Indigenous Intestinal Microbiota and Disease

different approaches to characterize the composition
and products of the human microbiota

Merja Rautio

Department of Microbiology
National Public Health Institute
and
Division of General Microbiology
Department of Biosciences
University of Helsinki

Helsinki, Finland

Academic Dissertation

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Helsinki 2002
Supervised by

Research Professor Hannele Jousimies-Somer, Ph.D.
Anaerobe Reference Laboratory
Department of Microbiology
National Public Health Institute, Helsinki

Docent Anja Siitonen, Ph.D.
Laboratory of Enteric Pathogens
Department of Microbiology
National Public Health Institute, Helsinki

Reviewed by

Professor Airi Palva, Ph.D.
Department of Basic Veterinary Sciences
University of Helsinki

Docent Maria Saarela, Ph.D.
VTT Biotechnology
VTT, Espoo

Opponent

Docent Pentti Huovinen, M.D., Ph.D.
National Public Health Institute, Turku
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications and a manuscript, which are referred to in the text by their Roman numerals:


In addition, some unpublished results are included.
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>CFA</td>
<td>cellular fatty acid</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DGGE</td>
<td>denaturing-gradient gel electrophoresis</td>
</tr>
<tr>
<td>EIA</td>
<td>enzyme immuno assay</td>
</tr>
<tr>
<td>FAME</td>
<td>fatty acid methyl ester</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescent <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>GLC</td>
<td>gas-liquid chromatography</td>
</tr>
<tr>
<td>IBS</td>
<td>irritable bowel syndrome</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>MAF</td>
<td>the mucosa-associated flora</td>
</tr>
<tr>
<td>MIC</td>
<td>minimal inhibitory concentration</td>
</tr>
<tr>
<td>MIS</td>
<td>Sherlock® Microbial Identification System</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PRAS</td>
<td>pre-reduced anaerobically sterilized</td>
</tr>
<tr>
<td>PY</td>
<td>pre-reduced peptone yeast extract broth</td>
</tr>
<tr>
<td>PYG</td>
<td>pre-reduced peptone yeast extract glucose broth</td>
</tr>
<tr>
<td>rDNA</td>
<td>ribosomal deoxyribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>SCFA</td>
<td>short chain fatty acid</td>
</tr>
<tr>
<td>TGGE</td>
<td>temperature-gradient gel electrophoresis</td>
</tr>
<tr>
<td>UC</td>
<td>ulcerative colitis</td>
</tr>
</tbody>
</table>
INTRODUCTION

Awareness of the variations in composition of the normal microbiota, its uniqueness for individuals, the distributions of the microbiota, and the biological succession that occur in neonates is important in explaining the occurrence of disease due to components of the microbiota (Tannock, 1999b). Even in the absence of disease, the presence of large numbers of viable, and hence presumably metabolizing, microbial cells in human intestinal tract must have major influences on the host (Tannock, 1999b,c).

Mostly, the human host is unaware of the presence of the normal microbiota because the microbes and the host have attained a balanced relationship. When this balance is disturbed, some of the microbial components of the microbiota become the etiological agents of disease and we can see the serious effects of disease such as pseudomembranous colitis as a consequence of antimicrobial usage. Furthermore, debilitating intestinal disorders such as inflammatory bowel disease and colonic cancer are thought to have a bacterial involvement (Chadwick & Chen, 1999; Birkbeck, 1999). Members of the microbiota of the human large intestine are capable of provoking serious infectious processes within the peritoneal cavity of humans. Virtually all such infections are polymicrobial and involve both aerobic and anaerobic bacterial species (Finegold, 1995; Onderdonk, 1999). Morbidity and mortality due to such infections is substantial, as is the cost to the health-care delivery system. In order to appreciate the complexity of these infections, it is first important to understand some of the complexity of the human intestinal microbiota.

Technical difficulties associated with the investigation of such a large and complex microbial collection are obvious and hard to overcome in research concerning the intestinal microbiota. The applications of useful procedures that help identify the gut microbial diversity as well as allow effective tracking of microbiota changes and differences are needed. Studies of the maintenance or controlled manipulation of the intestinal microbiota should also benefit from useful technologies, and these studies will in turn be of benefit in the prophylaxis and treatment of disease related to the activities of the intestinal microbiota.
REVIEW OF THE LITERATURE

1. THE NORMAL MICROBIOTA OF THE HUMAN INTESTINAL TRACT

The "normal microflora" is the term most commonly used in the medical literature for many decades when referring to the microbial community that consistently inhabits the body of a healthy animal. Other terms used are "normal flora" and "indigenous microbiota". Of these, strictly correct term is "indigenous microbiota", since it refers to a collection of microscopic creatures that are native to the body (Tannock, 1999b), and "flora" and "microflora" have an unfortunate botanical connotation.

The normal microbiota is comprised of a diverse collection of microbial species, mostly bacterial. It has been estimated that more microbial cells inhabit the human body than there are eucaryotic cells of which it is constituted \(10^{14} - 10^{13}\) (Luckey et al. 1972). The term "microbiome" has been used to describe the collective genome of our indigenous microbes. Assuming an average microbial genome size of 5 million base pairs and 4000 genes per genome, the intestinal microbiome alone may contain genes 50 to 100 times as many as our own genome (Hooper & Gordon, 2001).

**Figure 1.** The gastrointestinal tract
The human gastrointestinal tract (Figure 1), from mouth to anus, is remarkably complex organ with multiple functions and many different microbiological environments. The microorganisms that colonize this organ are both longitudinally and cross-sectionally distributed (Macfarlane et al., 1992; Berg, 1996). The different biochemical and physiological conditions prevailing in various regions of the intestinal tract provide environments in which certain species of microbes can flourish while other microbial types, lacking appropriate properties, can not. Due to the effects of gastric acid, and rapid passage of digesta through the stomach and small bowel, the numerically dominant areas of permanent colonization of the human gastrointestinal tract are terminal ileum and large intestine (Table 1).

**Table 1.** Numbers of culturable bacteria per gram luminal contents at various subsites of the human gut (Hill, 1995a)

<table>
<thead>
<tr>
<th>Site</th>
<th>Counts of bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouth</td>
<td>$10^7$ – $10^8$</td>
</tr>
<tr>
<td>Stomach</td>
<td>$&lt; 10^3$</td>
</tr>
<tr>
<td>Jejunum</td>
<td>$&lt; 10^3$</td>
</tr>
<tr>
<td>Terminal ileum</td>
<td>$10^5$</td>
</tr>
<tr>
<td>Colon</td>
<td>$10^{11}$</td>
</tr>
</tbody>
</table>

**Table 2.** Relative proportions of culturable anaerobes and aerobes at various subsites of the human gut (Hill, 1995a)

<table>
<thead>
<tr>
<th>Site</th>
<th>Counts of anaerobic bacteria : aerobic bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouth</td>
<td>10-100 : 1</td>
</tr>
<tr>
<td>Stomach</td>
<td>$&lt; 1 : 1$</td>
</tr>
<tr>
<td>Jejunum</td>
<td>1-10 : 1</td>
</tr>
<tr>
<td>Terminal ileum</td>
<td>1-100 : 1</td>
</tr>
<tr>
<td>Cecum</td>
<td>100-10 000 : 1</td>
</tr>
<tr>
<td>Colon</td>
<td>100-1 000 : 1</td>
</tr>
<tr>
<td>Colonic mucosa</td>
<td>1-10 : 1</td>
</tr>
</tbody>
</table>
Normal oral cavity microbiota varies according to age and from site to site. Thus, tooth surfaces, gingival crevices, the surfaces of the tongue, roof of mouth, cheek surface and pharynx all have a different and characteristic microbiota that is composed of organisms that adhere most efficiently to these surfaces (Gibbons & van Houte, 1975; Könönen, 2000). The salivary microbiota is derived from that on the oral surfaces. The oral microbiota is dominated by gram-positive rods and cocci (Hill, 1995a).

The stomach provides a less complex part of this ecosystem and bacterial diversity is low. The normal stomach receives bacteria from the mouth by saliva and food but in humans is thought not to support a normal microbiota, because the normal resting gastric juice pH is below 3 (Hill, 1995a; Tannock, 1999a). Hypochlorhydric stomach supports bacterial growth in gastric juice (Väkeväinen et al. 2000). However, studies on the stomach-colonizing pathogen Helicobacter pylori, and its association with gastric and duodenal ulceration, has been a major area of research (MacGowan et al., 1996). Although the common concept admits association (Lee, 1999), there are those who still believe this bacterium is not a true pathogen, rather it is an opportunistic colonizer that inhabits already damaged gastric mucosa (Graham, 1995).

The normal small bowel contains a very sparse microbiota of transient organisms. The principal microbial types cultured from the upper small intestine (duodenum and jejunum) are lactobacilli and streptococci (Berg, 1996). The upper small intestine is colonized from above when the gastric contents enter during digestion. However, many biliary and pancreatic secretions are bactericidal and help to sterilize the material entering (Holzapfel et al., 1998). Further, there is extensive fluid secretion from the mucosa which prevent colonization of the mucosal layer. Small bowel transit time is only two to four hours and this is a further barrier to small bowel colonization (Holzapfel et al., 1998). The distal small bowel (ileum) is colonized as a result of reflux of cecal contents through the ileocecal junction (Nord & Kager, 1984) and the microbiota of the terminal ileum is similar to that of the cecum (Hill, 1995a). However, this area of the gut is extremely difficult to sample with confidence and therefore the extent of colonization is not known.

Although bacteria can be found along the entire length of the gastrointestinal (GI) tract, the largest number of bacteria reside within the large intestine (colon) (Table 1 and 3). The entire length of the colon in adult humans is about 150 cm. It consists of the cecum (to which the appendix is attached), the ascending colon, transverse colon, descending colon, the sigmoid colon and rectum (Macfarlane & Cummings, 1991). The large intestine of an adult contains about 220 g of contents (Banwell et al., 1981; Cummings et al., 1990; Goldin, 1990) and bacteria comprise approximately 55% of fecal mass (Stephen & Cummings, 1980). It is generally acknowledged that the colon is populated with $10^{11}$-$10^{12}$ viable bacterial cells per gram of content, which is approximately $10^{14}$ in total (Goldin, 1990). This does not include those organisms that may be intimately attached to the intestinal epithelial tissue, or that reside within the crypts formed by the intestinal mucosa (Onderdonk, 1999).
The human colonic microbiota consists of more than 400 bacterial species (Finegold et al., 1974; Moore & Holdeman, 1974a) of which anaerobes outnumber aerobes (Table 2) by a factor of $10^2$ to $10^4$ (Finegold & Sutter, 1978; Simon & Gorbach, 1984). Several studies have shown that the culturably dominant bacterial species present within the large bowel are members of the genus Bacteroides (Table 3) accounting for up to 30% of all isolates. In addition, members of the genera Peptostreptococcus, Eubacterium, Bifidobacterium, Clostridium and Fusobacterium are among the culturally numerically dominant organisms present in the large intestine. Facultative species commonly described as "enterics", such as Escherichia coli, are present at less than 0.1% of the total culturable population (Onderdonk, 1999).

Table 3. Overview of common culturable microbiota of the gastrointestinal tract (Nord & Kager, 1984)

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Number of microorganisms (CFU/ml or CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oropharynx</td>
</tr>
<tr>
<td>Total bacterial count</td>
<td>$10^8$-$10^{10}$</td>
</tr>
<tr>
<td><strong>Aerobic bacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Rare</td>
</tr>
<tr>
<td>Streptococci</td>
<td>$10^5$-$10^9$</td>
</tr>
<tr>
<td>Staphylococci</td>
<td>$0$-$10^3$</td>
</tr>
<tr>
<td>Yeasts</td>
<td>$0$-$10^3$</td>
</tr>
<tr>
<td><strong>Anaerobic bacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Bacteroides fragilis group</td>
<td>Rare</td>
</tr>
<tr>
<td>Prevotella spp.,</td>
<td>$10^6$-$10^8$</td>
</tr>
<tr>
<td>Porphyromonas spp.</td>
<td></td>
</tr>
<tr>
<td>Lactobacilli</td>
<td>$0$-$10^3$</td>
</tr>
<tr>
<td>Bifidobacteria</td>
<td>Rare</td>
</tr>
<tr>
<td>Fusobacteria</td>
<td>$10^4$-$10^8$</td>
</tr>
<tr>
<td>Peptostreptococci</td>
<td>$10^5$-$10^7$</td>
</tr>
<tr>
<td>Clostridia</td>
<td>Rare</td>
</tr>
<tr>
<td>Eubacteria</td>
<td>$10^2$-$10^3$</td>
</tr>
</tbody>
</table>

Intestinal surfaces are covered with mucus, which protects the tissue. This nutrient-rich layer provides an excellent niche for a range of bacteria well adapted to a viscous environment. Studies on animals have suggested the existence of a specific mucosal-associated flora (MAF) (Savage, 1970; Midvedt, 1986). Studies of MAF in humans have been few. Nevertheless, analyses of biopsy material and specimens of surgically excised tissue have shown that human colon has MAF which is distinct from that of
the gut lumen. One of the most important features of MAF is that the aerobe:anaerobe ratio approximates to unity (Marks et al. 1979; Peach et al. 1978; Hill, 1995a) (Table 2). Facultative bacteria belonging to the Enterobacteriaceae family are well represented (Marks et al. 1979; Peach et al. 1978), whereas anaerobes have been shown to be mainly Bacteroides species (Poxton et al. 1997). The amount of oxygen diffused to the colonic mucosa may be the predominant factor explaining the proportionally higher count of aerobic and facultative organisms in MAF than in the luminal microbiota (Hill, 1995a).

Even with optimal anaerobic conditions of sample processing and culture, however, the total colony forming units (CFU) count represent 88% of the total bacterial cell count obtained by microscopy (Moore & Holdeman, 1974b). Nowadays alternative methods are also used for analysis of intestinal microbiota (Franks et al., 1998; Suau et al., 1999; Sghir et al., 2000). The results of nucleic acid-based methods allow a more complete appraisal of the intestinal microflora to be made (Vaughan et al., 2000).

2. THE DEVELOPMENT OF INTESTINAL MICROBIOTA

Human infants born by vaginal delivery have few intestinal microorganisms at the time of birth (Tannock et al., 1990) and the initial fecal output, called meconium, is almost sterile (Gorbach, 1993). By day two there is acquisition of intestinal bacteria, which are similar to those in the mother’s vaginal and fecal microbiota (Long & Swenson, 1977; Rotimi & Duerden, 1981). By day three and four, the infant’s microbiota has Bacteroides species and E. coli present. If the child is born by the Cesarean section, there is a delay in the acquisition of intestinal microbiota (Long & Swenson, 1977; Bennet & Nord, 1987) and primary gut microbiota, especially acquisition of Bacteroides fragilis, may be disturbed for up to 6 months after the birth (Grönlund et al. 1999). Depending on how the infant is fed, the microbiota can be quite different. In the breast milk-fed infant, the microbiota contains mostly Bifidobacterium species (Benno et al., 1984; Kleessen et al., 1995; Harmsen, 2000; Favier et al., 2002). When intestinal microbiota of infants develop, bifidobacterial species, change in time (Satokari et al., 2001). In formula-fed infants significantly higher bacterial counts of enterococci and clostridia (potential pathogenic organisms) are detected compared to breast-fed infants (Kleessen et al., 1995). After weaning and switching to regular food the adult pattern of fecal microbiota gradually develops.

3. THE INFLUENCE OF AGE AND DIET ON INTESTINAL MICROBIOTA

The colonic microbiota of infants is generally viewed as being adult-like after the age of two years. The composition of the normal microbiota of adults has generally been considered to be stable as long as the host is not subjected to stressful circumstances or to the administration of antimicrobial drugs (Finegold et al., 1983). There are reports, however, that elderly subjects harbor fewer bifidobacteria and higher levels of fungi and enterobacteria compared to younger adults (Gorbach et al., 1967; Hopkins et al.,
2001). The frequency of isolation of *Clostridium difficile* has also been shown to be greater in elderly volunteers than in younger subjects (Ljungberg et al., 1990; Hopkins et al., 2001). Age related changes in the microbiota have been shown by Hopkins et al. (2001) using a polyphasic approach based on culture, together with molecular and cellular fatty acid profiling methodologies. Especially evident was the reduction of bifidobacteria with aging (Hopkins et al., 2001).

Dietary intake does not seem to cause significant changes in the bacterial composition of the intestinal microbiota. In studies done on adults fed random diets versus adults fed standard diets, there were essentially no differences in the two groups in terms of number of facultative organisms or anaerobic organisms in the intestinal microbiota (Gorbach et al., 1967). In another study of the microbiota of people eating a vegetarian diet or a meat diet only minor differences were noted (Finegold et al., 1974). When a group of people eating an omnivore diet was shifted to a vegetarian diet, or vice versa, there were slightly more anaerobes in the microbiota of people eating the meat diet, but the differences were not significant (Moore & Holdeman, 1974a). Another study compared the microbiota of omnivores in Los Angeles to that of Seventh-Day Adventists and Japanese people, and again, only minor differences were found (Finegold et al., 1977). In the intestinal tract individuals harbor a characteristic microbiota defined in terms of genera and species, but also of bacterial strains (McCartney et al., 1996; Kimura et al., 1997; Tannock et al., 2000).

Extensive studies of fecal microbiota by Finegold et al. (1983) show that only a few species are almost always present in substantial quantities. They studied various populations including strict vegetarians, Japanese on a diet free of ruminant meat, and Americans on a typical Western diet including beef. No class of bacteria was invariably present, but *Bacteroides*, anaerobic cocci, *Eubacterium* and *Clostridium* were usually found, and streptococci and gram-negative facultatives were isolated from over 85% of all subjects. While the bacterial spectrum may differ between individuals, diet seemed to have relatively little effect on the diversity of bacterial species present (Tannock, 1983).

Very little is known about how the host genome influences the composition of the gastrointestinal microbiota, but there is evidence that the composition of fecal microbiota is genetically regulated (van de Merwe et al., 1993; Zoetendal et al., 2001; Toivanen et al., 2001).

### 4. THE PHYSIOLOGICAL ROLE AND METABOLIC CAPACITY OF THE INTESTINAL MICROBIOTA

Most of the microbiota-associated influences on man relate to the intestinal tract. This is because the gastrointestinal tract harbors the largest number, and most diverse collection, of bacteria inhabiting the human body (Tannock, 1999b). Not only does the microbiota influence the host, but the culture vessel of the intestine is composed of living cells that actively secrete and take up components. These secretions, mucin,
sloughed off epithelial cells, and the diet of the host form nutrients for the ecosystem. The host immune system also contributes. The intestinal ecosystem can be roughly divided into three influential aspects: diet, host physiology, and the microbiota. In the healthy adult, a fine balance exists between these parameters (Conway, 1995). Development of molecular techniques will improve our knowledge about the influence of microbiota on human physiology. Recent analyses have, for example, shown that colonization of germ-free mice with Bacteroides thetaiotaomicron affects expression of host genes (Hooper et al., 2001). The primary functions of gastrointestinal microbiota include nutritive, metabolic, immunologic, and protective activities.

**Nutritive activities**

Colonic bacteria are capable of fermenting carbohydrates in a host’s diet that are not digested by the enzymes of the small intestines. This fermentation undoubtedly affects the host in a number of ways. It may contribute to human nutrition as fermentation products such as acetate, propionate, and butyrate are absorbed from the colon and serve as energy sources for colonocytes, hepatocytes and peripheral tissues (Mortensen & Clausen 1996). The preference of colonocytes for butyrate as a fuel is more pronounced in the distal than proximal colon (Roediger, 1980) and may account for about 70% of total energy consumption (Scheppach, 1994).

The production of short-chain fatty acids (SCFAs) in healthy individuals accounts for 5-10% of the maintenance energy requirements in humans (Mortensen & Clausen, 1996). Colonic fermentation is also important in patients with small intestinal malabsorption of saccharides, e.g. lactose, as the bacterial degradation of the malabsorbed carbohydrates in the colon diminishes the caloric loss due to the absorption of the produced SCFAs (Holtug et al., 1992).

Many microbes synthesize nutrients essential for the host, such as vitamin K, biotin, and vitamin B₁₂ (Luckey 1977; Ramotar et al. 1984; Conly & Stein 1992). The degree to which these benefit man is a matter of debate (Mathers, 1995).

**Metabolic activities**

The gastrointestinal microbiota performs a wide variety of metabolic functions. Bacteria play a key role in the processes including carbohydrate fermentation, degradation of proteins, deconjugation of bile acids, steroid transformation, and metabolism of substances that enter the digestive tract. Although metabolites that are more toxic, carcinogenic, or mutagenic than the original compound may be formed, certain substances may be detoxified (Macfarlane & Macfarlane 1997). The intestinal microbiota may be considered as an additional “organ” within the host in spite of its multiorganism composition. The metabolic capability of the colonic bacteria in man has been estimated to be at least as great as that of the liver (Drasar & Hill 1974; Bingham, 1988) or even exceed that of the whole human body (Luckey 1977).

In the small intestine, where the environment is oxygenated, many of the microbial reactions are hydrolytic. The large bowel is anaerobic and biochemical reactions are usually reductive in nature. Significant differences in bacterial fermentation reactions
are also found in the different regions of the human large intestine (Macfarlane et al. 1992). The products of bacterial fermentation (SCFAs, gases, ethanol) are present in the highest concentrations in the cecum and ascending colon, where substrate availability is greatest. In contrast, products of protein fermentation (ammonia, branched-chain fatty acids, phenolic compounds, and volatile sulfur compounds) progressively increase from the ascending to the descending colon (Macfarlane et al. 1992).

Substrates for these various biochemical reactions are either endogenous or exogenous. They can come from mucin and compounds secreted in bile or across the intestinal mucosa as well as from nutrients and other substances in the food supply (Gorbach, 1993). Many of these bacterial enzymatic activities are inducible so that increasing concentrations of a substrate can induce higher enzymatic levels in bacteria over time (Gorbach, 1993).

Immunologic activities
Perhaps one of the greatest enigmas concerning the normal microbiota is the mechanism by which huge number of microbial cells can persist in intimate association with the mucosal surfaces without inducing a marked inflammatory or immunological response on the part of the host (Tannock, 1999b). In general, it has been accepted that low titers of antibodies reactive with indigenous bacteria can be detected in the sera of healthy humans (Hoiby & Hertz, 1979; Sirisinha & Charupatana, 1971). Paradoxically, a complex microbiota is likely to be crucial to both the development and homeostasis of the immune system of the host (Gordon & Pesti, 1971; Moreau & Coste 1993; Isolauri et al., 2001). There is evidence that for example probiotic lactobacilli, Lactobacillus rhamnosus strain GG, have an immunostimulating effect on oral rotavirus vaccination (Isolauri et al., 1995).

Protective activities
The colonic microbiota – as do peristalsis, mucin production and epithelial shedding – participate in the host defense of gastrointestinal tract (Duncan & Edberg 1995). The colonic microbiota provides colonization resistance and serves as a barrier against translocation of potentially pathogenic bacteria (Van der Waaij & van der Waaij 1990) such as Clostridium species and E. coli, some strains of which may become pathogenic. Antimicrobial agents disturb the protective barrier of the intestinal microbiota permitting, for example, invasion of the bowel by exogenous microorganisms including microbial pathogens (Lipson, 1976).

In addition to being a source of calories and providing energy-yielding substrates to the colonic mucosa (colonocytes) (Clausen & Mortensen, 1994 and 1995), SCFAs influence gastrointestinal function by increasing or modifying intestinal mucosal growth (Mortensen et al., 1991). Butyrate regulates differentiation of cultured cells and inhibits tumor growth in vitro and possibly also inflammation (Mortensen & Clausen, 1996; Luehrs et al., 1999). SCFA production also increases colonic blood flow (Mortensen et al., 1990) and promotes sodium and water absorption (Mortensen & Clausen, 1996).
5. IMPLICATION OF INTESTINAL MICROBIOTA IN DISEASES

It is of interest to note that of the hundreds or even thousands of potential phenotypes present as a part of the normal large intestinal microbiota only a few are legitimate pathogens. This suggests that those species capable of causing disease once outside their normal environment of the bowel have special virulent attributes such as polysaccharide capsules that allow them to survive and proliferate within, for example, the peritoneal cavity (Onderdonk, 1999). *B. fragilis* is the most common obligately anaerobic member of the intestinal microbiota associated with such infections and sepsis arising from fecal contamination (Patrick, 1993). In addition, *E. coli*, the culturally most common facultative member of the intestinal microbiota, is also the most common cause of urinary tract infections (Sobel & Kaye, 1995). The role or contributions of microbes or their products in noninfectious functional gastrointestinal disorders is less clear, but more data is accumulating to support their confounding importance in certain conditions (Chadwick & Anderson, 1995).

5.1 Appendicitis

Acute appendicitis is the most common surgical emergency in childhood. Although various aerobic and anaerobic bacterial groups have been isolated from inflamed appendices, the etiology and pathogenesis of acute appendicitis remains obscure. The enteric microbiota is considered important in pathogenesis, but whether the primary cause is infection is unclear. Whether specific bacterial species, groups or combinations are responsible or contribute to the inflammation has remained unresolved. Both anginosus streptococci (former *Streptococcus milleri* group) and *B. fragilis* have been frequent findings in acute appendiceal inflammation of adults and have been implicated in the pathogenesis. (Poole & Wilson G, 1977; Werner et al., 1975). Certain virulent *E. coli* serotypes may also contribute to the development of appendicitis (Saxén et al., 1996). Suggested non-bacterial etiologies or cofactors for acute appendicitis include mechanical obstruction (Pieper et al., 1982), inadequate dietary fiber (Burkitt, 1971) and factors associated with improved socioeconomic conditions (Barker et al., 1988).

5.2 Lactose intolerance

Milk is an important component of the Finnish diet but the prevalence of lactose maldigestion in Finland is 17% (Sahi, 1994). Lactose maldigestion has been under intensive research and its causes, genetic as well as those related to gut diseases, have been well established (Saavedra & Perman, 1989; Enattah et al., 2002). The small intestinal brush border membrane contains enzymes essential for dietary disaccharide digestion. The lactase enzyme hydrolyses lactose to the monosaccharides glucose and galactose which in turn are actively absorbed from the small intestine. In lactase deficiency and subsequent lactose malabsorption, unabsorbed lactose is transited to the large intestine and metabolized there by intestinal bacteria to short chain fatty acids and gases. The bacterial metabolism of unabsorbed lactose may lead to typical
symptoms of lactose intolerance such as abdominal pain, loose stools, bloating and flatulence (Vesa, 1997). In spite of low lactase activity in some lactose maldigesters the oral intake of even considerable amounts of lactose does not necessarily lead to any symptoms. During lactose tolerance test there is a negative correlation of the symptoms with the rise of blood glucose. However, the symptoms and the blood glucose concentration are not correlated with the level of lactase activity in duodenal biopsy (Nieminen et al., 1996). Therefore, differences in intestinal microbiota (the composition and the metabolic activities) have been suggested to be the plausible explanation for individual variation in abdominal symptoms resulting from lactose malabsorption.

5.3 Irritable bowel syndrome

Another common cause of gastrointestinal problems is irritable bowel syndrome (IBS) (Camilleri, 2001). The IBS is defined on the basis of the recently modified Rome criteria (Drossman et al., 1999). The syndrome can be divided into four subcategories according to whether the predominant symptom is abdominal pain, diarrhea, constipation, or constipation alternating with diarrhea (Horwitz et al., 2001). The symptoms of IBS resemble those of lactose intolerance, and can easily be confused (Vesa et al., 1998; Horwitz et al., 2001). Some authors have suggested that an underlying IBS might explain some of the symptoms associated with lactose intolerance (Hammer et al., 1996; Suarez & Levitt, 1996). The prevalence of IBS in Finnish population has not been studied, but in other industrialized countries it is estimated to be approximately 10% (Camilleri, 2001).

Altered bowel motility, visceral hypersensitivity, psychosocial factors, an imbalance in neurotransmitters, and infection have been proposed as playing a part in the development of the IBS (Horwitz et al., 2001). Food products have variously been reported as causing, perpetuating or treating IBS (Dunlop & Spiller, 2001). A range of fermentable substrates (Ravich et al., 1983; Phillips et al., 1995) as well as inhibitors of carbohydrate digestion (Reuser & Wisselaar, 1994) may provoke gastrointestinal symptoms. The syndrome may follow gastroenteritis (Gwee et al., 1996; Spiller et al., 2000; Thornley et al., 2001), and long-term duration of symptoms (Neal et al., 1997) as well as toxigenicity of Campylobacter species are associated with a higher risk of developing IBS (Thornley et al., 2001). Helicobacter pylori infection has been suggested to modify the risk of IBS, but no clear association has been shown (Locke et al., 2000). IBS has also been associated with an abnormal intestinal microbiota (Nobaek et al., 2000; Pimentel et al., 2000). The probiotic therapy has been efficient in changing the composition and biochemistry of fecal microbiota and decreasing symptoms in patients with IBS (Nobaek et al., 2000; Brigidi et al., 2001; Niedzielin et al., 2001). Bacterial fermentation may be an important factor in the pathogenesis since colonic gas production (mainly hydrogen) is greater in patients with IBS than in controls. Both gas production and symptoms reduce during an exclusion diet matched for macronutrients (King et al. 1998).
5.4 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is an umbrella term used to describe three conditions: Crohn’s disease (CD), ulcerative colitis (UC) and non-specific colitis. It affects 0.1% of the population in Western societies (Hooper & Gordon, 2001). Both CD and UC are chronic relapsing inflammatory disorders of the intestine, and non-specific colitis may be an intermediate form of these conditions. Although CD and UC are very often confined to the intestine, several extraintestinal conditions are associated with CD. These complications may occasionally antedate the bowel disease and may implicate the intestinal microbiota in the disease pathogenesis (Chadwick & Chen, 1999).

The intestinal bacterial microbiota or their products, such as SCFAs, may play an important role in these conditions (Marteau, 2002). Higher concentrations of mucosal bacteria are found in patients with bowel inflammation than in controls (Swidsinski et al., 2002). The colonization of anaerobic bacteria, especially Bacteroides spp., may be initiating and promoting cytokine responses that has been seen mediating in development of chronic colitis resembling human IBD in rat model (Hata et al., 2001). In patients with IBD, significant systemic antibody response to Bacteroides ovatus has been found (Saitoh et al., 2002). In situ hybridization techniques have shown the presence of Mycobacterium avium subsp. paratuberculosis DNA even in more than 70% of diseased tissue samples from CD (Sechi et al., 2001; El-Zataari et al., 2001; Hulten et al., 2001). Interestingly, H. pylori infection seems to modify the development of IBD and may have a protective effect (Väre et al., 2001).

Further evidence of bacterial involvement is supported by response to treatment with broad-spectrum antibiotics of both CD and UC (Freitas & Rocha, 2000; de Boer & Perelson, 1997). Butyrate irrigation has also been successfully used as a treatment to reduce inflammation in distal UC (Scheppach et al., 1992) and it can also increase mucin biosynthesis in vitro in normal and UC tissue (Finnie et al., 1995).

5.5 Colon cancer

Several studies have suggested that interactions between the bacterial colonic microbiota, certain dietary factors, and the colonic epithelium are involved in the development of colon cancer (Moore & Moore, 1995; Scheppach et al., 2001). In both UC and CD, a strong association exists between the duration and extent of mucosal inflammation and the cancer risk (Rhodes & Campbell, 2002).

Several bacterial enzymes have been implicated in generating mutagens, carcinogens, and various tumor promoters: β-glucuronidase, β-galactosidase, nitroreductase, azoreductase, 7-α-hydroxy-steroid dehydrogenase, glycocholic acid hydrolase, and cholesterol dehydrogenase (Gorbach & Goldin, 1990; Goldin, 1996; Kim & Jin, 2001). SCFA butyrate is known as an effective inducer of cell differentiation. Large number of investigations have shown that butyrate has marked effects on human colorectal cancer cell lines (Scheppach et al. 1995; Mortensen & Clausen, 1996). The protective
effect of dietary fiber has thus been associated with their production of butyrate into the colonic contents, which possibly decreases the risk of neoplasia in colonocytes (Mortensen & Clausen, 1996). It has also been shown that some probiotics may decrease the fecal concentrations of enzymes that may be involved in colon carcinogenesis (Ling et al., 1994; Goldin et al., 1996; Salminen et al., 1998b; Hirayama & Rafter, 2000; Wollowski et al., 2001).

Haines et al. (1977) reported a considerably increased incidence of methanogenesis in patients with colorectal cancer (80 vs. 40% in controls). However, it is known that rural African populations are mostly methanogenic (over 90%) but have a very low incidence of colorectal cancer compared to western populations (Segal et al., 1988). The association between methane producers and bowel cancer may be attributed to slow growth of these bacteria and a decreased transit time of the patient. Physical obstructions such as tumors would contribute to a prolonged residence time of gut content including colonic bacteria (Levitt et al., 1995).

5.6 Others

Complex fermentations carried out by bacteria in the colon produce a large number of metabolites, which can be detrimental to the health of the host (Simon & Gorbach, 1982). The in vitro study by Roediger and Moore (1981) was the first to provide evidence that SCFAs are a potent stimulus to net sodium absorption in the human colon. They suggested that this mechanism could explain why much greater absorptive capacity is observed in human colon when normal colonic contents are present rather than when colon is flushed out in perfusion studies which employ NaCl solutions (Debougje et al. 1978). This mechanism may operate in the pathogenesis of a variety of diarrheal conditions.

The colonic mucosa may be especially vulnerable during starvation and malnutrition, as luminal fuels (SCFAs) make a great contribution to energy metabolism (Scheppach, 1994). Lack of luminal nutrients may impair the morphology (atrophy, colitis) and function (sodium absorption, gut barrier) of colonic epithelial cells (Roediger, 1990; Scheppach, 1994). This hypothesis has stimulated considerable research regarding the role of SCFAs in maintaining the health of the colonic mucosa and in preventing and treating colonic diseases (Breuer et al., 1991; Scheppach et al., 1992; Mortensen & Clausen, 1996).

6. MODIFICATION OF THE NORMAL MICROBIOTA

Environmental factors, diet, medication, and stress can all adversely affect the composition and/or activity of the intestinal microbiota (Fuller & Gibson, 1998). The deficiencies created can be repaired either by added viable organisms (probiotics) or by stimulating specific components of the microbiota with chemical supplements (prebiotics). One of the most important and best-documented areas is that of antibiotic-associated diarrhea (Fuller & Gibson, 1998). C. difficile is recognized as a causative agent of pseudomembranous colitis, which often follows administration of antibiotics,
especially broad-spectrum preparations. The condition can be difficult to resolve by conventional means but yeast probiotics containing *Saccharomyces boulardii* have given encouraging results (Surawicz et al., 1989; McFarland et al., 1995).

**Probiotics** have been defined in several ways (Fuller, 1989; Havenaar *et al.* 1992; Guarner & Schaffsma, 1998; Salminen *et al.*, 1998a; Schrezenmeir & de Vrese, 2001). According to Havenaar *et al.* (1992) probiotics are defined as viable cultures of microorganisms that benefit the host by improving the properties of the indigenous microbiota. This definition has been emended by Schrezenmeir & de Vrese (2001) to include products in addition to microorganisms: “a preparation or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonisation) in a compartment of the host and by that exert beneficial health effects in this host”. Guarner and Schaffsma (1998) have stated “health benefits beyond inherent basic nutrition” and a European Commission concerted action program have defined probiotics as “a live microbial food ingredient that is beneficial to health” (Salminen *et al.*, 1998). Probiotics are often incorporated in food, especially dairy products, but they cover a wide variety of different products comprising tablets, powders, fermented milks (yogurts) and liquid suspensions (Fuller & Gibson, 1998).

The study of the intestinal microbiota is a crucial aspect of probiotic research and development. Much effort has been devoted to screen bacterial isolates for properties deemed to be appropriate for a probiotic strain, with characteristics enabling the microbe to survive passage through the digestive tract (Saxelin, 1996). It has been shown that lactic-acid-excreting bacteria, such as lactobacilli and bifidobacteria, are especially suitable for probiotics (Salminen & von Wright, 1998). Probably the biggest obstacle to allaying skepticism is that the probiotic concept is based on a very poor understanding of the intestinal microbiota. Despite for hundreds of publications only a few seem to contribute convincingly to our knowledge of health effects in humans (Bengmark, 1998; Fuller & Gibson, 1998). However, promotion of resistance to diseases offers a great deal of promise and it is important that well-conducted trials are carried out (Fuller & Gibson, 1998). Well-documented outcomes are: lower frequency and duration of diarrhea associated with antibiotics (*C. difficile*), rotavirus infection, and traveller’s diarrhea; stimulation of immunity; decrease in unfavorable metabolites and procarcinogenic enzymes in the colon (Schrezenmeir & de Vrese, 2001). Recently probiotics have even been shown to prevent atopic disease (Kalliomäki *et al.*, 2001; Rautava *et al.*, 2002).

**Prebiotics** are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Gibson & Roberfroid, 1995). Among low-digestible carbohydrates, fructo-oligosaccharides occupy a key position by their ability to stimulate selectively the growth of bifidobacteria (Gibson *et al.*, 1995; Kleessen *et al.*, 1997; Bouhnik *et al.*, 1999). A number of health benefits may be associated with this bifidogenesis, but contribution to human health still remains to be proven (Roberfroid, 1997).

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The term **synbiotic** is used when a product contains both probiotics and prebiotics (Gibson & Roberfroid, 1995; Collins & Gibson, 1999; Schrezenmeir & de Vrese, 2001).

7. SPECIMENS USED TO STUDY INTESTINAL MICROBIOTA

There are major difficulties in studying the microbiota in their natural habitat. Most of the work has been performed on feces but this does not necessarily reflect the proximal colonic contents, particularly from a quantitative viewpoint (Macfarlane et al., 1992; Tannock, 1999b). Examination of the fecal microbiota is an appropriate way of assessing the composition of the colonic luminal microbiota but almost certainly does not provide insights into the specific mucosally associated microbiota (Tannock, 1999b). The involvement of the MAF in pathological processes is potentially far greater than the luminal microbiota.

Unless fecal samples are used, any sampling of the colon is invasive and involves intubation or collection during operation. Both methods can have undesirable effects, failure to represent natural state and even rise ethical issues. Intubation may result in contamination with bacteria from another region, while sampling at operation will inevitably mean that the host is anesthetized and fasted. These factors will obviously have an effect on the microbes, and the results may be difficult to interpret because slowed peristalsis resulting from the anaesthetic can permit backflow (Borriello et al., 1978). Furthermore, the patient may also have undergone pre-operative antibiotic treatment. Available are a range of endoscopes that are capable of sampling also the small bowel contents at number of sites; the exact location of the sample can be checked radiologically. Enterotest capsules can also be used in sampling small bowel (Gracey et al. 1977). Indirect tests may involve the analysis of jejunal juice aspirates, urine or breath samples, depending on the metabolite to be assayed.

Unfortunately, sampling of these ecosystems inherently perturbs the system and one is always concerned about how representative a sample is (Convay, 1995). Whatever the sampling technique, samples usually must be collected and transported under anaerobic conditions. Ideally, the samples should be analyzed immediately but if analyses are delayed the fecal samples should be stored below -40°C (Ballongue, 1997).

8. METHODS USED TO STUDY INTESTINAL MICROBIOTA

Changes in the gut microbiota can be detected either by classical bacterial methods including microscopic and culture methods followed by isolation, identification and enumeration of different species, or by molecular techniques (Vaughan et al., 2000). Changes can also be detected by measuring biochemical components of the microbiota, including enzyme activities (Gorbach, 1986), SCFAs (Topping & Clifton,
2001), gas production (Oufir et al., 1996; Hertzler et al., 1997), and total cellular fatty acids (Peltonen et al., 1992).

8.1 Direct microscopic analysis

The light microscope has been a valuable tool for estimating the total number of bacteria in fecal samples (Langendijk et al. 1995; Holdeman et al. 1976). Direct microscopic analysis is a good aid for assessing how effective a culture methodology may be for analyzing the intestinal microbiota. However, the microscopic technique itself is not infallible and may significantly underestimate the true numbers. The technique generally used involves heat fixation and staining (Holdeman et al. 1977) and detachment of cells is likely to occur, especially during washing. Furthermore, bacteria tend to stick together and form clumps.

8.2 Culture methods

8.2.1 Cultivation and isolation

The study of the composition of the intestinal microbiota has relied almost exclusively on the quantitative culture of microbes from fecal samples. This approach is still the mainstay for studies on human intestinal ecosystem (O’Sullivan, 2000).

The culturing techniques involve plating out fecal or intestinal material on both selective and non-selective media and incubating at conducive atmospheric and temperature conditions. Before plating, samples are generally homogenized in a sterile liquid (O’Sullivan, 2000). Examples of non selective media that have been used in fecal studies are rumen fluid-glucose-cellobiose agar (Moore & Holdeman, 1974a), modified medium 10 (Wilson & Blitchington, 1996), plate count agar (Alander et al., 1997), and brucella blood agar supplemented with sheep blood, vitamin K1, and hemin (Langendijk et al., 1995).

Enumeration of specific bacterial genera is generally achieved by plating on selective media. For example, several selective agents such as bile, esculin and antibiotics can be used for the selective cultivation of Bacteroides species. However, the use of these selective agents is thought to inhibit many colonic strains and would therefore underestimate the Bacteroides count (Corthier et al., 1996). Bifidobacterium is another culturally dominant genus found in human intestine and a number of selective media for their enumeration have been developed (Mitsuoka et al., 1965; Tanaka & Mutai, 1980; Resnick & Levin, 1981; Muñoa & Parens, 1988; Beerens, 1991; Hartemink et al., 1997). However, the analysis of some commonly used selective media found that none were fully selective and that they generally contained toxicity against some bifidobacteria (Silvi et al., 1996). Other intestinal bacteria such as lactobacilli, clostridia, enterococci and Enterobacteriaceae can also be isolated using selective media (O’Sullivan, 2000).

The predominant components of the large bowel microbiota are obligately anaerobic bacteria and thus gastrointestinal bacteriology is largely study of anaerobic organisms.
Although this subject has made great progress during recent years it still requires specialized technology (Summanen et al., 1993). The overwhelming problem is oxygen sensitivity of obligately anaerobic bacteria. This single fact controls the methods used in sampling and transporting as well as culture techniques employed (Finegold et al. 1992).

8.2.2 Identification of isolates

Classical methods
Upon isolation of colonies it is necessary to identify a strain to the genus and sometimes further to the species or even type and subtype level. Traditionally, descriptive and diagnostic bacteriology has been based on phenotypic characterization of the organisms, such as cellular form (gram stain and cell morphology) and function (biochemical reactions). This characterization requires a battery of biochemical tests described in several manuals (Krieg & Holt, 1984; Sneath et al., 1986; Murray, 1999; Holdeman et al., 1977; Summanen et al., 1993; Jousimies-Somer et al., 1999; Jousimies-Somer et al., 2002). The reproducibility of the tests routinely used for the identification can be improved with the use of several microsystems, like the API system (bioMeriex), that are currently commercially available.

Cellular fatty acid analysis
Analysis of the cellular fatty acid (CFA) composition of the isolates can be done in conjunction with other tests or sometimes as the main determinant in identification. CFA analysis is a chemotaxonomic technique where the main requirement is the proper instrumentation. In practice, whole cells of bacteria or yeasts are treated to release their fatty acids, which are then converted to methyl ester derivatives and analyzed by gas-liquid chromatography (GLC). Definitive identification of fatty acids can be made only by mass spectrometry. However, for most applications, mass spectrometry is unnecessary, because the number of unidentified fatty acids is limited.

The CFAs are long-chain fatty acids (9-20 carbon atoms) and structural components of bacterial cell membranes. Different bacterial species have different cellular fatty acid composition and contents and, if cultures are grown under standardized conditions, the fatty acids remain sufficiently consistent so that their profiles may be used to generate a database from which unknown patterns can be retrieved and compared (Moore et al., 1994). The usual profile of most bacteria features 5 to 15 CFAs in significant amounts. Most bacteria synthesize fatty acids with chain lengths of 10 to 19 carbon atoms, and the most prevalent fatty acids are those with 16 or 18 carbon atoms (Welch, 1991).

The only commercially available GLC system dedicated to the identification of bacteria and yeasts by CFA analysis is the Sherlock® Microbial Identification System (MIS; Microbial ID, Inc., Newark, Del.). Software libraries for the identification of a large number of anaerobes (Moore Broth Library) have been compiled largely by the Virginia Polytechnic Institute Anaerobe Laboratory. Libraries for aerobes, mycobacteria, and yeasts have also been developed and updated (MIS, 1995).
Variables such as culture media and growth conditions must be controlled as carefully as possible, since the CFA composition of cells varies, at least quantitatively, according to the culture conditions used (Rose, 1988; Suutari & Laakso, 1994).

Molecular methods
The advent of molecular tools has greatly expanded the ability to reliably identify isolates and also to calculate the evolutionary relatedness between strains. Accurate species determination of unknown isolates is now achieved by sequence analysis of 16S ribosomal RNA (rRNA). Woese and coworkers (Woese 1987) first developed this tool for classifying organisms and evaluating their evolutionary relatedness. The available databases of rRNA sequences are now extensively covered and electronically accessible by several gene banks such as GenBank (Benson et al., 1993) and the ribosomal database project (Maidak et al., 2001), which allow detailed studies to be made on the phylogenetic position of unknown isolates. Technically, this is very feasible as polymerase chain reaction (PCR) can be used to amplify the 16S rRNA gene directly from colonies using primers, which are directed at universally conserved regions at both ends of the gene. The entire PCR amplicon, which is about 1.5 kb can then be directly sequenced and compared to the rRNA database (Amann et al., 1995).

Analysis of other molecule than 16S rRNA has also been used. The region between the 16S and 23S rRNA genes, termed the internal transcribed spacer, has been used for a more detailed analysis of bifidobacteria (Leblond-Bourget et al. 1996). The sequence analysis of this molecule was found to be much more sensitive than the rRNA analysis. More recently, Tannock et al. (1999) demonstrated its usefulness for identification of intestinal Lactobacillus spp. In addition, a short segment of the recA gene has emerged as a potential candidate for sensitive molecule for determining intrageneric phylogenetic relationships (Eisen 1995; Karlin et al. 1995). The recA gene encodes the RecA protein, which plays vital roles in recombination, DNA repair and SOS response (Roca & Cox 1997). In a study by Kullen et al. (1997) this concept was applied to the genus Bifidobacterium. The molecule was obtained from the type strains and intestinal bifidobacterial isolates and the phylogenic relationship obtained by sequence analysis of the recA gene compared favorably with the analysis from the complete rRNA gene.

8.2.3 Further characterization of isolates

Awareness of the composition of the microbiota at level of bacterial strains is also often important. The recent use of genetic fingerprinting methods to analyze the composition of particular populations that are part of the fecal microbiota of humans have demonstrated the diversity of strains that colonize the intestinal tract of different humans. For example, serotyping studies of E. coli isolates from fecal samples have shown that the collection of the serotypes changed over a period of time (Mason & Richardson, 1981).

The molecular typing methods used for detailed fingerprinting of the intestinal isolates include multilocus enzyme electrophoresis, pulsed field gel electrophoresis,
ribotyping, and random amplification of polymorphic DNA (O’Sullivan, 2000; Tannock, 2001).

8.3 Molecular technologies

Molecular biological methods are increasingly being applied to study the GI tract ecology (Collins & Gibson, 1999; O’Sullivan, 2000; Vaughan et al., 2000). The molecular methods involve the amplification by PCR of the 16S ribosomal RNA genes (16S rDNA) in microbial DNA extracted directly from the samples. The amplified sequences are cloned and should contain copies of the gene from all of the species present in the sample. The 16S rDNA clones are screened and representative clones are sequenced. Because 16S rDNA sequences are one of the cornerstones of the microbial taxonomy, alignment of the sequences with those stored in databanks permits the recognition of species, including those that cannot be cultivated by conventional methods.

Species may subsequently be enumerated directly in samples by means of oligonucleotide probes based on the 16S rDNA sequences (in situ hybridization). Since 16S rRNA sequences for thousands of bacterial species are now available, highly specific probes are relatively easy to design. These oligonucleotide probes can be readily synthesized on automated DNA synthesizers for nominal cost. The specificity of the probe can be adjusted to fit any taxonomic ranking, from kingdom to subspecies (Giovannoni et al., 1988; Amann et al., 1990; Raskin et al., 1994). Numerous genus- and species-specific PCR primers and probes have been developed for bifidobacteria (Yamamoto et al., 1992; Langendijk et al., 1995; Kaufmann et al., 1997; Matsuki et al., 1998; Matsuki et al., 1999) and recently genus-specific primers have also been designed for lactobacilli (Satokari, et al., 2001).

The bound probe is detected by radioactive, enzymatic, fluorescent or chemiluminescent means depending on the compound used for labeling the probe. If these probe molecules are labeled with fluorescent dye, the procedure is termed fluorescent in situ hybridization (FISH) (Manz et al., 1993; Welling et al. 1997; Franks et al. 1998; Harmsen et al. 1999). Currently, the lowest level of detection (microscopy) using FISH is $10^6$ bacterial cells per gram (Tannock, 1999c). For quantification purposes the method of fluorescent whole-cell hybridizations can been used (Langendijk et al., 1995). Combination of this method and automated microscopy is expected to have $10^7$ bacterial cells per gram as the lower limit of detection (Jansen et al., 1999).

Dot blot hybridization technique is useful for measuring the specific 16S rRNA in a mixture relative to the total amount of rRNA. Total DNA and RNA are isolated from the sample, bound to a filter using a dot or slot manifold device and hybridized with labeled oligonucleotide probes. The amount of label bound to the filter is the measure of the specific rRNA target present, and the relative amount of rRNA may be estimated by dividing the amount of the specific probe by the amount of the labeled
universal probe hybridized under the same conditions (Dore et al., 1998; Sghir et al., 1999; Sghir et al., 2000; Vaughan et al., 2000).

Temperature-gradient gel electrophoresis (TGGE) and denaturing-gradient gel electrophoresis (DGGE) are being developed as an additional molecular means of analysis of the intestinal microbiota (Muyzer et al., 1993; Muyzer & Smalla, 1998). The TGGE technique has been applied for example to monitor the most predominant bacterial populations in human fecal samples (Zoetendal et al. 1998) and DGGE technique in qualitative analysis of complex bifidobacterial populations of feces (Satokari et al., 2001) and monitoring of succession of bacterial communities in human neonates (Favier et al., 2002). In these techniques, 16S rDNA is amplified by PCR from DNA of microbial cells in a sample. The various molecular forms (from different microbial species) of the 16S rDNA in the sample can be separated from one another by use of TGGE or DGGE. A temperature or chemical gradient is established in polyacrylamide gel in parallel to the electric field. The DNA samples migrate through the gradient from low to high temperature (or low to high chemical gradient). At point in the gradient where partial denaturation of the double-stranded DNA occurs the migration of the fragment is drastically retarded and sequences of the same length, but of different thermal or chemical stability, can be separated (Riesener et al., 1992). 16S rDNA bands can be eluted from the gel for further amplification by PCR and then sequenced in order to provide identification or characterization of the microbe from which it was amplified.

Quantitation of rRNA that is isolated directly from the ribosomes may be used to reveal the metabolically most active members of a bacterial community. This approach involves competitive reverse transcription-PCR of community 16S rRNA and known concentrations of standard, and subsequent separation of the amplicons by, for example, TGGE or DGGE and quantification of the band intensities (Felske et al., 1998; Vaughan et al., 2000).

The application of molecular analytical methods does not, however, solve the problems of bias and lack of sensitivity (Wintzingerode et al., 1997). Amplification of 16S rDNA requires that the microbial cells in the sample first be lysed for the extraction of DNA. There is a vast difference in the susceptibility of the cells of different microbial species to lytic procedures. Gram-positive bacterial cells such as lactobacilli are more difficult to permeabilize than many others (Welling et al., 1997; Wintzingerode et al., 1997). Additionally, PCR are known to amplify rDNA molecules from mixed populations with different efficiency (Reysenbach et al., 1992; Rainey et al., 1994; Farell et al., 1995; Suzuki & Giovannoni, 1996). It has also been reported that PCR-based analysis of fecal samples is difficult to perform due to the presence of multiple inhibitors of the polymerase enzyme reaction (Satake et al., 1997). Analysis of PCR amplicon by TGGE, for example, visualizes only the dominant fraction of the population and hundreds of bacteria which represent a numerically important part of the community do not form visible bands in the TGGE profiles (Zoetendal et al., 1998). When the TGGE profile represents only 90 to 99% of the total bacterial community, it means that bacteria which reach levels of 10^9 cells or
fewer per g of feces will not be visualized, assuming that 1g of feces contains $10^{11}$ cells (Zoetendal et al., 1998).

Although the development of DNA probes has provided investigators with a major breakthrough for bacterial detection and identification, the hybridization assays have been limited by the number of probes that can be tested simultaneously with large number of samples. There are at least 30-40 numerically predominant species of bacteria in human feces (Drasar & Barrow, 1985; Tannock 1999b) and application of such a large number of oligonucleotide probes to each sample does not seem feasible at present, but may be facilitated in the future by DNA chip technology (Tannock, 1999c). Therefore, it is more convenient to have probes specific for major genera and groups present in the gut. To characterize the major groups of fecal bacteria, Hopkins and coworkers hybridized fecal ribosomal RNA by the three oligonucleotide probes targeted against bifidobacteria, enterobacteria and bacteroides-porphyrmonas-prevotella (Hopkins et al., 2001). Manz et al. (1996) designed a panel of four 16S rRNA-targeted probes specific for bacteria of the phylum cytophaga-flavobacter-bacteroides to analyze the structure and community composition in complex environments like human fecal microbiota. Sghir and coworkers used six probes and that application showed additivity of 70% of the total 16S rRNA detected by the bacterial domain probe (Sghir et al., 2000).

The probes are designed on the basis of currently available 16S rRNA sequences (Maidak et al., 2001), and the specificity is not guaranteed for unknown intestinal bacteria (Amann & Ludwig, 2000).

8.4 Cellular fatty acid (CFA) profiling of the community

Bacterial CFA profiles produced by GLC directly from stool samples represent the composition of bacterial cell walls in the sample and thus indirectly reflects its microbiota composition. In a stool sample, the CFA profile represents all bacteria; viable and nonviable, cultivable and noncultivable, present in the sample. The method has been used in detecting overall changes in the fecal microbiota of stool samples (Peltonen & Eerola, 1992; Peltonen et al. 1992; Eerola et al. 1994; Peltonen et al. 1997; Hopkins et al., 2001).

Previous studies have identified several CFAs that might be of use as signals for the occurrence of particular bacterial groups in the bowel (Newton et al., 1998; Hopkins et al., 2001). CFA profiles are reproducible and community fatty acid profiles can be used to assess the relative similarities and differences of microbial communities that differ in composition (Haack, 1994; Hopkins et al., 2001).
8.5 Enzyme/metabolite analysis

8.5.1 Bacterial enzymes

Measurement of specific enzymes and metabolites in fecal samples can indirectly give information on the presence of specific microbiota, or to be more precise, on the metabolic activities of specific groups of microbiota (Tannock, 1999b). This indirect approach is quite rapid and therefore allows the analysis of a large number of samples (O’Sullivan, 2000). It also gives important functional information on metabolic activities of the microbiota (Gorbach, 1986).

Several biochemical assays used in analysis of the microbiota of the intestinal tract involve measurements of fecal bacterial enzymes such as β-glucuronidase, azoreductase, and nitroreductase that can convert indirectly acting carcinogens to proximal carcinogens (Gorbach & Goldin, 1990).

Increases or decreases in specific enzymes in feces can also point to the metabolic activities of certain groups of bacteria. For example, β-glucuronidase, a common enzyme in E. coli and some Bacteroides spp. which has been implicated in colon carcinogenesis (Goldin & Gorbach 1984), has been shown to be significantly reduced in humans during ingestion of Lactobacillus rhamnosus GG (Ling et al. 1994). Also, a significant correlation has been implicated between the levels of fecal β-galactosidase and numbers of bifidobacteria (Favier et al. 1997). Wolin et al. (1998) developed a detection method for $^{13}$CH$_3^{13}$COOH from $^{13}$C-Glucose, which is an end product characteristic of bifidobacterial glucose fermentation in the fecal suspensions of infants. Striking differences have also been reported in the activities of reductive enzymes associated with the cecal or fecal microbiota of many laboratory animals (Rowland 1986). However, at present it is generally not feasible to accurately correlate fecal enzymes with the presence of specific microbiota. Further studies are needed to identify signature metabolites for intestinal microbiota (O’Sullivan, 2000).

There is no single way to quantify the enzymatic activity of the gut bacterial microbiota. Direct assays can be made using homogenates of feces or cecal contents and more detailed data can be obtained using pure strains of bacteria.

8.5.2 Short-chain fatty acids

Short chain fatty acids (SCFA), such as formic, acetic, lactic, butyric and propionic acids, are end products of metabolism by intestinal bacteria. In mixed bacterial populations, such as those found in the gut, formate is readily degraded by formate lysate to CO$_2$ and H$_2$. The major SCFAs found in the human colon are acetate (C$_2$), propionate (C$_3$), and butyrate (C$_3$) accounting for 90 to 95% of total SCFAs (Mortensen & Clausen, 1996). Longer fatty acids and branched-chain acids like isobutyrate (iC$_4$) and isovalerate (iC$_5$) also occur in the large intestine, though in smaller proportions (Mortensen & Clausen, 1996), and it has been suggested that they
originating from protein and polypeptide breakdown (Mortensen et al., 1988; Rasmussen et al., 1988).

Measurement of these acids in feces can be correlated with specific bacterial metabolism in the intestine (Rowland 1989). Changes in SCFA concentrations can be used as an indicator of perturbation of the intestinal microbiota. For example, a drug such as clindamycin, which is very active against the anaerobic components of the microbiota, causes more suppression of SCFA production than ampicillin (Hoverstad et al. 1986). Increase in SCFA which is considered a desirable trait can point to increase in metabolic activities of primarily lactic acid bacteria. For example, Lactobacillus rhamnosus GG fed to children with an intestinal infection significantly increased the total SCFA concentration (Sigur et al. 1996). In another study, supplementing the subjects' diet with bifidobacteria was found to result in a significant increase in acetate production (Jiang & Savaiano 1997).

Human experimentation has been confined largely to fecal measurements, which are of limited value as >95% of SCFAs are absorbed within the colon (McNeil et al., 1978; Ruppin et al., 1980; Roediger & Moore, 1981). Peripheral venous blood acetate has also been used to monitor large bowel events (Pomare et al., 1985), but blood acetate alone is of little value as an indicator of SCFA (Topping & Clifton, 2001). Propionate and butyrate concentrations are so low in blood that measurement is difficult without considerable sample concentrations (Muir et al., 1995; Wolfever et al., 1997). In vitro fermentation method is useful in studies concerning SCFA production from foods and ingredients by human fecal homogenates. Generally, batch cultures have been used (Topping & Clifton, 2001), and one large European collaborative study has evaluated fermentation under standardized conditions of incubation and dietary fiber analysis (Barry et al., 1995). Animal models have also been used for human large bowel SCFA metabolism, but it seems preferable to use studies in humans whenever possible (Topping & Clifton, 2001).

8.5.3 Gas production

The major end products of bacterial fermentation of polysaccharides besides short-chain fatty acids are gases; hydrogen (H₂) and carbon dioxide (CO₂). The volumes of hydrogen and carbon dioxide are reduced in subjects who have colonic microflora producing methane (CH₄) (Gibson et al., 1990). In about 50% of a European and North American population and in 90% of rural black Africans methane is generated from H₂ and CO₂ (Segal et al., 1988; Christl et al., 1995). In methane-negative individuals sulfate-reducing bacteria utilize H₂ to reduce sulfate to sulfide and considerable volume of hydrogen can be catabolized even in the absence of methanogenesis (Gibson et al., 1988). Methanogenesis and sulfate reduction are usually mutually exclusive (Gibson et al., 1993). Some fecal bacteria may also form acetate from hydrogen and carbon dioxide (Lajoie et al., 1988). Of the most common bacterial groups in the intestine, bifidobacteria are the only ones that do not produce any gases (Holdeman et al., 1977).
Methane is an inert gas and has probably no direct effect on man. The metabolities of sulfate reduction (mercaptides, hydrogen sulfide) are toxic and hydrogen sulfide is supposed to play a role in the pathogenesis of ulcerative colitis (Roediger et al., 1993; Pitcher & Cummings, 1996).

The gaseous products of colonic fermentation are either expelled per rectum or absorbed and exhaled. Measurement of breath excretion of methane and hydrogen has been widely used in clinical and physiological studies. Clinical breath tests are qualitative but the excretion of hydrogen in the breath is not in a constant proportion to that produced in the colon (Christl et al., 1992; Hammer, 1993). Breath methane excretion reflects methanogenic bacterial activity in the colon only in individuals colonized by particular organism (Methanobrevibacter smithii) at >10^6 CFU/g dry feces (Miller & Wolin, 1986).

Rectal gas excretion (flatus) can also be measured. Rectal tube provides quantitative collection but is uncomfortable, tends to plug, and cannot be used for prolonged periods (Hammer, 1993; Furne & Levitt, 1996). Christl and coworkers have described a technique in which the subject is housed in an airtight chamber, and the excretion rates of H₂ and CO₂ are determined from the increase in their concentrations in the air supply to the chamber (Christl et al., 1992).

Gas production by fecal samples during incubation have also been measured (Ross & Shaffer, 1989; Mazur et al., 1993; Hartemink & Rombouts 1997; Jiang et al., 2001)

8.6 Use of laboratory animals

The intestinal ecosystem can be studied in a very controlled way by using animal models (Gustafsson 1984). Major insights into the role of the intestinal microbiota has come from the comparative studies of germ free (absence of a microbiota) and conventional (presence of a microbiota) animals. The germfree animal can be monooassociated with single bacterium or polyassociated with multiple microorganisms or even with entire GI microbiota (Berg, 1996). Germfree animals colonized purposefully with known microorganisms are called gnotobiotics, meaning "known life" (Berg, 1996).

The gut microbiota tends to be characteristic for each animal species, and the microbiotas of laboratory rodents differ in types, number and sites of activity from that of man. The difficulty can be partly resolved by the use of rats or mice born germ free and then associated with a microbiota of human origin. The most notable differences, however, lie in the stomach and small intestine, which harbor only a transient microbiota in human but are quite heavily colonized in most laboratory rodents (Hill, 1995a). This may result in greater microbial transformation of ingested substance in these animals than in humans.
8.7 Chemostat models

Because of the complexity of the intestinal ecosystem, there is a real need to utilize *in vitro* models to allow controlled studies of individual components. Anaerobic continuous-flow cultures are more easily manipulated than conventional or gnotobiotic mice (Berg, 1996).

Chemostat culture studies can be carried out in batch culture with feces as the source of inoculum, conditions where a realistic assessment of *in vivo* effects is limited because growth of the mixed population cannot be controlled directly (Rumney and Rowland, 1992). An alternative approach is the use of chemostats where bacterial selection occurs in a constant and controlled environment in response to varying external parameters. In an attempt to reproduce some of the different physical and nutritional characteristics of the proximal and distal colons, a multiple stage continuous culture system has been described by Macfarlane *et al.* (1989).

The use of models for studying the human colonic microbiota has been reviewed by Rumney and Rowland (1992). Numerous models have been developed and they vary considerably in complexity ranging from simple batch cultures to semicontinuous systems adapted from a system described by Miller and Wolin (1981) and multistage continuous-culture fermentors (Macfarlane *et al.*, 1998). Close collaboration among five European laboratories has substantiated the validity of the *in vitro* fecal system whereby saccharides of different types can be classified in degree, rapidity and quality of fermentability (Barry *et al.* 1995).
AIMS OF THE STUDY

The aim of the present study was to evaluate different methods for their applicability to characterize the bacterial microbiota of the human intestine in appendicitis and some functional gastrointestinal disorders, and as a result of antimicrobial therapy.

More specifically, the present study had the following aims:

1. to profile bacterial enzymatic and metabolic activities as well as the whole community-derived bacterial fatty acids in order to find potential methodological candidates for further studies on microbiota parameters in lactose malabsorbers in relation to lactose tolerance

2. to determine whether antimicrobial agent induced changes in fecal microbiota are detectable by culture and bacterial cellular fatty acid profiling

3. to investigate whether the quantitative composition of gas produced by fecal samples of lactose malabsorbers is traceable to the severity of symptoms of lactose intolerance

4. to characterize bacterial microbiota in appendiceal tissue in relation to the inflammation by culture and advanced identification methods

5. to phenotypically and genotypically characterize an anaerobic gram-negative rod often isolated from appendix samples but not conforming to any previous descriptions
MATeRIALS AND METHODS

Table 4 compiles the specimens, subjects and methods used in this study.

**Table 4.** Specimens, subjects and methods used

<table>
<thead>
<tr>
<th>Study</th>
<th>Specimens</th>
<th>Number of samples</th>
<th>Subjects</th>
<th>Number of subjects</th>
<th>Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>stool sample</td>
<td>13</td>
<td>volunteers without GI disorders</td>
<td>3</td>
<td>enzyme profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>lactose maldigesters</td>
<td>10</td>
<td>biochemical profiling</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CFA analysis</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CFA analysis</td>
</tr>
<tr>
<td>II</td>
<td>stool sample</td>
<td>6</td>
<td>volunteers without GI disorders</td>
<td>6</td>
<td>CFA analysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>after antimicrobial exposure</td>
<td></td>
<td>culture</td>
</tr>
<tr>
<td>III</td>
<td>stool sample</td>
<td>21</td>
<td>lactose maldigesters with severe symptoms</td>
<td>10</td>
<td>gas production</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>lactose maldigesters with mild symptoms</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>appendix tissue</td>
<td>41</td>
<td>suspected acute appendicitis</td>
<td>41</td>
<td>culture</td>
</tr>
<tr>
<td>V</td>
<td>isolated strains</td>
<td>31</td>
<td></td>
<td></td>
<td>phenotypic characterization</td>
</tr>
<tr>
<td>VI</td>
<td>isolated strains</td>
<td>23</td>
<td></td>
<td></td>
<td>16S rRNA sequencing</td>
</tr>
</tbody>
</table>

1. SUBJECTS

**Study I** dealt with three healthy subjects and 10 persons with biopsy-proven lactose intolerance.

**Study II** dealt with six healthy male volunteers with an age range of 21-31 years. None of the subjects had received any antimicrobial agents for the four weeks preceding the study and were not using other drugs during the study days.
Study III dealt with a subgroup of 21 patients out of the original study population, which included 43 biopsy proven hypolactasic patients who registered their symptoms on a questionnaire during a six-hour follow-up period after oral challenge of 50g lactose in water coloured with carmine red. Patients were asked to determine the intensity of bloating or distension, abdominal pain, nausea, flatulence, and diarrhea. In addition, the time of stools coloured by carmine red was recorded to determine the bowel transit time. Measure round the waist was used as quantitation of bloating. The symptom score was calculated by combining parameters from the questionnaire, the bowel transit time, and the increase of waist measurement for each patient. Subjects from both extreme ends of the symptom score scale (min 18.2, max 90.0), were chosen for further studies. Consequently, 10 patients representing with the most severe (score >52) and 11 patients with the mildest (score <29) symptoms were included.

Study IV dealt with 41 children with suspected acute appendicitis admitted to the pediatric surgical unit of Aurora Hospital, Helsinki, aged from 1 to 16 years (median age 12 yrs). More of the patients were males (25 M vs.16 F) reflecting the sex related incidence of the disease. The patients received 500 mg of tinidazole per rectum 0.5 hours before the operation. No other preoperative antibiotics were given.

Studies V and VI dealt with anaerobic bacterial strains that did not conform to known species (31 and 23 strains, respectively), including isolates from samples of subjects in the study IV.

2. SPECIMEN COLLECTION AND HANDLING

In study I the fecal samples were frozen at -70°C within 24 hours of collection. At first, all methods were standardized by using fecal samples from three healthy subjects. Proper range of dilutions (wet weight/volume) - allowing recording of qualitative and quantitative differences in enzymatic and metabolic reactivity patterns between individuals – were determined by serially diluting these fecal samples. The dilutions were subsequently applied in a pilot study characterizing the intestinal microbiota of 10 persons with lactose intolerance.

In study II the volunteers received 750 mg ciprofloxacin (Ciproxin, Bayer AG, Leverkusen, Germany) orally twice a day for seven days. Fecal samples were collected before any medification was administered and immediately after the 7 day course of ciprofloxacin. The samples were frozen to –70°C within 6 hours of collection. Six pairs of the samples were analyzed by culture and by direct gas-liquid chromatography of bacterial CFAs.

In study III the fecal samples (total volume of defecation) were collected from patients with biopsy-proven hypolactasia and aliquots from the last passed portion were selected and frozen at -70°C within 24 hours of collection. Gas production (H₂,
CO₂, methane) by intestinal microbiota of the two different patient groups was analyzed.

The study IV material included 41 appendix tissue samples obtained at emergency surgery. The tissue specimens were sectioned transversely into two samples, approximately 1 cm³ each, one for bacterial cultivation and the other for histopathological examination. Microbiological samples were placed in sterile plastic containers, immediately sealed in a self-generating anaerobic pouch (GasPak pouch, BBL) and sent at room temperature to the laboratory, where they were processed within 24 h of collection. Samples that arrived later were excluded from the study.

In Study V 31 bile-resistant, pigment-producing, anaerobic bacterial strains were isolated from the appendix of 25 patients included in Study IV. The strains were stored in 20% skim milk at -70°C as stock cultures for detailed bacteriologic characterization.

Study VI was a phylogenetic description of 23 strains; 16 isolates from appendix tissue samples included in Study V and 7 isolates from unrelated fecal samples. In addition, the type strains of Bacteroides putredinis (ATCC 29800) and Rikenella microfusus (ATCC 29728) were included.

3. ENZYME PROFILING (I)

The enzyme profiles were determined with the API ZYM panel (bioMerieux, Marcy l’Etoile, France), which includes 19 different enzyme substrates and with Rosco diagnostic tablets (Rosco, Taastrup, Denmark) for urease, trypsin, β-galactosidase (ONPG), β-glucuronidase, β-glucosidase, and α-fucosidase activities.

Direct dilutions of 10⁻³ and 10⁻⁴ of the fecal samples in saline were used to inoculate API ZYM panels. The panels were incubated aerobically at 37°C for 4 hours, ZYM A and ZYM B reagents (bioMerieux, Marcy l’Etoile, France) were added, and the reactions were semiquantitatively interpreted with the help of a color chart. The approximate number of the nanomoles may be known from the color intensity: 1 corresponds to the liberation of 5 nanomoles, 2 to 10 nanomoles, 3 to 20 nanomoles, 4 to 30 nanomoles and 5 to 40 or more nanomoles. Zero corresponds to a negative reaction.

Rosco diagnostic tablets were added to tubes containing 0.3 ml of fecal dilutions of 10⁻³, 10⁻⁴, and 10⁻⁵. After 4 and 24 hours of incubation at 37°C, appropriate reagents were added and the results were interpreted according to the manufacturer’s instructions.
4. BIOCHEMICAL PROFILING (I)

The biochemical data were collected by inoculating API 50 CH panels (bioMeriéux), which provide 49 carbohydrate fermentation reactions. The samples were diluted in saline and added to API 50 CHE medium to give the final dilutions of $10^{-3}$ and $10^{-4}$. The reactions were interpreted after 24 and 48 hours of aerobic incubation at 37°C.

5. SHORT-CHAIN FATTY ACID ANALYSIS (I)

Short-chain fatty acids (SCFAs) were detected directly in the fecal samples and in preincubated samples by gas-liquid chromatography (GLC). To detect the SCFAs directly in the fecal samples, 1 g of the sample was suspended in 9 ml of peptone-yeast extract (PY) medium. For the preincubation study, $10^{-1}$ dilution of the feces was made in PY. Of this dilution, 100 μl was inoculated into 5 ml of PY medium and peptone-yeast extract-glucose (PYG) medium and incubated for 24 hours at 37°C.

Both volatile and nonvolatile SCFAs (a total of 16 different fatty acids in our standard solutions) were examined for in both types of samples by GLC using a Hewlett Packard 5890 gas chromatograph (Hewlett Packard, Palo Alto, California, USA) equipped with a SP-1000 glass column and a flame ionization detector.

6. BACTERIAL CELLULAR FATTY ACID ANALYSIS (I, II)

6.1 Sample pretreatment

The bacterial material was first separated from dietary fiber and whole eukaryotic cells as follows. First, 100 mg of the fecal sample was weighted, suspended into 5 ml of PY medium, gently mixed, and allowed to sediment for 2 h at 4°C. Then the sample was remixed and allowed to sediment further for 15 min. The supernatant contains free fatty acids, whole bacterial cells and fragments of cell membranes from eukaryotic cells. The supernatant was removed (3 ml) and bacterial phase was harvested by centrifugation at 1000 × g for 15 min at room temperature to produce a pellet. The sedimentation force was designed to separate bacterial cells as heavier particles from free fatty acids and the cell membrane fragments. The ensuing pellet was then processed further for GLC analysis or frozen at −20°C for further processing.

6.2 Gas-liquid chromatography of CFAs

Saponification, methanolysis and extraction of the cellular acids of the pellet were performed as recommended in the operating manual of the Microbial Identification System (MIS) software package by MIDI (Microbial ID, Inc., Newark, DE, USA). CFA profiles were generated by using a model HP-5890 series II gas chromatograph (Hewlett-Packard, Palo Alto, California, USA) equipped with an HP U2 cross-linked 5% phenyl methyl silicone fused silica capillary column (25m × 0.2mm i.d.), a flame
ionization detector, a model HP 7673 automatic sampler, and a computer 486DX with 16 MB of RAM. Hydrogen was used as a carrier gas. The temperatures used were 250°C for the injection port and 300°C for the detector. After injection of 2 μl fatty acid extract, the oven temperature was increased from 170°C to 260°C at a rate of 5°C/min, and then to 310°C at a rate of 40°C/min, held at 310°C for 1.5 min, and then returned to 170°C before the next sample was injected. The calibration mixture (Calibration Standards Kit HP 19298-60500 Rev. B.) was analyzed before the fatty acid extracts and re-analyzed after every 12th sample. The quantitative data obtained from the fatty acid profiles were used as the basis for numerical analysis performed with the help of the MIS software package by MIDI. The compounds were identified by using the Moore Broth Library, Version 3.9 (Microbial ID).

6.3 Fatty acid nomenclature

Fatty acids are designated in terms of the total number of carbon atoms:number of double bonds, followed by the position of the double bond from the methyl end of the molecule, and c or t represents cis or trans geometry, respectively. The prefixes a and i indicate anteiso- and iso-branching, and cyc refers to cyclopropane fatty acid. In addition, the location of hydroxy (OH) group may also be noted. Sum in feature is used in case more than one fatty acid has a particular retention time and acids cannot be resolved reliably from each other in analysis. DMA stands for dimethyl acetal and ALDE for aldehyde.

7. GAS PRODUCTION (III)

A modification of the method described previously (Ross 1987, Ross & Shaffer 1989) was used to measure the fermentation capacity of fecal microbiota in vitro. The accuracy and reproducibility of the method was tested by performing all the measurements in triplicate and validating by variance analysis.

Frozen stool samples were thawed, weighed and suspended to prepare fecal suspensions (0.2% w/v) in carbohydrate PY broths containing 1% lactose, glucose or galactose. Solutions were prepared in an anaerobic cabinet filled with a gas mixture of 90% N₂, 5% H₂, and 5% CO₂. Unincubated bottles were used as baseline controls for gas measurements. In average, 1.9% of hydrogen and 4.6% of carbon dioxide was measured from uninoculated bottles. The bottles were turned upside down during the incubation to prevent any escape of gas. After incubation (24 h, 35°C) in gas-tight bottles stoppered with butyl rubber caps and sealed with aluminum crimps (Sun International Trading, Ltd., Wilmington, NC USA), the amounts of hydrogen, carbon dioxide, and methane produced were determined. Oxygen (air) was also measured to control atmospheric conditions of incubation. The fermentation studies were done in triplicate and fermentation was stopped by adding 200 μl of 95% sulphuric acid and by moving the bottles into the refrigerator, where they were stored until analyzed.
The gas samples were taken from headspace with a gas tight syringe (2.5 ml) and analyzed by gas chromatography with a thermal conductivity detector (Hewlett Packard GC model 5890 equipped with stainless steel columns Porapak N and Molecular Sieve). The carrier gas was helium (flow 20 ml/min), injector temperature 150°C, oven temperature 45°C, and detector temperature 200°C. Samples were flushed through a 0.25 ml sampling loop fitted to a gas sampling valve.

8. CULTURE AND IDENTIFICATION OF ORGANISMS (II, IV, V)

8.1 Sample pretreatment and cultivation

The fecal samples in study II were thawed, and one gram of each specimen was suspended and serially diluted (tenfold) from 10^1 to 10^6 in PY broth. Undiluted sample and an aliquot of 10 μl of appropriate dilutions were inoculated and spread on selective and nonselective agar media for the enumeration and isolation of aerobic and anaerobic bacteria and yeasts (Table 5). The lower limit of detection was 10^3 microorganisms per g (wet weight) of feces.

Bacteriological appendix tissue specimens in study IV were processed within 24 h of collection. The specimens were ground in glass tissue grinders in pre-reduced liquid thioglycolate medium (dilution 1:1). Volumes of 10 μl of the homogenates were inoculated with a calibrated loop semiquantitatively onto various selective and nonselective media (Table 5). The plates were immediately placed in an anaerobic jar for incubation. Reduced media were prepared by storing the plates in an anaerobic jar; reducing agents were also added in some media.

8.2 Incubation

The plates for the total counts of the aerobes were incubated at 35°C in an atmosphere containing 5% CO₂ for up to 5 days in study II and for two days in study IV. The other aerobic plates or broth were incubated in the ambient air for 1-5 days except the CIN plates which were incubated at 30°C and CAMP plates which were incubated at 42°C in an atmosphere containing 5% O₂ and 10% CO₂ in jars. Agar plates for anaerobes were incubated in anaerobic jars filled with the evacuation replacement method with a gas mixture (90% N₂, 5% CO₂, 5% H₂) for 5-7 days before the first inspection and further up to 14 days before the final inspection.

8.3 Identification

In study II we used selective culture media to describe intestinal microbiota. The counts detected from these selective media represented mainly the bacteria on genus or group level but not the absolute counts of different bacterial species. The aim was to identify major differences between the sample groups.
Table 5. Culture media used

<table>
<thead>
<tr>
<th>Study</th>
<th>Media</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>For anaerobes</em></td>
<td></td>
</tr>
<tr>
<td>II, IV</td>
<td>Vitamin K and hemin supplemented Brucella blood agar&lt;sup&gt;1&lt;/sup&gt;</td>
<td>total counts of anaerobes</td>
</tr>
<tr>
<td>II</td>
<td>MRS (de Mann-Rogosa-Sharpe) agar (Difco)</td>
<td><em>Lactobacillus</em> spp.</td>
</tr>
<tr>
<td>II</td>
<td><em>Bifidobacterium</em> medium&lt;sup&gt;2&lt;/sup&gt;</td>
<td><em>Bifidobacterium</em> spp.</td>
</tr>
<tr>
<td>IV</td>
<td>PEA (phenylethyl alcohol agar)&lt;sup&gt;1&lt;/sup&gt;</td>
<td><em>Peptostreptococcus</em> spp.</td>
</tr>
<tr>
<td>II, IV</td>
<td>KVLB (kanamycin-vancomycin laked blood) agar&lt;sup&gt;3&lt;/sup&gt;</td>
<td><em>Prevotella</em> spp., <em>Porphyromonas</em> spp.</td>
</tr>
<tr>
<td>II, IV</td>
<td>BBE (bacteroides bile esculin) agar&lt;sup&gt;1&lt;/sup&gt;</td>
<td><em>Bacteroides</em> spp., <em>Bilophila wadsworthia</em></td>
</tr>
<tr>
<td>II, IV</td>
<td>NV (neomycin-vancomycin) agar&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>TSBV (tryptic soy-serum-bacitracin-vancomycin) agar&lt;sup&gt;1&lt;/sup&gt;</td>
<td><em>B. ureolyticus</em> -like organisms, <em>Fusobacterium</em> spp.</td>
</tr>
<tr>
<td>II, IV</td>
<td>NEYA (neomycin-egg yolk agar)&lt;sup&gt;1&lt;/sup&gt;</td>
<td><em>Clostridium</em> spp.</td>
</tr>
<tr>
<td>II</td>
<td>CCFA (cycloserine-cefoxitin fructose agar)&lt;sup&gt;1&lt;/sup&gt;</td>
<td><em>Clostridium difficile</em></td>
</tr>
<tr>
<td>IV</td>
<td>CFAT (cadmium-fluoride-acriflavine-tellurite) agar&lt;sup&gt;1&lt;/sup&gt;</td>
<td><em>Actinomyces</em> spp.</td>
</tr>
<tr>
<td></td>
<td><em>For aerobes</em></td>
<td></td>
</tr>
<tr>
<td>II, IV</td>
<td>Blood agar (5% sheep blood)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>total counts of aerobes</td>
</tr>
<tr>
<td>II, IV</td>
<td>Chocolate agar&lt;sup&gt;2&lt;/sup&gt;</td>
<td>total counts of aerobes</td>
</tr>
<tr>
<td>II, IV</td>
<td>CLED (cystine-lactose-electrolyte deficient) agar&lt;sup&gt;4&lt;/sup&gt;</td>
<td><em>Enterobacteriaceae</em> spp.</td>
</tr>
<tr>
<td>II</td>
<td>BE (bile-esculin) agar (Difco)</td>
<td><em>Enterococcus</em> spp.</td>
</tr>
<tr>
<td>II</td>
<td><em>Staphylococcus</em> 110 agar (Oxoid)</td>
<td><em>Staphylococcus</em> spp.</td>
</tr>
<tr>
<td>IV</td>
<td>XLD (xylose-lysine-decarboxylase) agar&lt;sup&gt;4&lt;/sup&gt;</td>
<td><em>Salmonella</em> and <em>Shigella</em> spp.</td>
</tr>
<tr>
<td>IV</td>
<td>CIN (crystal violet-cefsulodin-irgansan-novobiocin) agar&lt;sup&gt;4&lt;/sup&gt;</td>
<td><em>Yersinia</em> spp.</td>
</tr>
<tr>
<td>IV</td>
<td>CAMP (<em>Campylobacter</em> blood-free selective) agar&lt;sup&gt;4&lt;/sup&gt;</td>
<td><em>Campylobacter</em> spp.</td>
</tr>
<tr>
<td>II</td>
<td>SabA (Sabouraud dextrose agar with chloramphenicol) (Difco)</td>
<td>Yeasts</td>
</tr>
<tr>
<td>IV</td>
<td>Selenite broth&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Enrichment of <em>Salmonella</em> spp.</td>
</tr>
<tr>
<td>IV</td>
<td>Thioglycollate broth&lt;sup&gt;1&lt;/sup&gt;</td>
<td>enrichment of aerobic and anaerobic organisms</td>
</tr>
</tbody>
</table>

In study IV, the aim was to concentrate on identifications to comply with recent changes in taxonomy and to pinpoint all species present in samples. All colony morphotypes were recorded, enumerated semiquantitatively and isolated for further study. Thioglycollate broths were subcultured only if plated media yielded no growth. Isolation and identification of organisms were performed by established methods (Holdeman et al. 1977, Summanen et al. 1993; Jousimies-Somer et al. 1999; Murray et al. 1999). Anaerobic bacteria were tested for fermentation and biochemical reactions by use of pre-reduced anaerobically sterilized (PRAS) media; the end products of glucose metabolism were determined by gas-liquid chromatography (Hewlett Packard).

Preformed enzyme tests such as API ZYM and Rosco disks were also used (Durmaz et al., 1995). Also bacterial cellular fatty acid analysis was performed on selected strains. The species identifications of Prevotella nigrescens and Prevotella intermedia were confirmed by hybridization with species-specific oligonucleotide probes, analysis of the electrophoretic mobilities of glutamate and malate dehydrogenases and arbitrarily primed polymerase chain reaction (AP-PCR) as described previously (Mättö et al. 1996). E. coli isolates were serotyped and characterized as described previously (Siitonen, 1992; Siitonen et al., 1993; Saxén et al. 1996).

9. DETERMINATION OF 16S rRNA GENE SEQUENCES AND PHYLOGENETIC ANALYSIS OF ISOLATES (VI)

In order to verify the precise taxonomic position of the unusual pigmented organism we determined its complete 16S rRNA sequence and compared it to the deposited sequences in the Ribosomal Database Project and the GenBank (Benson et al., 1993; Maidak et al., 2001). Bacterial DNA was extracted from pure cultures and the 16S rRNA gene was amplified as described by Jalava & Eerola (1999). The DNA sequencing was done using ABI PRISM 310 Genetic Analyzer and ABI PRISM DNA sequencing kit. About 300 nucleotides long fragments of the gene were sequenced of all isolates. In addition, the PCR product of two isolates (AHN 2437 and AHN 2438) was sequenced as nine partly overlapping segments in order to determine the sequence of the whole gene. The PCR and the sequencing primers have been described by (Jalava et al., 1995). The compared sequences were aligned using the PILEUP program of the GCG package (Devereux et al., 1984). The distance matrix was calculated using the DNADIST program of the PHYLIP package (Felsenstein, 1993). The alignment was resampled 1000 times using SEQBOOT. The phylogenetic tree was constructed from the distance matrix using the neighbour-joining method (Saitou & Nei, 1987).

10. STATISTICAL METHODS (II, III, IV)

In study II the statistical significance of the differences before and after the ciprofloxacin intake was analyzed by Student’s paired t test. Logarithmic transformation was performed when appropriate. Chi square and two-tailed t tests were
used to determine the significance of any differences between the histopathology groups in study IV. In study III the reliability of the method for determining the amount and composition of the gas produced by fecal specimens was tested by variance analysis. A $p$ value of less than 0.05 was considered statistically significant.

11. ETHICAL CONSIDERATIONS (I, II, III, IV)

The studies with patients or human volunteers were approved by the Ethical Issues Committee at the Helsinki University Central Hospital.
RESULTS

1. ENZYMATIC AND METABOLIC PROFILES (I)

Our findings in the pilot study to profile the net bacterial enzymatic and metabolic activities of fecal suspensions showed differences between individuals that could be exploited in future analyses when metabolic differences will be researched for various groups of subjects.

In the API ZYM tests at a dilution of $10^{-3}$, nine of the 19 tests were always positive at various intensities (corresponding to enzyme concentrations of 20 to 40 or more nanomoles), and five tests were negative in all samples. Thus five remaining reactions (leucine arylamidase, valine arylamidase, $\alpha$-galactosidase, $\beta$-glucuronidase, and $\alpha$-fucosidase) were of discriminative value. Dilution of the specimens to $10^{-4}$ demonstrated more qualitative differences in the enzymes present: eight tests were negative in all samples and ten reactions had variable results.

With Rosco diagnostic tablets activities of $\beta$-glucuronidase, $\beta$-glucosidase, and $\alpha$-fucosidase were detectable in all samples at a dilution of $10^{-3}$. Some reactions became positive only after 24 hours of incubation. At a dilution of $10^{-4}$, these glycosidases were mostly detected as weakly positive reactions, and no positive reactions were detected at a dilution of $10^{-5}$. Trypsin and urease activities were detected in only a few samples (four of 10 and 2 of 10, respectively) at a dilution of $10^{-3}$.

In the API 50 CH test after 24 hours of incubation, there were 20 positive reactions at a dilution of $10^{-3}$, and four of these remained positive at dilution of $10^{-4}$ in all samples. After 48 hours of incubation, 26 of the 49 carbohydrates were fermented at a dilution of $10^{-3}$ and 12 were fermented at a dilution of $10^{-4}$, thus revealing the discriminatory value of two dilutions. Reactions of only five sugars were always negative at both dilutions: erythritol, xylose, xylitol, lyxose, and D-fucose. The remaining reactions had variable results.

The SCFAs detected directly in all samples included large amounts of acetate and smaller amounts of propionate, butyrate, and succinate. The other detectable SCFAs were present in smaller quantities and only occasionally. Five fatty acids, including isocaproic, caproic, malonic, fumaric and phenylacetic acid, were not detected at all.

The SCFA profiles of all the preincubated samples consisted of acetate in both media tested. In PY medium without fermentable carbohydrate, small quantities of propionate and butyrate were also detected. PY medium (which is rich in peptones) yielded isoacids, whereas PYG medium (which provides a fermentable carbohydrate) yielded large amounts of lactate.
2. FINDINGS IN CFA ANALYSES (I, II)

From the samples collected from three healthy volunteers and ten lactose maldigesters in study I, 39 different fatty acids were found (average, 23 per sample; range, 15-31 per sample). Palmitic acid (16:0 FAME) and stearic acid (18:0 FAME) predominated and were the only fatty acids present in >3% of total CFAs in all samples. The proportions of the CFAs varied in the samples, thereby allowing recording of differences between individuals.

The results of cellular fatty acid profile analysis of six volunteers in study II showed differences in profiles between samples taken before and after ciprofloxacin medication. Of the cellular fatty acids detected, 16 different fatty acids were detected only in samples taken before medication. The average number of different fatty acids (diversity) detected was 26.5 in samples taken before ciprofloxacin and 15.0 in samples after the medication. Importantly, the prevalence and relative proportions of five individual fatty acids (DMA-C14:0, ALDE-C16:0, C15:0, DMA-a-C15:0 and DMA-C18:11) decreased significantly (p<0.05; see table 3 in study II) during medication.

3. GAS PRODUCED BY STOOL SAMPLES (III)

When the gas produced by fecal contents of lactose maldigesters with different degree of symptoms was analyzed, no significant difference was found between the three carbohydrates (lactose, glucose, galactose) used. The patients with severe symptoms produced on average 5.3% H₂ (percentage of the bottle’s head space gas volume) and 10.2% CO₂, compared with 3.6% H₂ and 8.6% CO₂ produced by patients with mild symptoms.

Substantial intragroup variations in gas production were recorded among patients with severe (H₂ in galactose ranging from 0.9% to 8.3% and CO₂ from 5.9% to 15.9%) as well as with mild symptoms (H₂ from 0% to 8.3% and CO₂ from 1.4% to 14.5%). Qualitative analysis also showed that three patients produced detectable amounts of methane (two patients with severe and one with mild symptoms).

The individual symptoms, i.e. flatulence or abdominal pain, did not directly correlate with gas production but abundant gas production was associated with increased waistline measures after lactose provocation.

The reliability of the method for determining the amount and composition of the gas produced by fecal specimens proved well reproducible by variance analysis. The triplicate measurements of the same sample gave same results; when 0-hypothesis was that all three measurements equal 1:1:1, this was true.
4. FINDINGS BY PHENO- AND GENOTYPIC METHODS (II, IV, V, VI)

In study II the effect of ciprofloxacin on intestinal microbiota was studied by fecal bacterial culture. A significant, ciprofloxacin-induced change in the fecal microbiota was observed. Ciprofloxacin treatment for seven days clearly decreased the total counts of fecal aerobic bacteria from \(1.5 \times 10^8\) CFU/g to \(8.4 \times 10^8\) CFU/g \((p = 0.03)\). All Enterobacteriaceae and Enterococcus species totally disappeared or decreased to undetectable levels \(<10^3\) CFU/g wet weight) from stool samples during medication. The total counts of anaerobic bacteria also declined significantly from \(4.0 \times 10^9\) CFU/g to \(7.1 \times 10^8\) CFU/g \((p=0.01)\). This was mainly due to a drop in prevalence and counts of Bifidobacterium and Clostridium species. B. fragilis group bacteria dominated in samples after the medication. The prevalence and concentrations of B. fragilis group bacteria and lactobacilli stayed nearly unchanged through the medication. Yeasts were originally present in three subjects in low numbers just above the detection limit \((10^3\) CFU/g), but appeared at higher levels after ciprofloxacin administration and one originally yeast-negative subject, became yeast-positive. No C. difficile were recovered.

Culture method was also used in study IV to determine the bacterial etiology of appendicitis in children in relation to the histologic tissue pathology. Aerobic and anaerobic species were isolated from 40/41 (98%) appendix tissue samples; on average, 14.1 isolates per specimen (10.4 anaerobes and 3.7 aerobes). Specimens from patients with gangrenous appendices yielded significantly higher numbers of anaerobic isolates per specimen than did specimens from patients with healthy appendices (11.7 vs. 7.7 ; \(p < .01\)). Bacteria belonging to the B. fragilis group were the most frequently isolated anaerobic microorganisms (95% of specimens). Other organisms frequently isolated in all histology groups were Micrococcus (former Peptostreptococcus) micros (66%), Bilophila wadsworthia (63%), Fusobacterium nucleatum (44%), Eggerthella lenta (44%), and a hitherto undescribed bile-resistant, pigment-producing gram-negative rod (41%). Of the aerobes E. coli (88%) and S. anginosus group (former S. “milleri” group) organisms (61%) were the most frequent findings.

The rate of isolation of the "bile-resistant pigmented" was 33% from normal, 32% from inflamed, and 56% from gangrenous appendices. The extensive phenotypic characterization of this unusual rod showed that this organism exhibited properties distinct from any previously described species (V). The additional 16S rRNA sequencing (IV) verified that this unusual organism should be recognized as a new species, probably in the same genus with B. putredinis. The phylogenetic analysis of the partial 16S rRNA sequences of the 23 isolates gave sequence information of an average of 323 bases at positions between 538 and 925 (positions numbered according to Brosius et al. (1981)). On the basis of partial sequence similarity it was clear that the strains form two homogenous groups and constitute two separate 16S rRNA populations. These two groups revealed different base profiles at positions 611, 620, 648 and 649; group I (12 isolates) C, T, A, G and group II (11 isolates) A, C or T, G, A. The two strains chosen as representatives from different groups, with complete...
sequences determined, showed the highest level of sequence similarity with *B. putredinis* (94.6 and 95.1%) and *Rikenella microfusus* (87.6 and 87.0%).
DISCUSSION

1. BASIC METHODOLOGICAL CONSIDERATIONS

This study exploiting various phenotypic methods to assess the composition and metabolic as well as enzymatic activities of microbial communities in the gut showed certain inherent limitations but also useful features of the methods used. Especially, the non-culture-based methods used may augment culture or molecular based techniques in the future. Table 6 compiles the evaluation of methodologies used in this study.

**Table 6. Comparison of phenotypic techniques**

<table>
<thead>
<tr>
<th>Method</th>
<th>Viable bacteria needed</th>
<th>Bacterial composition/function</th>
<th>Technically difficult</th>
<th>Labor intensive</th>
<th>Rapid * technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymatic profiling (pre-formed enzymes)</td>
<td>no</td>
<td>function</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Metabolic profiling (fermentation, degradation)</td>
<td>yes</td>
<td>function</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>CFA analysis (derived fatty acid methyl esters of cell wall)</td>
<td>no</td>
<td>composition</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Gas production (fermentation)</td>
<td>yes</td>
<td>function</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Culture method and isolation technique (growth)</td>
<td>yes</td>
<td>composition</td>
<td>yes/no</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>

* Indicates if the technique can easily be completed in 1-3 days

1.1 Enzymatic and metabolic profiling in characterization of the intestinal microbiota

Fecal microbiota was studied using commercial test kits to ensure a broad-spectrum coverage of reactions and to provide a reproducible and cost-effective methodologic reference. Enzymatic and metabolic profiling yielded clear differences in profiles
between individuals and showed that these methods offer a rapid tool for studying changes and differences in the fecal microbiota. The dilutions used gave adequate numbers of both positive and negative reactions for comparison. The dilutions were chosen to best disclose interindividual differences in reactivity but also so that the baseline colour of the samples did not interfere the results. Interpretation of several reactions from two or three sample dilutions after two different incubation times resulted in a very comprehensive profile. Since the amount of data obtained by various profiling methods is exhaustive, computer-assisted methods are required.

Detection of preformed enzymes and metabolic fatty acids (SCFAs) directly in the fecal samples has the benefit of not requiring viable bacteria for testing. However, detection of SCFAs from fecal samples is rather limited as most of the SCFAs are absorbed within the colon (McNeil et al., 1978; Ruppin et al., 1980; Roediger & Moore, 1981).

Biochemical data and SCFA profiles from the pre-incubation studies reflect the activity of viable bacteria in fecal samples, but may select for rapidly growing species and groups and finally those most resistant to acidic conditions.

The in vivo findings have demonstrated that the microbiota is more metabolically active in the proximal colon compared to the distal colon (Macfarlane et al., 1992). This is a reflection of relative availability of fermentative substrates in the two sites and is of significance with respect to the analysis. Biochemical changes occurring in the contents of the proximal colon may not be detectable in the distal colon or in feces (Tannock, 1999a). However, the aim of the present study was not to find out the actual situation inside the intestinal tract but rather to find differences that could reflect differences in microbial compositions.

It is also known that diet may have an effect on the activity of fecal enzymes in various ways (Hill, 1995b). Diet may contain components that induce the enzymes, or stimulate or inhibit their activity (Hill, 1995b). It is difficult to simulate gut conditions in in vitro metabolic profiling assays because nutrient medium is used and the pH is usually adjusted to the pH optimum of the enzyme being tested. Our methods therefore measured the maximum potential induced enzyme activity but gave no information on the actual enzyme activity in vivo in the individual being studied.

1.2 Applicability of CFA profiling method to detect changes and differences in the intestinal microbiota

Fatty acid analysis is a powerful tool because of its low cost, quick and simple method, and extent of automation. Because there are variations in carbon length and fatty acid types (e.g. straight chained, varying degrees of unsaturation, iso- an anteiso-branched, cyclopropane, 2- and 3-hydroxylated etc.), these molecules form a rich pool of markers for analysis. Cellular fatty acid profiling has also an advantage over viable counting techniques in that cells need not be in a culturable state to be detected. One limitation of the GLC method is that it measures only relative changes in the amounts
of different fatty acids (and thus the bacterial species). If there are changes in the total amount of intestinal bacteria without relative changes it cannot be detected by the GLC method.

Most of the fatty acids are found in a wide range of organisms and provide little information about community structure. Therefore, taxonomic interpretations e.g., associations with special species or groups are difficult to assign from community level fatty acid profiles (Haack et al., 1994; Hopkins et al., 2001). No database exists which correlates specific fatty acids with the taxa they occur in. Valuable compendia of microbial lipids, such as that of Ratledge and Wilkinson (1988), are indexed by taxa not by fatty acids. The CFA technique requires further development to determine whether quantitative measurements of signature fatty acids can provide a useful description of specific bacterial populations in the gut.

Phospholipid contents of microorganisms vary across taxa, and fatty acid profiles of individual bacteria are known to vary quantitatively and qualitatively with changes in environmental factors (Rose, 1988). Cell fatty acid composition is especially sensitive to medium composition, but also incubation temperature, cell age, and degree of anaerobiosis (Moore, 1993). However, dietary intervention does not always alter fecal CFA profiles, since no differences in CFA or bacteriological composition were observed in rats fed a restricted diet (Henderson et al., 1998). In colonic environment the dietary changes are not nearly as extreme as what is imposed on the bacteria in different culture media, where most of the nutrition comes from a single or few substrates (Peltonen & Eerola, 1993).

1.3 Measurement of gas production by intestinal bacteria

The measurement of gas production proved to be a very reproducible and useful method for potential further studies. Gas production was not significantly different from the three carbohydrates tested (lactose, glucose, galactose). That finding was in line with previous studies by Ross and Shaffer (1989).

The small inocula (0.2%) used in this study may have selected for the rapidly growing bacteria and the results thus mainly may have represented their gas production. The inoculum used in in vitro batch fermenters to study dietary fiber fermentation by intestinal bacteria have been as high as 16.7% (Barry et al., 1995).

Hydrogen-utilizing metabolism reduces the gaseous volume in the colon and thus prevents flatulence. In patients with pneumatosis cystoids, methanogenesis is absent and sulfate reduction is insignificant. This deficiency provides an explanation for the massive hydrogen excretion in those patients and for their symptoms (Florin, 1997). Hydrogen consumption by bacteria during methanogenesis, acetogenesis or sulfate reduction reactions in our batch culture system used was not probably as efficient as it is in vivo as we have found that methane production by fecal suspensions is clearly suppressed in our system if pH drops (Blom et al., 2000).
Other studies (Ross & Shaffer, 1989; Wang & Gibson, 1993; Bouhnik et al., 1997) that have used fecal inocula have determined gas production by using different techniques (batch vs. fermentors). Thus, differences in techniques, growth media and preparation of inocula makes comparison of the results difficult.

1.4 Usefulness of culture and isolation techniques

Culture methods have been the gold standard and used extensively by the leading anaerobic bacteriology laboratories all over the world. Because no single or combined non-cultural method comprehensively cover intestinal microbiota findings (Wintzingerode et al., 1997, O’Sullivan, 2000) culture will remain a major reference method (Vaughan et al., 2000).

Non-selective media are generally used to estimate total numbers of both aerobic and anaerobic microbiota. However, while these media contain no known selective agent, they do select against some bacteria from the human intestine (Moore, 1987; Sheppard et al., 1990; O’Sullivan, 2000). For example, bacteria that need extra requirements or bacteria that grow on the media but are in a physiological state which does not support the growth, may not grow directly from samples (O’Sullivan, 2000).

Enumeration of particular microbial genera or species relies on the use of selective media. The inability to culture all of the microbes present in sample and the use of a limited range of reliable selective media doubtless introduces bias into analyses of the composition of the microbiota (Drasar & Barrow 1985). Selective media have the inherent disadvantage of not being absolutely selective and on the other hand being relatively toxic against certain strains within the selected genus. Analysis of some commonly used bifidobacterial selective media found that none were fully selective and that they generally contained toxicity against some bifidobacterial strains (Silvi et al., 1996; Hartemink et al., 1997; Hartemink & Rombouts, 1999).

The use of selective media will increase the yield of different species (Finegold et al., 1993; Jousimies-Somer et al., 1999). There are very few selective culture media that permit the growth of only one group of intestinal bacteria and therefore accurate enumeration of particular bacterial genus. However, members of the B. fragilis group, enterococci, enterobacteria, lactobacilli, bifidobacteria and yeasts can be quite reliably enumerated (Tannock, 1999c). Bifidobacterial numbers, for example, are identical whether determined by culture or by oligonucleotide probe hybridization (Langendijk et al., 1995). We used selective culture media to characterize the bacterial population in intestinal microbiota of the subjects before and after ciprofloxacin administration. The counts detected from these selective media represented mainly the bacteria on genus or group level, not the counts of different bacterial species. Nevertheless, this method was able to identify major differences between the sample groups.

The colon is an anaerobic environment and consequently a sample collected from the colon by intubation, or at operation, must be introduced into an oxygen-free
environment as rapidly as possible. In addition, the time and temperature of storage and the nature of the transport medium will influence the outcome of subsequent culture work (Borriello et al., 1978). Qualitative and quantitative changes in the microbiota have been seen to occur in samples stored for a relative short period of time (Bonten et al., 1997; Ballongue, 1997). In the present study the samples were transported under anaerobic atmosphere and no transport media where used that might support outgrowth of certain bacteria and thus give a false picture of the bacteriological content in samples.

Specimens were processed and inoculated on the bench and immediately placed in an anaerobic environment produced by the evacuation replacement method, which produces anaerobiosis very rapidly in jars (Summanen et al. 1999). When processing the specimens on the bench instead of inside an anaerobic chamber, it is essential that the media used are not saturated with oxygen. Reduced media were prepared by storing the plates in an anaerobic jar; reducing agents were also added in some media.

A high proportion of anaerobic organisms grow slowly. Consequently inspection of the plates at, for example, 24 hours not only fails to reveal such organisms but also exposes them to oxygen. Wren (1977) showed that if the anaerobic jars were not opened until 48 hours after plating nearly three times as many types of organism could be isolated. We did not examine the plates for anaerobes until 5 days after inoculation, and always continued the incubation up to 14 days before the final inspection.

The aim was to isolate as many different types of organisms as possible, and that was achieved by the using a wide range of freshly prepared non-selective, selective and differential media. Many anaerobes are pleomorphic and present as a variety of apparent morphological types. The colonies of the same strain were usually picked from several media.

The confidence level of the bacterial species identification will increase with the number of tests that is carried out but even the most sophisticated array of tests can lead to uncertainties in the classification of the isolates (Collins & Gibson, 1999). Further, the metabolic plasticity of the organisms and subjectivity of the operator brings unreliability into analysis (Collins & Gibson, 1999). The identification can also be difficult because of poor discrimination when strains, which are considered to represent the same species, show considerable variations in biochemical attributes. Differentiation of the isolates into species, especially in the case of the obligate anaerobes, is always logistically difficult (Finegold et al., 1993; Summanen et al., 1993). In addition, their further identification and characterization by classical methods is labor intensive (Finegold et al., 1993).

Many microbes in different ecosystems cannot be cultivated by standard culture techniques (Ward et al. 1990). Comparative analysis of 16S rRNA sequences amplified from human feces indicated that 24% of the molecular species identified corresponded to known organisms, suggesting that most of the bacteria in the gut have yet to be described (Suau et al. 1999). Thus, classical culture based methods do not
provide an accurate representation of the intestinal microbiota and this may explain why the etiological agents of some inflammatory bowel diseases have remained unknown.

Interestingly, Wilson and Blitchington (1996) compared conventional culture and 16S rDNA sequence analysis as a method to analyze human fecal samples. They found that, overall there was a good agreement in the biodiversity of the samples analyzed by these two methods when culture-based method was done optimally. Well in line with that is our novel finding of "bile-resistant pigmenter" from appendix samples. This suggests that the number and diversity of micro-organisms potentially recoverable from patients with appendicitis is greater than previously reported.

Despite the limitations, culture techniques are very powerful and absolutely essential to obtain a complete picture of the diversity and role of the intestinal microbial ecosystem (O’Sullivan, 2000). To study such a complex ecosystem, the combination of both culture and some non-culture based technique are required (Palleroni, 1997).

2. MICROBIOTA CHARACTERIZED BY DIFFERENT METHODS

2.1 Intestinal microbiota of subjects with lactose intolerance

Several bacteriological parameters of intestinal microbiota in lactose maldigesters were analyzed in this pilot study. The proportions of the fatty acids varied in the samples from lactose maldigesters thereby allowing recording of differences between individuals. In our further studies the fecal bacterial CFA profiles of lactose maldigesters experiencing most severe symptoms during lactose tolerance test differed from those of the patients with mild symptoms and healthy controls (Rautio et al. 1997 and unpublished results).

We also investigated whether the gas produced by fecal contents of lactose maldigesters was traceable to symptoms of intolerance. In this relative small patient group we could not demonstrate a clear association between intestinal microbiota-derived gas production and any single symptom except for slight association between abundant gas production and increased waistline measure after lactose provocation.

Gas production by intestinal microbiota of lactose maldigesters with severe symptoms was higher than of those with mild symptoms. This difference reflects the differences in fecal bacterial composition and supports the hypothesis of the role of intestinal microbiota in lactose intolerance described also by Vesa (1997). Increased production of gas alone is unlikely to be the cause of symptoms. Other fermentation products – such as SCFAs – may be important. Individual physiologic differences in elimination of gases through circulation and expiration, as well as tolerance to abdominal distension, may partly explain the lack of correlation of gas production and symptoms.
Only minor differences in gas production between patient groups with mild and severe symptoms were found. Factors that influence colonic gas absorption include transmucosal pressure gradient and the specific absorptive capacity for various gases. Conversely, it has been suggested that ineffective colonic gas absorption is rather the consequence than the cause of high colonic accumulation rate after ingestion of non-absorbable carbohydrates (Hammer, 1993). According to this concept, the interindividual differences in colonic gas accumulation are most likely due to differences in bacterial production and catabolism of gas.

2.2 Effect of antimicrobial administration on intestinal microbiota of subjects without GI disorders

The intestinal microbiota composition was radically disturbed by the ciprofloxacin medication and this change was also detectable as a diminished diversity of bacterial CFAs after challenge. However, no direct but some suggestive connection between the change in fatty acids with specific groups of organisms was found.

The effect of ciprofloxacin medication on aerobic bacteria was the most pronounced. It eliminated (or reduced under detection level) the members of the Enterobacteriaceae family and Enterococcus species in all volunteers. Reports of other authors have shown similar findings for Enterobacteriaceae (Nord, 1995; Enlund & Nord, 1999), but not for enterococci (Enlund & Nord, 1999). However, the daily dosage in those studies was lower.

In contrast to the reports showing that only the aerobic microbiota is suppressed by ciprofloxacin administration (Shah et al., 1987; Enlund & Nord, 1999), in the present study with higher dosage also the anaerobic microbiota, with exception of B. fragilis group and lactobacilli, was affected. The effect was best seen as reduced prevalence and count of Bifidobacterium and Clostridium species.

2.3 Microbiota in suspected appendicitis

In the study investigating the detailed aerobic and anaerobic bacterial etiology of appendicitis in children, special attention was paid to carefully collect, transport, process and culture the specimens in a manner to optimise the recovery of even the most fastidious anaerobic species. Furthermore, bacteriologic results were compared to the histological findings of appendiceal samples in order to recognize possible differences that may correlate with the degree of inflammation. The bacteriology of appendicitis has been carefully studied in adults but only fragmentarily in children (mainly anaerobes) (Baron et al., 1992; Bennion et al., 1990 a, b; Schumacher et al., 1997).

The shift from histologically normal towards gangrenous appendices was clearly associated with markedly elevated number of bacterial species. The number of different isolates present in the appendix increased from about 12 isolates in normal to 14 in inflamed and to 16 in gangrenous appendices. The difference was statistically
significant between normal and gangrenous appendices and most marked among the anaerobic species.

In spite of these quantitative differences, however, we were unable to demonstrate any qualitative differences in the bacterial composition in relation to the histopathologic degree of inflammation. This finding is well in concordance with the results of Baron et al. (1992) who detected significantly higher numbers of species in gangrenous or perforated than in acute cases of appendicitis in adults, and also with those of Schumacher et al. (1997) who recovered also higher numbers of anaerobes from acute or complicated cases of appendicitis than from non-inflamed or chronically inflamed appendices in children.

The results clearly demonstrated the presence of mixed aerobic and anaerobic microbiota in both normal and inflamed appendices in children, a finding in line with the report by Schumacher et al. (1997). Furthermore, these results are in conformity with previous findings in adults (Baron et al., 1992; Bennion et al., 1990 a, b). The most striking difference, however, was the frequent isolation of the “bile resistant pigmented”; this finding has not been reported previously from any infections in children or adults. The rate of the isolation of this “bile resistant pigmented” was similar from normal (33%) and inflamed (32%) appendices but clearly, although not significantly, higher from gangrenous appendices (exceeding 56%). The omission of this bacterium by earlier studies is most probably due to the bacterium’s tiny, slow growing colonies that are easily overlooked.

The high frequency of B. wadsworthia isolates was also a unique feature compared with the results of previous studies in both children (Schumacher et al., 1997) and adults (Baron et al., 1992; Bennion et al., 1990a,b). The absence of Porphyromonas gingivalis in samples taken from children in all histology groups was also surprising; the trend that was also seen with P. intermedia. This fact points to the negligible carriage of periodontopathogens by children in contrast to adults (Mättö et al., 1997).

Although the clinical significance of these bacteriologic findings remains to be completely elucidated, it is apparent that the number and diversity of micro-organisms potentially recoverable from patients with appendicitis is greater than previously reported (Baron et al., 1992; Bennion et al., 1990a,b; Schumacher et al., 1997). The relative or synergistic distribution of different species, such as M. micros and S. anginosus group organisms, in the pathogenesis of appendicitis deserves further studies involving larger patient material.
SUMMARY AND CONCLUSIONS

The intestinal microbiota, their metabolic capabilities and products play an important role in human health. Although tremendous strides have been made, our knowledge of the ecology of the human intestinal microbiota is largely still in its infancy. The main goal is to develop an understanding of both the composition and function of the microbial community of the human intestine. Increased understanding of safe ways to manipulate the gastrointestinal microbiota may have much potential, for example in therapy, in the future. There are technical difficulties associated with the investigation of such a large and complex microbial collection. However, complementation of the traditional approaches with newer, especially molecular methods shows enormous promise for obtaining insight into the intestinal microbiota. This study was carried out in order to investigate some methods for their applicability to characterize the bacterial populations present in intestine of humans with and without certain gastrointestinal pathologies.

The key findings of the present study were:

1. The use of commercial test kits in enzymatic and metabolic profiling enabled a broad spectrum of reactions to be recorded cost-effectively when the intestinal microbiota was characterized as a mixed microbial population in terms of its biochemical properties.

2. Bacterial CFA profiles detected directly in the fecal samples collected from lactose-intolerant patients showed that there were differences between individuals’ microbiota and that the method was applicable for further studies.

3. The intestinal microbiota composition was radically disturbed by the administration of ciprofloxacin. This change was detectable in species diversity and microbial counts by culture and in diversity of bacterial CFAs in stool samples. Furthermore, the change was reflected by prevalence and relative shares of five fatty acids. However, no direct connection between any specific groups of organisms and individual fatty acids were conclusively found.

4. Gas production by intestinal microbiota of lactose maldigesters with severe symptoms was higher than that in patients with mild symptoms. However, the difference did not reach statistical significance. The reliability of the method for determining the amount and composition of gas produced by fecal specimens proved to be very reproducible.

5. The etiology of acute appendicitis in children was found to be polymicrobial as in adults but some differences in main findings were clearly evident. In spite of differences in prevalence of different bacterial isolates, we were unable to associate
any single species with the histopathologic degree of inflammation. As a new finding we frequently isolated an unusual anaerobic pigmented gram-negative rod that no previous study group has reported.

6. Detailed characterization of the unusual anaerobic pigmented gram-negative rod isolated from normal and inflamed appendiceal tissue samples showed, that this organism exhibited phenotypic properties distinct from any previously described species. The study gave a scheme to facilitate the recognition and identification of this organism. Our phylogenetic findings on this unusual organism warrant its classification in a new genus and species.
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