parameters can give valuable insights into states of health and disease[92,93].

1.5 Determination of microbial community functions

Metagenomics not only enables us to determine what organisms are present in a community but, as it provides a catalogue of the genes present, also furnishes us with information concerning the range of activities that the community has the potential to undertake. Such studies have revealed that the intestinal microbiota is enriched in gene categories (i.e. clusters of orthologous groups of proteins; http://www.ncbi.nlm.nih.gov/COG; Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg) involved in carbohydrate metabolism, energy metabolism and storage, generation of SCFAs, amino acid metabolism, biosynthesis of secondary metabolism and metabolism of cofactors and vitamins[94].

Other technologies that have been developed to provide information about community function rather than composition include metatranscriptomics, metaproteomics and metabolomics.

Metatranscriptomics involves extracting and sequencing the mRNA molecules present in the microbial community, thereby identifying which of the genes present are actually being expressed[95]. For example, a metatranscriptomic analysis of the faecal microbiota has demonstrated that the main functionalities expressed were carbohydrate metabolism, energy production and synthesis of cellular components[96].

Metaproteomics involves analysing all of the proteins present in a microbial community and is an approach that is developing very rapidly due to improvements in protein separation techniques combined with highly accurate, high-throughput mass spectrometry[97]. In a study of the feces from three healthy females, a core metaproteome (i.e. those proteins shared between different individuals) was identified[98]. This consisted of 1216 proteins among which glutamate dehydrogenases, pyruvate formate lyases, phosphoenolpyruvate carboxykinases, GroEL chaperonins and a NifU protein with chaperone function were highly abundant. These core functions have also been identified in other intestinal metaproteome studies. Current studies of the intestinal metaproteome emphasize the importance of

proteins involved in carbohydrate metabolism, in maintaining protein integrity and in coping with the low redox potential in the gut. Specifically, flagellins, glutamate dehydrogenase and glyceraldehydes-3-phosphate dehydrogenase appear to be essential proteins for maintaining bacterial life in the intestinal tract.

Metabolomics involves analysing (usually by gas chromatography-mass spectrometry) the metabolites produced by a microbial community. In a recent metabolomics study of feces obtained from healthy and cirrhotic patients, a total of 9,215 metabolites were detected[99]. Six major groups of metabolites (bile acids, bile pigments, lysophosphatidylcholines, aromatic amino acids, fatty acids and acylcarnitines) were found to be significantly altered in the cirrhotic patients compared with healthy controls.

Data from studies using the above culture-independent techniques, along with data from culture-dependent studies such as those described previously in this chapter, has contributed enormously to our understanding of the composition and function of the indigenous microbiota of humans. Progress in the field has been rapid thanks to the efforts of research consortia such as the MetaHIT (Metagenomics of the Human Intestinal Tract; www.metahit.eu) project supported by the European Union and the Human Microbiome Project funded by the National Institutes of Health in the USA (http://www.hmpdacc.org). Contemporary views of the nature of the microbial communities found at various body sites will be described in subsequent chapters (see chapters 3, 4, 5, 16 and 24).

1.6 Closing remarks

Imagine the surprise and excitement, as well as an undoubted feeling of trepidation, three and a half centuries ago at the discovery of little "animalcules" wriggling around inside our mouths and intestines. Since then, our microbial companions have never ceased to surprise and amaze us. First they became something to be feared as we discovered that they can cause a range of diseases, some of which (e.g. meningitis, pneumonia) are life-threatening. Then they became something to be cherished as we found that they are essential for our development, are involved in protecting us from exogenous pathogens and supply us with nutrients, energy and vitamins. Now, in the second decade of the 21st century, we are learning that they are also involved in determining our physique, behavior and mood. An historical appreciation of the development of our relationship with our microbiota can only teach us that we are in store for many more surprises.

TAKE-HOME MESSAGE

- A major driving force behind the resurgence of interest in the human microbiota is an increasing awareness of its importance in human development, nutrition, behavior, wellbeing and disease.
- Knowledge of the human microbiota has increased dramatically in the 21st century due to the development of technologies that can identify which microbes are present in a community, as well as determining their possible functions, without needing to grow them in the laboratory.
- We are only just beginning to understand the composition and functions of the microbial communities that inhabit humans.

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