Silja Mentula

Analysis of Canine Small Intestinal and Fecal Microbiota

Prevention of Ampicillin-induced Changes with Oral β-lactamase

Publications of the National Public Health Institute A 8/2005

National Public Health Institute
Helsinki, Finland
and
Department of Applied Chemistry and Microbiology
University of Helsinki, Finland

Silja Mentula

ANALYSIS OF CANINE SMALL INTESTINAL AND FECAL MICROBIOTA

PREVENTION OF AMPICILLIN-INDUCED CHANGES WITH ORAL β-LACTAMASE

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Agriculture and Forestry, University of Helsinki, for public examination in Auditorium XII, University Main Building,Unioninkatu 34, on June 9th, at 12 noon.

National Public Health Institute, Helsinki, Finland

and

Department of Applied Chemistry and Microbiology, University of Helsinki, Finland

Helsinki 2004

Supervised by

Professor Pentti Huovinen
Antimicrobial Research Laboratory
Department of Bacterial and Inflammatory Diseases
National Public Health Institute, Turku

Docent Eija Könönen
Anaerobe Reference Laboratory
Department of Bacterial and Inflammatory Diseases
National Public Health Institute, Helsinki

Reviewed by

Professor Charlotta Edlund
Södertörns högskola, University College
and
Department of Laboratory Medicine
Division of Clinical Bacteriology
Karolinska Institutet
Stockholm, Sweden

Docent Maria Saarela
VTT Biotechnology, Foods and Processes
VTT, Espoo

Opponent

Professor Ian Poxton
Division of Medical Microbiology
University of Edinburgh Medical School
Edinburgh, Scotland, UK

To my family

Publications of the National Public Health Institute
KTL A8 / 2005

Julkaisija-Utgivare-Publisher

Kansanterveyslaitos (KTL)
Mannerheimintie 166
00300 Helsinki
Puh. vaihde (09) 474 41, telefax (09) 4744 8408

Folkhälsoinstitutet
Mannerheimvägen 166
00300 Helsingfors
Tel. växel (09) 474 41, telefax (09) 4744 8408

National Public Health Institute
Mannerheimintie 166
FIN-00300 Helsinki, Finland
Telephone +358 9 474 41, telefax +358 9 4744 8408

ISBN 951-740-517-0
ISSN 0359-3584
ISBN 951-740-518-9 (pdf)
ISSN 1458-6290 (pdf)

Edita Prima Oy
Helsinki 2005

CONTENTS

ABBREVIATIONS

CFU	colony forming unit
DNA	deoxyribonucleic acid
GALT	gut-associated lymphoid tissue
IBD	inflammatory bowel disease
i.v.	intra venous
MAC	microflora-associated characteristics
MRSA	methicillin-resistant *Staphylococcus aureus*
NCCLS	National Committee for Clinical Laboratory Standards
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
p.o.	per oral
rRNA	ribosomal ribonucleic acid
SIBO	small intestinal bacterial overgrowth
TGGE	temperature-gradient gel electrophoresis
TEM	class of plasmid-mediated β-lactamases (first isolated from a patient named Temoniera)
TRBL	targeted recombinant β-lactamase
VRE	vancomycin-resistant enterococci

Silja Mentula, Analysis of canine small intestinal and fecal microbiota – prevention of ampicillin-induced changes with oral β-lactamase
Publications of the National Public Health Insitute, A8/2005, 136 Pages
ISBN 951-740-517-0; 951-740-518-9 (pdf-version)
ISSN 0359-3584; 1458-6290 (pdf-version)
http://www.ktl.fi/portal/suomi/julkaisut/julkaisusarjat/kansanterveyslaitoksen_julkaisuja_a/

ABSTRACT

Background: The intestinal microbiota affects many physiological and immunological functions of the body and is closely connected to the homeostasis and health status of an individual. Due to difficulties in obtaining samples from the intestine and studying complex microbial populations the knowledge of the composition, dynamics and metabolic potential of microbial populations in the upper gut is limited. Antimicrobials may disrupt the balance of the microbiota by causing quantitative and qualitative changes such as emergence and enrichment of antibiotic-resistant populations, and decrease in colonisation resistance against pathogens. The aims of this study were to analyse cultivable jejunal microbiota of a dog in detail and compare it to faecal findings, and to evaluate the ability of oral β-lactamase administration, targeted to degrade intestinal ampicillin residues, to prevent ampicillin-induced changes in the intestinal microbiota.

Material and methods: Jejunal and faecal samples were obtained from healthy laboratory beagles with permanent jejunal fistula randomised into 3 treatment groups receiving ampicillin, ampicillin + β-lactamase or placebo. Samples were collected before, during and after the treatment and analysed using bacterial culture and TGGE (temperature-gradient gel electrophoresis) for total bacterial counts and composition of the dominant microbiota. Susceptibility testing and PCR for detection of TEM gene were the methods for monitoring resistance rates. In addition, jejunal and serum ampicillin concentrations were determined.

Results: The relative quantities of predominant bacterial groups, proportion of anaerobes, intra- and inter-individual species variety and stability of the microbiota differed significantly between the jejunum and faeces. Oral β-lactamase inhibited the effects of ampicillin, monitored as significant differences in resistance rates and

bacterial composition during treatment between the groups receiving ampicillin with and without β-lactamase.

Conclusion: This study suggests that faeces with stable and rich microbiota does not represent jejunal microbiota that is characterised as harbouring few individually varying bacterial species at a time with fluctuating counts. Oral β-lactamase has potential in inhibiting the adverse effects of β-lactam therapy.

Keywords: gastrointestinal tract, jejunum, microbiota, β-lactams, susceptibility

Silja Mentula, Analysis of canine small intestinal and fecal microbiota – prevention of ampicillin-induced changes with oral β-lactamase
Kansanterveyslaitoksen julkaisuja, A8/2005, 136 sivua
ISBN 951-740-517-0; 951-740-518-9 (pdf-versio)
ISSN 0359-3584; 1458-6290 (pdf-versio)
http://www.ktl.fi/portal/suomi/julkaisut/julkaisusarjat/kansanterveyslaitoksen_julkaisuja_a/

TIIVISTELMÄ

Taustaa: Suolistomikrobisto vaikuttaa moniin kehon fysiologisiin ja immunologisiin toimintoihin ja siten myös kehon tasapainotilaan sekä yksilön terveyteen. Johtuen vaikeuksista näytteiden saamisessa suoliston eri osista sekä monimuotoisten mikrobipopulaatioden tutkimisen hankaluuksista, ymmärtämys suolistomikrobiston koostumuksesta, dynamiikasta ja metaboliikasta on vaillinaista. Antibiootit voivat häiritä mikrobiston tasapainoa aiheuttaen niin määrällisiä kuin laadullisia muutoksia mikrobistossa kuten antibiootille vastustuskykyisten mikrobien ilmaantumista ja rikastumista ja taudinaiheuttajamikrobien kolonisaatioresistenssin alenemista. Tämän tutkimuksen tavoitteena oli analysoida koiran ohutsuolen mikrobisto yksityiskohtaisesti viljelymenetelmää käyttäen ja verrata sitä ulosteen mikrobistoon. Lisäksi tavoitteena oli arvioida suun kautta annettavan, suolistoon kulkeutuvien β-laktaamiryhmän antibioottijäämien hajoittamiseen suunnitellun β-laktamaasivalmisteen tehoa ampisilliinin suolistomikrobistossa aiheuttamien muutosten ehkäisyssä.

Materiaalit ja menetelmät: Ohutsuolen sisältö ja ulostenäytteet kerättiin terveiltä beagle-koirilta, joille oli tehty pysyvä fisteli (permanent jejunal fistula). Koirat oli satunnaistettu kolmeen hoitoryhmään, jotka saivat joko ampisilliiniä, ampisilliiniä ja β-laktamaasia tai lumevalmisteita. Näytteet kerättiin ennen antibioottikuuria, kuurin aikana ja sen jälkeen ja analysoitiin käyttäen viljelyä ja TGGE-profilointia (temperature-gradient gel electrophoresis) bakteerien kokonaismäärien ja koostumuksen tutkimiseen. Antibioottiresistenssin esiintymistä seurattiin herkkyysmäärityksin sekä TEM-geenin (plasmidi-välitteinen β-laktamaasigeeni) esiintymistä PCR-määrityksin. Lisäksi seurattiin ohutsuolen sisällön sekä seerumin ampisilliinipitoisuuksia.

Tulokset: Vallitsevien bakteeriryhmien suhteelliset osuudet, anaerobisten bakteerien osuus, yksilöiden sisäinen sekä yksilöiden välinen lajivaihtelu ja mikrobiston stabiilius erosivat merkittävästi ohutsuolen ja ulosteen mikrobistojen välillä. β-laktamaasivalmiste ehkäisi ampisilliinin vaikutuksia: teho havaittiin merkittävinä eroina resistenssin esiintymisessä ja mikrobiston koostumuksessa ampisilliinia ja ampisilliiniä sekä β-laktamaasivalmistetta saaneiden ryhmien välillä.

Johtopäätökset: Tämä tutkimus osoittaa, että ulosteen melko stabiili ja runsas mikrobisto ei edusta ohutsuolen rajoittunutta, yksilöittäin, ajallisesti, määrällisesti ja laadullisesti vaihtelevaa mikrobistoa. β-laktamaasi on lupaava valmiste β-laktaamiantibioottien aiheuttamien epäsuotuisten vaikutusten ehkäisyyn.

Avainsanat: suolistomikrobisto, ohutsuoli, β-laktaamit, mikrobilääkeherkkyys

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Mentula S, Harmoinen J, Heikkilä M, Westermarck E, Rautio M, Huovinen P, Könönen E. Comparison between the cultured small intestinal and fecal microbiota in beagle dogs. Appl Environ Microbiol 2005, in press.

II Harmoinen J, Mentula S, Heikkilä M, van der Rest M, Rajala-Schultz PJ, Donskey CJ, Frias R, Koski P, Wickstrand N, Jousimies-Somer H, Westermarck E, Lindevall K. Orally administered targeted recombinant Beta-lactamase prevents ampicillin-induced selective pressure on the gut microbiota: a novel approach to reducing antimicrobial resistance. Antimicrob Agents Chemother 2004;48:75-79.

III Mentula S, Harmoinen J, Koski P, Westermarck E, Rautio M, Huovinen P, Könönen E. Inhibition of ampicillin-induced emergence of resistance in intestinal coliforms by targeted recombinant beta-lactamase. Int J Antimicrob Agents 2004;24:555-561.

IV Mentula S, Virtanen T, Kanervo-Nordström A, Harmoinen J, Rautio M, Huovinen P, Könönen E. Relatedness of *Escherichia coli* strains with different susceptibility patterns isolated from beagle dogs during ampicillin medication. (submitted for publication)

Author's contribution

Publication I

Silja Mentula (SM) was responsible for the microbiological experimental work and carried out identification of the strains, excluding 16S RNA gene sequencing. Collected and interpreted the results and wrote the paper.

Publication II

SM was responsible for the culture-based experimental work, including the selection of coliform isolates and collecting and modifying the susceptiblity data. Interpreted the culture-based results and wrote the paper together with the corresponding author.

Publication III

Responsibilities in experimental work were similar to publication II. SM analysed the resistance patterns, interpreted the results and wrote the paper.

Publication IV

SM was responsible for the microbiological experimental work, analysed and compared the typing data to susceptibility data, interpreted the results and wrote the paper.

INTRODUCTION

The intestinal microbiota is known to have a crucial impact on the health status of an individual. Microbes influence functions such as nutrition, development and maintenance of the immune system, and defence against pathogens. To understand the relationship between the intestinal microbiota and health, and the metabolic potential of the microbes, and to evaluate the effect of different diets, treatments or medical conditions on the microbiota, fundamental knowledge of the intestinal microbial populations is needed.

Because of the inaccessibility of the intestine, there are major difficulties in studying the intestinal microbiota in its natural habitat and most studies of the intestinal microbiota have been done using faecal samples. Due to low number of studies data on the differences between the microbiota of different parts of the gut are limited. The microbiota of the human upper gut and caecum has been reported to differ significantly from that of the colon and faeces (Gorbach *et al.* 1967, Justesen *et al.* 1984, Simon & Gorbach 1986, Marteau *et al.* 2001). However, the recoverability of small intestinal microbiota from faeces has not been fully analysed.

Bacterial composition, activities and concentrations can be severely distorted by outside agents such as antimicrobials. Exposure of gut microbiota to antimicrobials selects resistant bacteria that may enrich and spread within a given surrounding (Livermore 2003, Münnich & Lübke-Becker 2004, Drazenovich *et al.* 2004). Increasing rates of antimicrobial resistance following extensive use of antimicrobials has led to a worldwide interest in finding ways to inhibit the emergence of resistance.

The present study was done in order to make a detailed analysis on the canine jejunal microbiota and compare it with corresponding faecal microbiota in 22 beagle dogs with permanent jejunal fistula. The other aim of the study was to analyse the effect of i.v. ampicillin treatment on the intestinal microbiota, especially on the antimicrobial resistance of coliforms, and to analyse whether the adverse effects of ampicillin could be inhibited by simultaneous oral administration of β-lactamase, designed to degrade antibiotic residues in the gut. The analyses were performed using culture method and

various molecular tools (TGGE, quantitative PCR, PFGE). The ultimate aim of the study was to contribute to the better understanding of the composition and dynamics of the gut microbiota, and to the development of means to prevent antimicrobial resistance.

REVIEW OF THE LITERATURE

1. Gut microbiota in health and disease

1.1. The importance of microbes

Microorganisms colonise all mucosal surfaces forming a complex and dynamic entities. The intestine harbours the vast majority of the microbes of the body, and there the microbes also have the most prominent impact on our health. The gut microbiota can be described to be in a state of unstable stability with reference to the relatively stable composition of the host specific main bacterial groups and their proportions within an individual, and to the simultaneous constant flow of new bacterial clones co-existing and replacing the previous ones in a balanced and seemingly homologous living system (McCartney *et al.* 1996, Franks *et al.* 1998, Zoetendal *et al.* 1998, Falk *et al.* 1998). Gut microbiota can be regarded as a metabolically active organ of the body, beneficial to both microbes and the host. Compared to skin, the contracting gastrointestinal tract with villi and microvilli comprises 100-fold surface area (2 m^2 versus 150-200 m^2, respectively) providing a considerable amount of space for digestive and physiological processes (Van Dijk 1997, Waldeck 1990). The vital importance of the microbiota to the homeostasis of the body is obvious since the imbalance of the microbiota may cause several different disturbances of the gastrointestinal functions such as diarrhoea and impairs the health status of an individual.

Microbiota has crucial role in many physiological/biochemical and immunological functions of the body (Gordon & Pesti 1971, Berg 1996). The importance of the microbiota has been analysed by comparisons of germ-free (sterile) and conventional animals, most often mice, rats, or chickens. Individuals grown in a microbe-free (sterile) environment lack many features of the immune system, require 30% more calories to maintain body mass and die sooner than those grown in normal conditions (Gordon & Pesti 1971, Wostmann *et al.* 1983, Tanaka & Ishikawa 2004, Bäckhed *et al.* 2004). The features being altered by bacterial activity, differing between germfree and conventional mice, are collectively known as microflora associated characteristics (MACs) (Midtvedt 1989). In turn living microbes in the intestine are provided a niche

with abundance of nutrients and fermentable carbon sources originating from host secretions, diet, cast off epithelial cells and mucin. Additionally, the conditions are stable, warm and humid.

1.2. The effects of intestinal microbiota

To colonise different niches of the intestinal tract microbes have acquired distinct and appropriate adaptation strategies. The commensal and symbiotic (indigenous) microbes are those that are beneficial or at least not harmful to the host in contrast to the harmful pathogenic (transient) species. In addition to the immunopotentiation (maturation of the gut-associated lymphoid tissue, GALT) the commensal bacteria play a significant role in the nutrition by degrading otherwise non-digestible food compounds, producing vitamins and short-chain fatty acids, used as an energy source by the colonic mucosa, conversion of urobilin to urobilinogen, conversion of cholesterol to coprostanol, and in absorption of ions (Moore & Holdeman 1974, Mackowiak 1982, Simon & Gorbach 1986). The gut-associated lymphoid tissue (GALT) contains over 80% of all B-cells (antibody producing lymphocytes) of the body and more lymphoid cells than the spleen, peripheral lymph nodes and blood together (Brandtzaeg *et al.* 1989). The increasing frequency of atopic allergies has been linked to overly hygienic living conditions, referred to as the hygiene hypothesis, altering the colonisation patterns in infancy and leading to lack of tolerance to harmless food proteins and antigens (Wold 1998). Indigenous bacteria also stimulate vascularisation and development of intestinal villi, have an important role in the metabolism of endogenous and exogenous compounds (detoxification), and in prevention of colonisation and proliferation of pathogens and opportunistic microbes (Hooper *et al.* 2002, Stappenbeck *et al.* 2002). Term colonisation resistance was introduced by van der Waaij *et al.* (1971). It provides non-specific defence against infections and includes various bacterial mechanisms such as competitive exclusion (competition of living/adhesion space and nutrients) and production of substances harmful to pathogens (antimicrobial agents, bacteriocins) as well as anatomical and physiological features of the host (mucosa, secretions, gastrointestinal motility). Most effects of bacteria against other bacteria are strictly local since e.g. bacteriocins are degradable by host digestive enzymes (Guarner & Malagelada 2003).

1.3. Dysfunctions of the gut microbiota in humans

Clinical disturbances

Whether the causes for dysfunctions in the intestine are of physiological or exogenous origin, they are often associated with gastrointestinal tract infections with altered bacterial composition, bowel movements, and/or water and ion balance. The clinical terms for gastrointestinal tract infections are gastroenteritis, diarrhoea, dysentery and enterocolitis, each with distinctive set of clinical manifestations (Mims *et al.* 1998).

One of the most common type of chronic gut dysfunction, the inflammatory bowel disease (IBD), has been linked to loss of tolerance to commensal bacteria. Intestinal bacteria may be involved in maintaining the inflammation reaction in IBD (e.g. ulcerative colitis, Crohn's disease) since patients have increased mucosal secretion of IgG antibodies against several commensal bacteria and have higher amounts of bacteria attached to their epithelial surfaces compared with healthy people (Macpherson *et al.* 1996, Swidsinski *et al.* 2002).

Another serious clinical condition possibly linked to disturbances in gut microbiota is colon cancer. By production of potentially toxic or carcinogenic substances such as amines, phenols and indols, intestinal bacteria are most likely one of the factors modulating the risk of initiation of colon cancer (Smith & Macfarlane 1996, Macfarlane & Macfarlane 1997). Increase in the incidence and growth rate of colonic tumors have been shown to be associated to some *Bacteroides* and *Clostridium* species while probiotic bifidobacteria bacteria prevent tumorigenesis in rats and mice (Onoue *et al.* 1997, Horie *et al.* 1999, Singh *et al.* 1997). Similarly, the presence of *Bacteroides vulgatus* and *Bacteroides stercoris* has been connected with a high risk of colon cancer whereas *Lactobacillus acidophilus, Lactobacillus* S06 and *Eubacterium aerofaciens* (currently *Collinsella*) associate with a low risk in humans (Moore & Moore 1995). Short-chain fatty acids produced by bacterial fermentation have been associated with reduction in the incidence of colon cancer and IBD (Wachtershauser & Stein 2000, Blottiere *et al.* 2003, Miyauchi *et al.* 2004).

Small intestinal bacterial overgrowth

In small intestinal bacterial overgrowth (SIBO), also called the contaminated small bowel syndrome, total bacterial counts exceed those of healthy subjects by 1-2 log difference, usually crossing the limit of 10^5 cfu/g (Finegold & George 1989, Stotzer *et al.* 1998, Corazza *et al.* 1990). Patients with SIBO may have no symptoms and no predisposing factors, or may suffer from clinical manifestations such as malabsorption of lipids, amino acids and carbohydrates, vitamin B_{12} deficiency, and disturbance in water and electrolyte transport in enterocytes (Gracey 1983). The causes and pathogenesis of the SIBO are poorly understood but several factors promoting manifestations have been identified. They include pathological abnormalities of the intestinal tract, stagnation of bowel contents, decreased gastric acidity, antimicrobial therapy and impaired immune system (Finegold & George 1989, Gracey 1983).

SIBO, as well as increased permeability of the intestinal mucosal barrier and deficiencies in host immune defences (e.g. mucin and secretory IgA production) are thought to promote bacterial translocation, i.e. the crossing of bacteria through the host epithelial mucosa (Berg 1999, Macpherson *et al.* 2000). Bacterial translocation enables bacteria to transfer within the body causing local infections, sepsis, shock, multisystem organ failure, or death of the host (Guarnier & Malagelada 2003). Delayed small intestinal transit times, possibly promoting small intestinal overgrowth, has been recorded in several conditions with high incidences of bacterial translocation such as obstructive pancreatitis, hepatic failure, and portal vein obstruction (Moody *et al.* 1995, Wang *et al.* 1994, Yi *et al.* 1999).

2. Composition of the human gut microbiota

2.1. General features

The mammal gut microbiota is composed of up to 1000 bacterial species with 30-40 species comprising the majority (99%) at a time, and with a particular individual combination of predominant species and unique strains distinct from other individuals (Savage *et al.* 1968, Savage 1977, Lee *et al.* 1971, Moore & Holdeman 1974, Xu & Gordon 2003).

The stability of the microbiota is challenged by gastric, pancreatic, and biliary acid secretions, peristaltic activity and continuous turnover of the epithelium and the mucus layer. On the other hand, peristaltic movement of the gut induces the exposure of different segments of the gut to the varied components of the intestinal contents ensuring the continuous beneficial interaction of microbiota, dietary molecules, epithelium and gut-associated immune system (Falk *et al.* 1998). The most numerous bacteria in the gut are obligatory anaerobic species accompanied by smaller amounts of aerobic/facultative bacteria with aerobes comprising only 1:100 - 1:1000 of the total count in the intestinal lumen (Finegold *et al.* 1983, Berg 1996). However, the proportion of aerobic species varies between the different regions of the intestinal tract.

Table 1. Microbial densities in different parts of the digestive tract (Simon & Gorbach 1984, Berg 1999, Marteau *et al.* 2001).

Site	**Bacterial count (cfu/ml, cfu/g)**
Oropharynx	10^{8-11}
Stomach	$0\text{-}10^{4}$
Small intestine	
Duodenum	$0\text{-}10^{4}$
Jejunum	$0\text{-}10^{5}$
Ileum	10^{4-8}
Caecum	10^{6-8}
Colon	10^{10-12}

Microbial densities are low in the stomach, and in the proximal and middle small intestine (duodenum, jejunum) because of the low pH and peristalsis (Table 1). The counts and diversity of the microbiota increase towards the distal small intestine (ileum) and colon (Savage 1977, Berg 1996). The small intestinal transit time is only 2-4 h compared with up to 60 h in the colon (Holzapfel *et al.* 1998, Drasar & Mill 1974). In the colon pH is higher than in the upper small intestine, the turnover is slower, redox potential is lower, short-chain fatty acid concentration is higher and the bacterial load is higher, indicating unique conditions in each gut compartment (Mackie *et al.* 1999). There is a shift in the biochemical atmosphere also within the colon between proximal colon with intense fermentation, rapid bacterial growth and pH of 5-6 and distal colon with lower fermentable substrate availability, putrefactive

processes, slow bacterial growth and neutral pH (Cummings & Macfarlane 1991, Tannock 1999a, Guarner & Malagelada 2003).

The conditions in the lumen differ also from those proximal to the mucosa. The gut can be divided crosswise into four different microhabitats: lumen, mucus layer, deep mucus layer and mucosal epithelial cells (Berg 1996). These microhabitats provide separate niches with unique properties. The predominant bacterial species associated to the jejunal, ileal and colonic mucosa differ significantly from the respective jejunal or ileal lumen and faecal bacterial community, and additionally bacterial counts between separate mucosal sites vary considerably (Bhat *et al.* 1980, Zoetendal *et al.* 2002, Wang *et al.* 2003). The mucosa-associated bacteria in colon and rectum are fairly similar in total counts and species composition (Poxton *et al.* 1997). The analysis of the mucosal microbiota is complicated by the undefined effects on the bacteria caused by the evacuation of the intestine preceding the biopsy collection and the possible washing procedures at sampling. A very limited number of studies has focused on the differences between the bacterial composition and activities in the different gut compartments.

2.2. Species composition

The composition of the microbiota is known to be affected by several factors concerning the host such as age, diet, medication, health status, microbes in the environment and stress, as well as factors concerning the microbes such as growth rate, nutritional and adherence characteristics and microbial interactions (Holzapfel *et al.* 1998, Mitsuoka 1992). In addition, also host genetics have been reported to have significant correlations between bacterial communities (van der Merwe *et al.* 1993, Toivanen *et al.* 2001, Zoetendal *et al.* 2001). Once established, the microbiota remains relatively constant over time in adults (Finegold *et al.* 1983). Community shifts within the microbiota occur throughout the lifespan, especially in infants and in elderly people (Mitsuoka 1992, Hopkins *et al.* 2001, Favier *et al.* 2002).

As the composition of the microbiota is influenced by external factors such as acquisition of microbes in the diet, manipulating the microbiota from outside in order to improve the health of the host has drawn much attention (Mackie *et al.* 1999). The

concept of bacterial therapy using microbial cocktails has been introduced and it has shown to be a promising method to treat and prevent various respiratory and gastrointestinal tract infections (Huovinen 2001).

The cultivable dominating fecal bacteria belong to genera *Bacteroides, Bifidobacterium, Eubacterium* and related genera, *Clostridium, Peptococcus, Peptostreptococcus* and related genera, *Fusobacterium* and *Ruminococcus*, whereas the subdominant family and genera comprise of facultative anaerobes (aerobes) such as *Enterobacteriacae, Streptococcus, Enterococcus, Lactobacillus* and *Proteus* (Table 2) (Simon & Gorbach 1984, Tannock 1999c). Some of these genera, especially *Eubacterium*, anaerobic gram-positive cocci and *Clostridium* have gone through extensive taxonomic revisions on the basis of new molecular methods (DNA-DNA hybridisation, 16S rRNA sequencing) that are replacing phenotypic methods as means to classify microbes. Molecular methods have also provided new aspects to the quantitative relations between different bacteria by determining proportions of group specific rRNA of total rRNA of a microbiota (Table 3). Attempts to combine culture-based data with molecular methods-based data has not yet provided us an accurate picture of the numerically predominant active species in the gut. Based on the partial sequencing of 16S rRNA, most of the bacteria found in the human colon fall into four phylogenetic clusters consisting of (1) *Bacteroides*, (2) *Bifidobacterium* and other gram-positive bacteria with high G+C content, (3) *Clostridium coccoides* and relatives (*Coprococcus, Eubacterium, Lachnospira, Ruminococcus*), and (4) *Clostridium leptum* and relatives, fusobacteria, and the *Atopobium* group (Wilson *et al.* 1997).

Table 2. Examples of common cultivable inhabitants of the adult human intestine (Moore & Holdeman 1974, Holdeman *et al.* 1976, Finegold et al. 1983, Sneath *et al.* 1986, Murray *et al.* 2003).

Genus / Group	Common representatives
Anaerobic	
Bacteroides	*B. fragilis* group, *B. splanchnicus*
Clostridium[a]	*C. perfringens, C. sordellii, C. bifermentans, C. innocuum*
Bifidobacterium	*B. adolescentis, B. longum B. infantis, B. breve*
Eubacterium[b]	*E. rectale, Eggerthella lenta, Collinsella aerofaciens, Mogibacterium timidum*
Gram-positive cocci[c]	*P. anaerobius, P. micros, P. asaccharolyticus, Ruminococcus*
Gram-negative cocci	*Veillonella parvula, Acidaminococcus fermentans, Megasphaera elsdenii*
Fusobacterium	*F. necrophorum, Faecalibacterium prauznitzii*[d]
Facultative/ aerobic	
Lactobacillus	*L. acidophilus, L. casei, L. rhamnosus*
Enterobacteriacae	*Escherichia coli, Klebsiella, Enterobacter*
Streptococcus	*S. bovis, S. anginosus, S. salivarius*
Enterococcus	*E. faecalis, E. faecium*
Corynebacteria	*Corynebacterium, Arcanobacterium, Actinomyces, Rothia*
Yeasts	*Candida albicans, Saccharomyces, Geotrichum*

[a] Divided into phylogenetic clusters (*C. coccoides, C. leptum*)
[b] Genera *Atopobium, Collinsella, Eggerthella, Mogibacterium, Slackia* have been reclassified from *Eubacterium*.
[c] Anaerobic gram-positive cocci includes genera *Anaerococcus, Coprococcus, Finegoldia, Micromonas, Peptococcus, Peptoniphilus, Peptostreptococcus, Ruminococcus*.
[d] Formerly *Fusobacterium prausnitzii*

Table 3. Mean proportions of different bacterial groups of the total microbial population in human feces detected by 16S rRNA hybridisation with group specific probes.

Bacterial group (probes)	Matsuki *et al.* 2002	Sghir *et al.* 2000	Rigottier-Gois *et al.* 2003	Franks *et al.* 1998
Bacteroides (Bacto1080, Bfa602, Bdis656, Bact338)	39%	37%	42%	20%
Clostridium coccoides (Erec482)	22%	14%	22%	29%
Clostridium leptum (Clept1240)	8%	16%		
Low GC (Strc493, Lacto722)		1%		12%
Bifidobacterium (Bif228, Bif164, Bif1412)	7%	<1%	3%	3%
Enterobacteria (Enter 1432)		<1%	1%	
Prevotella (Prevo0803)	6%			
Faecalibacterium prausnitzii (Fprau645)			9%	

2.3. Upper gut microbiota

In contrast to the heavily and steadily colonised lower regions of the intestinal tract, stomach and upper small intestine (duodenum, jejunum) harbour microbes in more time dependent manner. Both are practically free of cultivable living microorganisms when they are empty but harbour small numbers (10^{1-3} cfu/ml) of organisms at least transiently with highest numbers shortly after eating. The sparse microbiota in the upper small intestine is thought to consist mostly of oropharyngeal origin with major components being gram-positive acid tolerant lactobacilli, streptococci, fungi and staphylococci, and a minority of gram-variable veillonella, fusiforms, clostridia, actinomycetes, corynebacteria and haemophilus (Gorbach *et al.* 1967, Justesen *et al.* 1984, Berg 1996, Sullivan *et al*. 2003). The number of bacteria attached to the epithelia is also lower and consists of more gram-positive organisms compared to large intestine (Plaut *et al.* 1967, Guarner & Malagelada 2003). Whether the bacteria

found are only transient passengers or represent true colonisers of the upper small intestine is unclear.

In the distal small intestine (terminal ileum) the counts (10^{4-8} cfu/ml) and diversity, including appearance of substantial numbers of coliforms and bacteroides, increase drastically (Drasar & Mill 1975, Hentges 1993). The predominantly gram-positive bacteria of the upper small intestine shifts to gram-negative predominance towards the colon (Gorbach *et al.* 1967). In addition to declining acidity, the microbiota in the ileum is affected also by reflux of caecal contents (Gorbach *et al.* 1967, Nord & Kager 1984). The small intestine has been thought to harbour a distinct and characteristic microbiota but the composition of it has not been fully described. Ileal samples obtained by intubations and small intestinal samples obtained by needle aspiration grew total counts of 10^{3-6} cfu/ml, with *Bacteroides* and anaerobic gram-positive rods such as *Bifidobacterium* dominating over enterococci and coliforms (Gorbach *et al.* 1967, Finegold *et al.* 1983). In the caecum aerobic species such as *Escherichia coli*, enterococci, and lactobacilli, comprise a significantly larger part of the total bacterial count (10^{8} cfu/ml), covering 50% of total bacterial rRNA, compared to faeces where aerobes cover 7% of the total count (10^{10} cfu/ml), respectively (Marteau *et al.* 2001).

2.4. Opportunistic commensals

Opportunistic commensals are the bacteria that are frequently present as permanent members of the microbiota but under favourable circumstances may proliferate vigorously and turn pathogenic. Although the vast majority of the normal microbiota consists of anaerobic bacteria most endogenous infections are caused by aerobes such as enterobacteria and enterococci (Vollaard & Clasener 1994). Common anaerobic genera such as *Bacteroides* and *Clostridium* include species with known pathogenicity; *B. fragilis* is the most frequent clinical isolate (Namavar *et al.* 1989) and *C. difficile* can exist as a harmless commensal in infants but cause disease especially in hospitalised elderly (Stark *et al.* 1982, Poxton 2005). Opportunistic bacteria may produce toxic metabolites causing disorders when accumulated. Some bacteria and fungi, e.g. species of *Neisseria, Bacteroides, Streptococcus* and *Candida* have the ability to change surface antigenicity in order to evade host immune responses (Deitsch *et al.* 1997, Kuwahara *et al.* 2004). Bacteria that are seldom

associated with infections such as *Bifidobacterium, Eubacterium* and *Lactobacillus* species can also occasionally cause serious illnesses (Brook & Frazier 1993).

Coliforms

One of the predominating bacterial groups in the human and animal intestine is coliforms, i.e. gram-negative aerobic fermentative rods, with *E. coli* being the most common and most studied. It is also considered an indicator of faecal contamination in food and water. Although *E. coli* may not cover more than a fraction (<1%) of the total count of the intestinal bacteria, it is significant as a pathogen being one of the main causes of nosocomial infections, main causative of urinary tract infections and an important cause of diarrhoea (Eisenstein & Zaleznik 2000, Whittam *et al.* 1993, Maslow *et al.* 1995). *E. coli* has acquired several pathogenic mechanisms by which diarrhoeagenic strains can be divided into four distinct groups: enterotoxigenic (ETEC), enteroinvasive (EIEC), enterohaemorrhagic (EHEC), and enteropathogenic (EPEC) *E. coli* (Mims *et al.* 1998). On the other hand, non-pathogenic *E. coli* may contribute to the preservation of colonisation resistance and has been given as bacterial probiotic therapy to patients with ulcerative colitis and in prevention of colonisation of antibiotic-resistant *E. coli* strains in premature babies and in prevention of neonatal calf diarrhea (Rastegar *et al.* 1990, Rembacken *et al.* 1999, Kruis *et al.* 2004, von Buenau 2005).

Fungi

Fungi, most often yeasts, are normally present in small amounts in the intestine and faeces of mammals. In immunodeficient patients or when bacteria are suppressed by antimicrobials, yeasts (*Candida*, *Cryptococcus* spp.) may overgrow the intestine and act as pathogens causing diarrhoea, abdominal cramps and infections in other body sites (Krause *et al.* 2001, Tortorano *et al.* 2004). Yeasts can also be probiotic. In treating antibiotic associated diarrhoea *Saccharomyces boulardii* serine protease has been demonstrated to inhibit the pathogenic effects of *C. difficile* toxins A and B on human colonic mucosa (Castagliuolo *et al.* 1999).

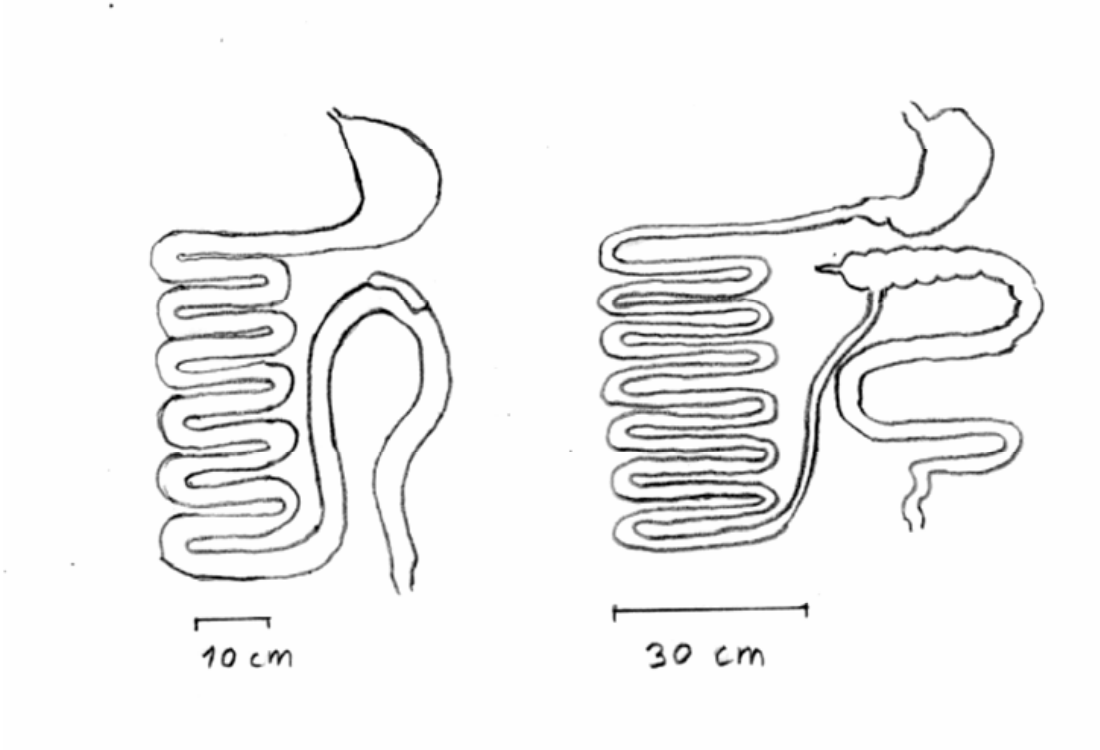
3. The digestive tract and intestinal microbiota of dog

The development of microbiota begins at birth when the sterile foetus is colonised in the birth canal and by the immediate environment. The bacterial succession is similar in the human and canine intestine with the very first colonisers originating from the mother, followed by microbes benefiting from breast-feeding and then drastically changing towards obligate anaerobes and greater diversity as solid foods are introduced (Benno *et al.* 1992a, Mackie *et al.* 1999). The most numerous colonisers in beagles during the first year are bacteroides, eubacteria, bifidobacteria, lactobacilli and anaerobic cocci while clostridia and streptococci increase later (Benno *et al.* 1992a).

Since dogs are carnivorous the total length of their intestine in relation to the body length is somewhat shorter and the motility slower than in humans (Figure 1). As a whole the digestive tract of dog, however, resembles that of humans and the physiology is similar in many ways. Like humans dogs utilise intestinal microbiota in their physiology and both are homothermic mammals. The pH values in the different compartments of the digestive tract are also comparable to those of human: the pH in the dog stomach is 3, in duodenum and jejunum 6, in ileum 7.5, in colon 6.5 and in feces 6.2 (Smith 1965).

The main cultivable bacterial groups and most common findings in humans and dogs are the same including clostridia, bacteroides, streptococci, coliforms, enterococci, lactobacilli and veillonellae with increasing counts towards the large intestine (Smith 1965, Davis *et al.* 1977, Benno *et al.* 1992b, Greetham *et al.* 2002, Buddington 2003, Rinkinen *et al.* 2004). Most intestinal pathogens cause similar clinical symptoms in dogs as in humans (*C. difficile*, multiresistant gram-negatives, enterococci, staphylococci). Due to the ability of microbes to adapt and specify to niches with unique characteristics there are differences between the bacterial species level composition in different mammals. The most common intestinal dysfunction in dogs is diarrhoea. Another common disturbance, small intestinal bacterial overgrowth, composing of *E. coli*, enterococci and clostridia is associated with raised serum folate, reduced serum vitamin B12 concentrations and altered mucosal permeability and function (Batt *et al.* 1983, Rutgers *et al.* 1996).

Figure 1. Gastrointestinal tract of dog and human including stomach, small intestine, caecum and large intestine (Adopted from Stevens & Hume 1998).



Dog
(*Canis familiaris*)
Body length 90 cm

Human
(*Homo sapiens*)
Body length 180 cm

4. Obtaining samples from the intestine

4.1. Getting representative samples from the upper gut

Studies of microbiota *in vivo* carry some limitating factors associated to the complexity of the ecosystem leading to difficulties in identifying and determining the functions of all members or subpopulations. These include the unknown dynamic interactions within the microbiota and between microbes and host, inherent differences in the microbiota between study subjects, natural variability of the microbial strain composition with time, bacterial contamination during intervention trials between controls or staff and study group, and differences in human and experiment animal microbiota/physiology (Corpet 1987, Zoetendal *et al.* 2004).

Apart from general difficulties in analysing complex microbial populations, the difficulty in studying the gut microbiota lies in the inaccessibility of the microbiota in its natural habitat. Samples from intestinal contents from living healthy subjects obtained without anaesthesia and fasting, preoperative antibiotic prophylaxis, or laxatives/purgatives are simply not available. After any of these procedures the microbiota is no more in its natural state and may have gone through vast modification, and the sample size may also remain minimal. In addition, anaesthesia slows down the peristaltis, which makes backflow possible, and subjects studied at surgery have gastrointestinal dysfunctions and do not represent healthy individuals (Borriello *et al.* 1978). Attempts to get samples from the intestine without surgery include self-closing enterocapsules, tubes, endoscopes and, in animals, cannulation (Hirtzmann & Reuter 1963, Shiner et al 1963, Gracey 1977, Hill 1996, Stotzer 1998). Capsules that pass through the intestine or tube that remains in place allow bacteria to multiply before the sample is processed. Invasive techniques going through other segments of the digestive tract carry the risk of contamination on the way. Tubes may also alter the intestinal motility and the position of the tube may be difficult to determine (Donaldson 1964).

Thus most analyses of the intestinal contents or biopsies are from the caecum or colon and originate from diseased persons (Gillian *et al.* 1992), sudden death victims (Macfarlane 1992) or are focused on certain probiotics (Alander *et al.* 1997, Alander

et al. 1999, Johansson *et al.* 1993) or sulphate reducers (Zinkevich *et al.* 2000). Very few studies have analysed the small intestinal microbiota of healthy subjects, and limited data are thus available (Marteau *et al.* 2001).

Because of these difficulties in obtaining proper samples from different parts of the intestinal tract, especially from the upper gut, and ease in collecting faecal samples, faeces is often the chosen sample type in studies of the gut microbiota. However, the faecal microbiota represents merely the distal colon, not even the proximal colon or terminal ileum, and may not represent the upper intestinal microbiota or the metabolic activities of it. Moreover, faecal predominant species tend to overgrow subdominant bacteria and the selectivity of selective agar media may be poor which makes it very difficult to gain isolates for further identification and to characterise the true bacterial diversity in faecal samples. Molecular methods with higher sensitivity can be used to detect the subdominant microbes but for further characterisation bacterial isolates should be obtained by culture.

4.2. Jejunal fistula

To overcome the inaccessibility of the intestine, a dog fistula model was designed to gain an easy access to jejunal chyme without causing pain to the animal or disturbing the normal functions of the gut (Wilsson-Rahmberg & Johnsson 1997, Harmoinen *et al.* 2001). The fistula is created using a 25 cm segment of the dog's own intestine by intussusception. The free ends of the intestine are connected by end-to-end anastomosis, and the isolated intestinal segment is inserted by end-to-side anastomosis to the intestine extending through abdominal cavity to connect the intestine to the skin with proximal end of the segment facing the skin. Small intestinal content (jejunal chyme) can be collected pre- or post-feeding without aspiration by inserting a silicon tube through the nipple valve into the intestine. A nipple valve can be surgically inserted to desired site at the small intestine. The closer the fistula is to the pylorus the more running (liquid) the content is (Harmoinen 2004). Most productive samples are obtained shortly after feeding (1-4 h) depending on the site of the fistula. Small intestinal samples from permanent fistula operated dogs can be obtained without any sedation/anaesthesia while the dog is fully active and standing. The fistula operation is reported not to have any significant effect on the intestinal motility, measured as

transit percentage of barium-impregnated polyethylene spheres, or on the cultivable microbiota (Harmoinen *et al.* 2001).

5. Analysing the microbiota

5.1. Microscopic methods

Microscopic methods can be valuable in analysing bacterial numbers (Kepner & Pratt 1994). However, microscopic counts do not necessarily correlate with the plate count. Plate count detects only reproducible cells whereas with microscopy it is possible to enumerate dead and/or viable cells depending on a stain applied (Breeuwer & Abee 2000). On the other hand, the clumping of cells and loss of cells after fixation during staining may distort microscopic counts. Often microscopy is combined with fluorescent in situ hybridisation (FISH), which enables the identification and enumeration of selected bacteria (Amann *et al.* 1995).

5.2. Culture method

Traditionally culture method has been regarded as the golden standard for studying bacteria. It is notable that it is the only way to get bacterial isolates for further studies. The focus of the analysis of the intestinal microbiota has been on the enumeration and identification of numerically predominant cultivable faecal species (Holdeman *et al.* 1976, Finegold *et al.* 1983, Moore & Moore 1995). However, up to 60-80% of the mammal microbiota cannot be cultured and has not been characterised and identified (Moore & Holdeman 1974, Wilson & Blitchington 1996, Suau *et al.* 1999). The cultivable fraction is, however, relatively high compared to most other microbial ecosystems such as environmental biofilms and activated-sludge flocks (Amann *et al.* 1995). Uncultured microbes fail to grow on artificial agar media used in the culture as their suitable growth conditions, i.e. combination of nutrients, media, atmosphere, or necessary interactions with the host cells or other microorganisms, are not known or the cells are stressed or nonviable. In addition, the selective culture media used are either not selective enough or are too selective supporting the growth of only part of the microbes, and some microbes prevent the growth of others, and some are misidentified (Nelson & George 1995, Hartemink & Rombouts 1999). Thus the

culture result may not always give a truly representative picture of the microbiota studied. Culture method is also regarded as time-consuming and laborious. Despite the limitations, culture methods have successfully provided us the basic knowledge of clinically important microbes and intestinal ecosystem, and culture remains an indispensable tool for clinical microbiology.

The identification of isolated bacteria has traditionally relied on morphological, physiological and biochemical characteristics. This phenotypic characterisation based on cellular and colonial morphology, growth requirements and characteristics, fermentation profiles etc. has weaknesses connected to the poor reproducibility, ambiguity because of plasticity of bacterial growth and poor discriminatory power (McCartney 2002). Likewise, molecular-based genetic characterisation carries limitations. Thus there is often need to combine phenotypic with genotypic characterisation (polyphenic approach).

5.3. Molecular biological methods

Detection and identification

Alternatively, culture-independent molecular methods have been introduced and notable amounts of DNA originating from uncultivable bacteria have been extracted, analysed and classified. Molecular methods are recognised as a standard phylogenetic classification tool (Stackebrandt *et al.* 1994). They may offer more rapid and reliable identification than culture because the identification is based on the composition of nucleic acids rather than on the genomic expression under given culture conditions, and the DNA can originate from living or dead cells (Nissen & Dainty 1995, Tannock *et al.* 2004). The applicability of molecular methods ranges from identification or detection of single bacterial species to characterisation of compex microbiotas, and molecular techniques have been applied in analysing evolutionary relatedness of different bacteria, in bacterial diversity studies, enumeration of bacterial groups, especially of extremely fastidious organisms, monitoring specific bacterial populations, and identification of bacterial isolates (McCartney 2002).

Molecular methods are based mainly on detection of ribosomal RNA (rRNA) or ribosomal DNA (rDNA; DNA encoding the rRNA). The probes for target DNA

sequences can be designed for detecting bacteria on different phylogenetic levels from major genera or the group level to the species or even strain-specific level depending on the type of the study ranging from gut ecology studies to tracking specific probiotics or pathogens (Charteris *et al.* 1997, Franks *et al.* 1998). Highly conserved regions of ribosome can be used for designing universal probes and different variable regions for specific/targeted probes. Several thousands of 16S rDNA sequences including numerous uncultured bacteria are freely available in genomic databanks. The target DNA can be detected using various PCR-based methods or dot blot hybridisation with specific synthetic oligonucleotide probes or using fixed bacterial cells by fluorescent in situ hybridisation (FISH) combined with flow cytometry or microscopic analysis (Amann *et al.* 1995, Wang *et al.* 1996, Wilson & Blitchington 1996, Lin *et al.* 1997). Quantitative PCR allows quantification of all DNA fragments detected by PCR using specific controls of known quantity giving an estimate of the number of target microorganisms in the sample (Doungudomdacha *et al.* 2001, Sanz *et al*. 2004). Using multiplex PCR several target regions can be multiplied in single reaction with all necessary primers (García *et al*. 1998).

Population fingerprinting

In general, fingerprinting techniques for studying bacterial communities within ecosystem are based on PCR and generate profiles representing the sequence diversity within the population (Zoetendal *et al.* 2004). Molecular fingerprinting techniques allow rapid assessment of the predominant bacterial species present in a sample and simultaneous analysis of multiple samples, and is well suited to studying changes in individual microbial communities over time (Suchodolski *et al*. 2004). Studies using rRNA are thought to reflect also the metabolic activity of bacteria; in hydridisation the ribosomal number is thought to be comparable to the number of active cells (Felske *et al.* 1997, Rigottier-Gois *et al.* 2003). TGGE (temperature gradient gel electrophoresis) or DGGE (denaturing gradient gel electrophoresis) can be used for creating bacterial profiles that can be compared using similarity percentages and dendrograms (Satokari *et al.* 2003, Muyzer *et al.* 1993). TGGE has high sensitivity for detecting sequence differences (Muyzer *et al.* 1993). The composition of individual rDNA genes can also be sequenced and analysed using cloning libraries (Suau *et al.* 1999) or profiled using probing grids, checkerboard hybridisation (O'Sullivan 1999) or DNA microarrays

(Wilson *et al.* 2002). Sequencing of single PCR clones is a laborious and expensive procedure but allows immediate discrimination between bacterial species.

Like culture, molecular methods carry limitations such as PCR biases, laborious processing at the species level, requirements for a clone library or sequence information, semi-quantitative results, and expensive machinery and reagents (Zoetendal *et al.* 2004). The biggest challenge in molecular methods lies in the quantification of different microbial groups and determining their functionality, activity and dynamics within the diverse microbial populations (Sghir *et al.* 2000, McCartney 2002).

5.4. Molecular typing methods

Molecular methods are widely used for typing of cultured isolates. The most common molecular typing methods are plasmid profiling, pulsed-field electrophoresis (PFGE), multilocus enzyme electrophoresis (MLEE), ribotyping and random amplification of polymorphic DNA (RAPD-PCR, AP-PCR) (O'Sullivan 2000, Tannock 2001). These typing methods are applicable to numerous bacterial genera and can be used to distinguish bacterial isolates and to determine their relatedness.

For epidemiologically related organisms PFGE has been proven a powerful discriminating tool, especially in food-born outbreaks and nosocomial infections (Grif *et al.* 1998, Barrett *et al.* 1994, Diekema *et al.* 1997). PFGE has high discriminatory power and can differentiate e.g. between subtypes of O157:H7 associated with specific outbreak investigations, though for large-scale screening of O157:H7 isolates other fingerprinting methods such as repetitive extragenic palindromes (REP) and Box primers (rep-PCR), amplified fragment length polymorphism (AFLP) and ribotyping techniques are also applicable and less time consuming (Hahm *et al*. 2003). In PFGE the bacterial DNA is digested with restriction enzyme(s), the obtained fragments are separated electrophoretically and discrete banding patterns for each isolate are analysed. Strains typed with PFGE using standardised methodology can be compared internationally using clone libraries. The determination of relatedness of non-related organisms with PFGE is more tricky and cannot use the same criteria as for related organisms, since the relationship between restriction fragment banding

pattern and true genetic relatedness is poorly understood. In addition, PFGE includes always subjective decisions such as the selection of restriction enzyme(s), reading the restriction fragment banding pattern and choosing methods for analysing the data, and the interpretation of the results (Davis *et al.* 2003, Singer *et al.* 2004). Distracting variabilities in the patterns brought by transferable large plasmids can be eliminated by excluding fragments smaller than 160 kb from the analysis (Guyot *et al.* 1999).

5.5. Indirect methods for analysing microbiota

The microbiota, including cultivable and uncultivable species, can also be studied by indirect methods such as cellular fatty acid analysis (Peltonen *et al.* 1992), gas production (El Oufir *et al.* 1996), fermentation end product analysis (Topping & Clifton 2001) and enzyme activity profiling (Tannock 1999b, Gorbach & Bengt 1986). Analysis of microbial biochemical activities gives an insight to the functionality of the microbiota without having to go through culture, isolation and identification steps. The *in vitro* methods, such as continuous flow cultures or batch fermentations, may not always be predictive of the actual *in vivo* situation, since the complex and dynamic interactions between the gut and the microbiota are difficult to mimic. The analysis of microbiota using bacterial metabolites is also complicated by their unstability as they are used by other microbes or absorbed.

6. Antimicrobial agents

6.1. Classification of antimicrobial agents

Infectious diseases can be controlled by treating a disease with drugs (antimicrobial therapy), or by preventing it by vaccines (immunisation) and healthy environment (sanitation, hygiene, nutrition). The most important drugs for treating microbial diseases are antimicrobial agents, representing one of the great triumphs of modern medicine. Antimicrobial agents are chemical substances produced by microorganisms which are active against other microorganisms. In prokaryotes cell wall synthesis, protein synthesis (bacterial ribosome), nucleic acid synthesis and cell membrane function are sufficiently different from those in human eukaryotic cells so that they can be targets for antibacterial agents without inhibition of the equivalent mammalian cell target (Mims *et al.* 1998). Many antimicrobial agents are natural microbial metabolic products or derivates of them, chemically modified to improve their properties. There are also totally synthetic antimicrobial agents. Some bacterial species are inherently resistant to some classes of antimicrobials because they lack a susceptible target, they are impermeable to the agent or they produce inactivating enzyme (Brock *et al.* 2003). Antimicrobial agents can be divided on the basis of the target site, chemical structure or function (Table 3).

6.2. Emergence of antimicrobial resistance

Antimicrobial resistance is a worldwide problem and numerous strategies to avoid the emergence of resistance have been designed. New antimicrobials are being introduced but as most of them are similar in structure to the previous ones, the emergence of resistance is often only a matter of time. Despite of the development of a wide range of different types of antimicrobial agents, extremely adaptable bacteria seem to develop resistance to each new agent that comes along. Use of antimicrobials always causes selective pressure leading to reduction in efficacy, and the huge overuse induces bacteria to acquire resistance faster than expected (Livermore 2003). Inappropriate use of antimicrobials brings potential risk to the patient as risks of toxicity and opportunistic infection or superinfection, and to the community by selection of resistant organisms and increased health care costs. Both appropriate use

Table 3. Classification of antimicrobial agents on the basis of the target of action (Mims *et al.* 1998).

Inhibition of cell wall synthesis
β-Lactams (ampicillin, cefotaxime, imipenem, aztreonam, cloxacillin)
Glycopeptides (vancomycin, teikoplanin)
Inhibitors of protein synthesis
Aminoglycosides (gentamicin, streptomycin, kanamycin, neomycin, amikacin)
Tetracyclines (tetracycline, doxycycline)
Phenicols (chloramphenicol)
Macrolides (erythromycin)
Lincosamides (clindamycin, lincomycin)
Streptogramins
Fusidic acid
Inhibitors of nucleic acid synthesis
Sulfonamides
Trimethoprim
Quinolones (ciprofloxacin, norfloxacin, nalidixic acid)
Rifamycin (rifampicin)
Agents that affect DNA
Nitroimidazoles (metronidazole)
Inhibitors of cytoplasmic membrane function
Polymyxins (colistin)

of antimicrobials, including reduction in prescribing, and specific means to inhibit the emergence of resistance have been confronted. In addition to the mere use of antimicrobials the dissemination of resistance is connected to modern phenomena like large hospitals, institutional care of elderly, socialised care of children and youth in large units, and increasing travel (Holloway 2000, Sharma *et al.* 2005).

There is still a lot to learn about the dynamics in the emergence, spread, and maintenance of resistance. It remains unclear why some bacteria, called hyper-mutators, develop resistance very rapidly via mutation or DNA transfer while others remain susceptible, and why some resistant bacteria achieve epidemic spread while others do not. Potential factors in epidemic success include changes in bacterial properties such as adherence, desiccation and disinfectant tolerance, increased growth rate and adaptation to the fitness cost of resistance (Livermore 2003). When acquiring resistance by genomic alteration bacteria may lose some other character that may be

valuable when the antimicrobial selective pressure is absent. This burden or losing some feature is called fitness cost. Resistance mechanisms can be selected in both pathogenic and non-pathogenic bacteria, as shown in *E. coli* (Sunde & Sorum 1999). The largest reservoir of resistance has been thought to reside in the commensal gut microbiota (Guyot *et al*. 1999, Gulay *et al.* 2000, McDonald *et al.* 2001). Antimicrobial remains get into the intestine as residues that are not absorbed after oral administration, by diffusion from the surrounding tissue, and in bile via enterohepatic circulation (Nord & Heimdahl 1986b, Edlund & Nord 2000). After being enriched the resistance determinants may transfer within or between different bacterial genera e.g. between *Bacteroides* species and from gram-positive bacteria (*Clostridium, Streptococcus, Enterococcus*) to gram-negative bacteria (*Bacteroides*), and between bacteria originating from humans and animals (Nijsten *et al.* 1995, Shoemaker *et al.* 2001).

6.3. Mechanisms of resistance

As there are several mechanisms by which antibiotics influence bacteria, there are also several mechanisms by which bacteria acquire resistance. Resistance may result from a single random chromosomal mutation or a series of mutations leading to synthesis of altered protein. Spontaneous mutants that have competitive advantage in the presence of antibiotic pressure survive and multiply. Dissemination of resistance is, however, more often the result of acquirence of transmissible plasmids, transposons or gene cassettes in integrons carrying resistance genes, typically coding for resistance to several unrelated classes of antimicrobials (Hall 1997, Poole 2001). Mechanisms of resistance include target site alterations (lowered affinity), alterations in the access to the target site (impermeability, efflux pumps, alternative metabolic pathways) and inactivation of the drug (enzymes that modify or destroy the antimicrobial) (Brock *et al.* 2003).

6.4. Adverse effects of antimicrobial agents

An ideal antimicrobial agent would provide selective decontamination of the microbiota in a “gut friendly” manner, i.e. be active against pathogens and potentially pathogenic microbes but not against those that preserve the colonisation resistance.

Unfortunately only few, if any, available antimicrobials meet these criteria (Vollaard & Clasener 1994).

The use of antimicrobials leads to selection of resistant organisms among pre-existing variants and may radically reduce the number of commensal anaerobes susceptible to antimicrobials like *Bifidobacterium, Bacteroides* and *Lactobacillus* leading to reduction in colonisation resistance. This opens new niches for the remaining resistant opportunistic bacteria or transient pathogens that normally have restricted growth. They may overgrow the intestine or move to another anatomical site where the host is unable to tolerate the colonisation, and pathogenic microbes such as *C. difficile*, vancomycin-resistant enterococci, multidrug-resistant enterobacteria and *Candida* may proliferate (Berg 1996, Falk *et al.* 1998, Sullivan *et al.* 2001). In patients with *C. difficile*-associated diarrhoea treated with metronidazole the proportion of facultative bacteria was increased with higher enterobacterial and enterococcal counts but also with greater diversity of clostridia and lactobacilli compared to healthy subjects (Hopkins & Macfarlane 2002).

In addition to persisting infections and dissemination of resistant organisms, antibiotics predispose patients to serious clinical implications such as gastrointestinal disturbances and functional bowel symptoms, including diarrhoea, alterations in fermentation processes and reduction of short-chain fatty acid production (Bergogne-Berezin 2000, Sullivan *et al.* 2001, Maxwell *et al.* 2002). Antibiotic-associated reduced carbohydrate metabolism in turn may result in osmotic diarrhoea, poor absorption of fatty acids, water and electrolytes, altered bile acid balance and cholesterol metabolism (Hashimoto *et al.* 1996, Bergogne-Berezin 2000). Antimicrobials may also have allergic and toxic effects on the mucosa, and the pharmacologic effect that macrolides have on intestinal motility may affect the pathogenesis of antibiotic-associated diarrhoea (Bergogne-Berezin 2000). Antibiotics may also trigger release of LPS (lipopolysaccharides) endotoxins from gram-negative organisms with possible deleterious effects (Prins *et al.* 1985). A combination of oral *Candida albicans* administration and *Aspergillus fumigatus* spore exposure has been shown to induce allergic airway disease in mice treated with antibiotics (cefoperazone) but not in untreated mice (Noverr *et al.* 2004). In clinical practise, antimicrobial resistance increases mortality, morbidity and health care costs. If the

patient is not responding to the antimicrobial treatment, the length of hospital stay is prolonged or repeated physician visits are needed, and secondary antimicrobials are often more expensive than the first line choices (Livermore 2003).

Nosocomial infections

Cross-contamination of resistant bacteria between individuals is especially significant in hospital environment where there is simultaneous selective pressure from several antibiotics and pathogens with lowered antimicrobial susceptibility may be enriched. In addition, a lot of patients with predisposing factors such as impaired immunosystem are exposed (Blazquez *et al.* 2000). Both hospitalised individuals and outpatiens are reported to frequently carry resistant bacteria regardless of antibiotic usage (Levy *et al.* 1988). The most common organisms causing infections of the urinary tract, surgical wounds, skin, respiratory and gastrointestinal tract, as well as systemic infections are staphylococci including MRSA, enterococci including VRE, *E. coli, Klebsiella* spp., *Enterobacter* spp., *Pseudomonas* spp., *C. difficile* and *Candida* spp. (Schaberg *et al.* 1991, Farr 2002, Biedenbach *et al.* 2004, Hull & Beck 2004, D'Agata 2004). However, in many cases the infective strain originates from the patient's own microbiota, thus hygienic measures cannot alone completely prevent nosocomial infections (Flynn *et al.* 1987), which are a widely acknowledged problem in both human and animal care, and are known to contribute significantly to mortality and health care costs. In Finnish patients over 50 000 nosocomial infections are reported annually, contributing to 2000-5000 deaths (Ora 2005).

6.5. Use of antimicrobials in humans and animals

Common use of antimicrobials of human importance in cattle and pets may increase the risk of transferring resistance from animal to human microbiota. Controversial arguments about antibiotic use in animals are being debated (Piddock 1996, Cook 1997, Phillips *et al.* 2004, Guardabassi *et al.* 2004). Reductions in prescribing antimicrobials at a national level have been shown to reduce the prevalence of resistance within few years (Kristinsson 1997, Seppälä *et al.* 1997). However, reduction in antimicrobial usage does not always lead to reduced resistance. Resistant strains that are well adapted and carry minimal or no fitness cost because of the resistance determinant may not be displaced for a long period of time after the

exposure to antimicrobial agent has ceased. Strains that acquire resistance plasmid may initially grow more slowly than plasmid-free counterparts in the absence of selection pressure but after repeated subculture the difference may diminish as shown in tetracycline- and streptomycin-resistant *E. coli* and in tetracycline- and erythromycin-resistant *Bacteroides* (Bouma & Lenski 1988, Lenski 1997, Shoemaker *et al.* 2001).

6.6. β-Lactam antibiotics

One of the most important groups of antimicrobial agents is β-lactam antibiotics, which are widely used in human and veterinary medicine (Livermore 1998). β-Lactams account for 60% of antimicrobial consumption in Finland (National Medical Statistics, www.nam.fi). Most commonly used β-lactams include penicillins (ampicillin, piperacillin, cloxacillin), cephalosporins (cephalexin, cefuroxime, ceftriaxone), cephamycins (cefoxitin) and carbapenems (imipenem, meropenem). Their antimicrobial potential is based on their ability to interfere in the biosynthesis of the peptidoglycan layer of the bacterial cell wall.

Ampicillin

Ampicillin ($C_{16}H_{19}N_3O_4S$) is relatively non-toxic and acid-stable and can be given orally or parenterally, parenterally in sodium salt form. Absorption of ampicillin is slower and more irregular than that of some of the esters (talampicillin) and analogs (amoxicillin) developed from it. The serum half-life of ampicillin in normal adults is 0.7-1.4h (Neuvonen *et al.* 1994). Peak serum concentrations are obtained at about 2h after oral administration and right after parenteral administration. After an oral dose 30% of ampicillin is exreted in urine within 6h, and after parenteral administration 75% (Kucers & Bennett 1987). Low amounts are excreted also in bile. Unexcreted ampicillin is inactivated mainly in the liver (Kucers & Bennett 1987).

6.7. β-Lactamases

The most common mechanism of resistance to β-lactams is bacterial β-lactamases, produced by a number of both gram-negative and gram-positive bacterial species. In addition to clinically important enterobacteria, β-lactamase production has been described in various anaerobic genera of the normal microbiota such as *Bacteroides, Fusobacterium* and *Clostridium* (Nord 1986a). β-Lactamases inactivate the antibiotic by hydrolysing the amide bond in the β-lactam ring. Numerous different β-lactamases, both chromosomal and plasmid-mediated, have been described and they have been classified into four main groups and several subgroups on the basis of their function or structure (Livermore 1995, Bush *et al.* 1995, Bush & Jacoby 1997). Although hyperproduction of wild type β-lactamase induces resistance, the major contributors to resistance are thought to have arisen from single or multiple mutations in the structural β-lactamase genes (Reguera *et al.* 1991, Henquell *et al.* 1995, Belaaouaj *et al.* 1994, Brun *et al.* 1994). Resistance determinants are transferable and are found within a wide variety of plasmids (Thomson *et al.* 1993). In hospital environment ampicillin is considered a significant factor in maintaining selection pressure for the most prevalent plasmid mediated β-lactamases in gram-negative organisms (Burman *et al.* 1992).

The predominant β-lactamases in gram-negative bacteria are TEM-type β-lactamases, coded by bla_{TEM} genes, with TEM-1, TEM-2 and SHV-1 enzymes being the most common ones (Brinas *et al.* 2002). In *E. coli* TEM-1 is the predominating β-lactamase (Huovinen *et al.* 1988). These Ambler class A enzymes have traditionally been active against a broad spectrum of penicillins but evolution and selective pressure have conferred resistance to expanded-spectrum cephalosporins (extended-spectrum β-lactamases; ESBLs) or to β-lactam-clavulanic acid combinations (inhibitor-resistant TEM; IRT) or to both (complex mutant TEM; CMT) (Medeiros 1997, Sirot *et al.* 1997, Poirel *et al.* 2004).

6.8. Inhibition of β-lactam resistance

β-Lactamase inhibitors

A way to avoid the microbial breakdown of β-lactams is the use of β-lactamase inhibitors simultaneously with β-lactams. β-Lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam contain β-lactam ring as substrates for the β-lactamase to which the "suicide inhibitor" binds preventing the enzyme to destroy the antibiotic. By themselves β-lactamase inhibitors have only little therapeutic value. Bacteria are, however, acquiring mechanisms to overcome the action of many of these inhibitors and a constant designing of new inhibitors is required.

Degradation of intestinal β-lactam residues

Another strategy to fight the emergence of resistance is intra-intestinal inactivation of antimicrobials by degrading the leftover antibiotic accumulating in the intestine where it triggers resistance and quantitative changes within the abundant microbial population (de Vries-Hoepers *et al.* 1993, Van der Waaij & Nord 2000). By degrading the antibiotic residue the selective pressure is abolished and the gut microbiota and colonisation resistance remain unaffected during antimicrobial treatment.

One way to bring β-lactamase to the intestine is by administrating β-lactamase-producing bacteria into the gut. Many intestinal microbes produce β-lactamase and faecal preparations from healthy subjects have been shown to inactivate β-lactams with individual variation (Veringa & van der Waaij 1984, Welling & Groen 1990). Patients harbouring β-lactamase-producing intestinal bacteria have less β-lactam-induced alterations in their microbiota (van der Waaij *et al.* 1986, Chachaty *et al.* 1992, Chachaty *et al.* 1993, Stark *et al.* 1995). In gnotobiotic mice detectable amounts of β-lactamase and inhibition of ceftriaxone activity were produced in the intestine by colonising the mice with a mixture of four β-lactamase-producing anaerobes (*Bacteroides thetaiotaomicron, Clostridium clostridioforme, B. uniformis* E9, *B. uniformis* V4E3) (Leonard *et al.* 1989).

Administrating the β-lactamase as a purified enzyme enables accurate screening and evaluation of the effects of the administration providing knowledge of the dosage.

When given orally the enzyme should be in a form where it does not affect the therapeutic effect of the target antimicrobial, thus it should be active only in the intestine, and not be absorbed into the systemic circulation, and, on the other hand, resist proteolysis and remain active in the intestine. This can be achieved in oral administration by packing the enzyme in capsules that are targeted to release the enzyme in the lower gastrointestinal tract and not in the stomach, in e.g. pH-based manner. In the present study, we analysed the ability to inhibit antibiotic-induced changes in the gut with a product with the above-mentioned characteristics, called targeted recombinant oral β-lactamase (TRBL). TRBL has been shown to degrade ampicillin in the jejunum of beagle dogs in a dose dependent manner but not to enter the systemic circulation or affect the serum ampicillin levels after i.v. administration of ampicillin (Harmoinen *et al.* 2003). A commercially available β-lactamase is in use in animal husbandry to destroy penicillin residues in cow milk (Korycka-Dahl *et al.* 1985, Suomen eläinlääkkeet 2003)

6.9. Testing antimicrobial susceptibility

The antimicrobial susceptibility of bacteria can be determined *in vitro* by standardised methods such as disk diffusion method and broth or agar dilution assay (NCCLS 2002). Disk diffusion method is convenient in screening of resistance phenotypes (presence of the resistance genes) while dilution assay determines the precise level of resistance. In disk diffusion method the size of growth-inhibiton zone around a filter paper disk containing the antibiotic on agar plate determines the susceptibility of the culture, and in antimicrobial dilution assay minimal inhibitory concentration (MIC) is determined as inhibition of growth within a series of antibiotic dilutions in broth or on agar. MIC can also be tested on agar using commercial E-test strips containing stable increasing antibiotic gradient producing a growth-inhibition ellipse.

AIMS OF THE STUDY

The purpose of the study was to examine in detail the microbiota in the upper and lower part of canine intestine. The samples were collected before, during and after ampicillin treatment with or without simultaneous β-lactamase administration and analysed using both traditional culture method and molecular methods. When designing the study we hypothesised that (1) jejunal and faecal microbiota differ significantly from each other, (2) ampicillin administration has dramatic effects on the composition and counts of the microbiota and on the susceptibility of the coliforms, (3) oral targeted β-lactamase inactivates intestinal ampicillin, inhibits the ampicillin-induced changes and preserves colonisation resistance, (4) shifts in coliform population can be followed using resistance data and PFGE (pulsed-field gel electrophoresis) typing.

The specific aims of the study were to:

(**I**) Compare the cultivable canine microbiota in the jejunum to that in faeces.

(**II**) Analyse the ability of β-lactamase administration to prevent ampicillin-induced emergence of resistance in dogs during ampicillin treatment monitored by performing faecal bacterial culture combined to susceptibility testing, performing TGGE (temperature gradient gel electrophoresis) for dominant faecal bacterial groups and by determining the quantity of TEM-type β-lactamase-producing faecal bacteria by PCR and by following ampicillin levels in serum and jejunum.

(**III**) Characterise canine jejunal and faecal coliform populations, their species variability and resistance patterns in the three treatment groups.

(**IV**) Determine the genetic relatedness of coliform populations in the three treatment groups monitored by resistance patterns and PFGE typing.

MATERIALS AND METHODS

1. Study design

In the present study, healthy male laboratory beagles with permanent jejunal fistula were used. The dogs were obtained from Harlan-Winkelmann Gmbh (Borchen, Germany) and National Laboratory Animal Center (University of Kuopio, Finland). Their age was between 1 and 3 years and weight between 12 and 19 kg. The dogs were fistula operated and kept in the premises of the Department of Clinical Veterinary Sciences (Faculty of Veterinary Medicine, University of Helsinki). An intussuscepted nipple valve fistula was surgically inserted 170 cm distal to the pyrolus (Harmoinen 2004). The dogs were kept individually, fed with commercial dog food (Pedigree™, Waltham™, Masterfoods, Helsinki, Finland**)** and taken for walks in the same premises. Coprophagy was avoided by cleaning faeces from the premise after each dog had been taken out. The local ethics committee for animal experiment action in Helsinki, Finland approved the study.

For a pilot study 6 beagles were randomised into 3 treatment groups, 2 in each, receiving ampicillin and placebo, ampicillin and targeted recombinant β-lactamase (TRBL) or only placebo. For the final study a new group of 18 beagles were similarly randomised into 3 treatment groups, 6 in each, but instead of 20 mg/kg of ampicillin for 10 days they received 40 mg/kg for 14 days (Table 1). The baseline samples were collected on day 0 and the follow-up samples 2 weeks after the treatment period (Table 2). Ampicillin sodium (A-PEN™, Orion Pharma, Espoo, Finland) or placebo (Natrosteril™, Orion Pharma) was administered i.v. 4 times a day 30 min after feeding and TRBL or placebo capsules were administered 20 and 27 min after feeding. Because of the different dosing of the ampicillin, the pilot study samples were excluded from the resistance studies (**II, III, IV**) but all baseline samples and all consecutive samples from the dogs receiving only placebo were included in the comparison study (**I**).

Table 1. Dosages used in the resistance studies for the 18 beagles.

Treatment	Amp / Pla	Amp / TRBL	Placebo
Ampicillin (i.v.)	40 mg/kg	40 mg/kg	0
TRBL (p.o.)	0	0.6 mg/kg	0
Number of dogs	6	6	6

Table 2. Sample collection timetable.

Time point	Day
Baseline	0
During treatment	4, 10, 14
Follow-up	28

TRBL (targeted recombinant β-lactamase)

In the designing of the oral β-lactamase the recombinant β-lactamase (Pen P protein) of *Bacillus licheniformis* 749/C was used as a model enzyme (Neugebauer 1981). The purified and freeze-dried targeted recombinant β-lactamase (TRBL) enzyme (Ipsat Therapies Ltd., Espoo, Finland) was released in gastrointestinal tract from enteric-coated pellets in a time and pH-controlled form (Röhm GmbH & Co KG, Pharma Polymers, Darmstadt, Germany) (Harmoinen *et al.* 2003). The capsule is targeted to dissolve above pH 5.5, aimed to release the enzyme in the jejunum.

2. Study animals and sample collection

For study **I** jejunal and faecal baseline samples from 6 dogs in the pilot study and 16 dogs in the final study were included, 2 dogs from the final study with no jejunal samples were excluded. In addition, all samples from 7 of the dogs receiving placebo treatment were included. For study **II** all faecal samples from the 18 beagles recruited for the final study were included. For studies **III** and **IV** all jejunal and faecal samples from the 18 beagles recruited for the final study were included.

The serum and jejunal samples were collected 1 h after the first antibiotic administration of the day. The faecal samples were collected within 1 h of defecation. All samples were immediately frozen at -70°C. A total of 92 jejunal and 102 faecal samples were collected. The jejunal samples were not available from 2 dogs. The samples were processed in blinded manner, i.e. the Anaerobe Reference Laboratory personnel did not know from which treatment group the samples originated.

3. Study location

The dogs were operated and kept at the Department of Clinical Veterinary Sciences, Faculty of Veterinary Medicine, University of Helsinki, Finland. Parallel samples were analysed simultaneously using different methods in several different locations (Table 3). Bacterial culture, coliform identification, susceptibility testing and PFGE were performed at the Anaerobe Reference Laboratory, Department of Bacterial and Inflammatory Diseases, National Public Health Institute, Helsinki, Finland, whereas TGGE and PCR for TEM were performed at Microscreen BV, Groening, The Netherlands. Ampicillin concentrations in serum and jejunal samples were analysed at Yhtyneet Laboratoriot Oy (United Laboratories Ltd.), Helsinki, Finland. Statistical analyses were performed at each corresponding study location excluding Yhtyneet Laboratoriot.

Table 3. Methods used.

Method	Target	Sample material
Bacterial culture	Bacterial profiles, total counts, group/species level identification	Jejunal chyme, faeces
Susceptibility testing	Antibiograms	Jejunal and faecal coliform isolates
PFGE typing	Genetic relatedness	Jejunal and faecal *E. coli* isolates
TGGE	Bacterial profiles	Faeces
TEM-PCR	Amount of resistance gene	Faeces
High performance liquid chromatography (HPLC)	Ampicillin concentration	Jejunal chyme, serum

4. Bacterial culture

All samples from each animal were processed in parallel in order to ease the comparison between the 2 sample types and between consecutive samples. Small intestine fluid and faeces were thawed and homogenised, pH was measured (Benchtop 420 pH Meter, Orion) and the sample consistency, colour and odour were recorded. The homogenates were serially diluted (10^{-1}-10^{-7}) in prereduced peptone-yeast extract broth with pH 7.0. An extensive series of dilutions together with the undiluted sample (10 µl or 100 µl) were plated onto several non-selective and selective agar media and incubated at 35°C as appropriate (Table 4). Anaerobiosis was induced in anaerobic

Table 4. Culture media used. Presented ten-fold dilutions were used for faecal samples, for jejunal samples a limited set of dilutions at a lower dilution level was used.

Agar medium	Enrichment or selection	Bacteria	Dilutions	Atmosphere	Reference
Brucella	Sheep blood, hemin, vitamin K_1	Total anaerobic counts	0, -4, -6, -8	Anaerobic	Jousimies-Somer *et al.* 2002
BBE	Bile, esculin, gentamycin	*Bacteroides* and *Bilophila* spp.	0. -4, -6	Anaerobic	Jousimies-Somer *et al.* 2002
KVLB	Laked blood, kanamycin, vancomycin	Gram-negative anaerobes	0, -4, -6	Anaerobic	Jousimies-Somer *et al.* 2002
BIF	Tomato juice, hemin, maltose	Bifidobacteria	0, -4, -6	Anaerobic	Sutter *et al.*
MRS	Dextrose, ammonium citrate	Lactobacilli	0, -5, -7	Anaerobic	Jousimies-Somer *et al.* 2002
CCFA	Fructose, egg yolk, cycloserine, cefoxitin	*Clostridium difficile*	0, -3	Anaerobic	Jousimies-Somer *et al.* 2002
NEYA	Egg yolk, neomycin	*Clostridium* spp.	0, -4	Anaerobic	Jousimies-Somer *et al.* 2002
Blood	Sheep blood	Total aerobic counts	0, -3, -5, -6, -8	5% CO_2	MacFaddin 1985
CLED	Cystine, lactose, electrolyte deficient	*Enterobacteriaceae*, Streptococci	0, -4	Ambient air	MacFaddin 1985
BE	Bile, esculin	Enterococci	0, -4	Ambient air	MacFaddin 1985
Sabouraud	Chloramphenicol	Yeasts	0	Ambient air	MacFaddin 1985

jars filled by the evacuation-replacement technique (Anoxomat WS8000, Mart B.V., Lichtenvoorde, Netherlands) with gas mixture (90% N_2, 5% CO_2, 5% H_2). The exposure time to air was minimized throughout the procedure with careful planning and preparations, including the use of prereduced dilution broth and agar plates kept in anaerobic conditions 18 h prior to their use.

The total counts and main groups of aerobic and anaerobic bacteria and yeasts were enumerated (the detection limit 10^2 CFU/g), different colony morphotypes were recorded with a stereomicroscope and isolated and identified by established methods. The isolates were picked according to differing morphologies, not on the amount of colonies on the plate or assumed importance of a finding. The identification scheme included aerotolerance testing, gram staining, spot and screening tests (catalase, indole, nitrate, lecitinase, lipase, pigment), special antimicrobial potency disk patterns (vancomycin, kanamycin, colistin, sodium polyanethol sulfonate, penicillin, oxgall), biochemical tests (carbohydrate fermentation, enzyme detection) and metabolic end-product analysis by gas-liquid chromatography after peptone-yeast extract glucose broth culture (Jousimies-Somer *et al.* 2002, Murray *et al.* 2003). The presence of *C. difficile* common antigen and toxin A in faeces was determined with a commercial kit (Triage *C. difficile* Panel, Biosite, San Diego, CA) according to the manufacturer's instructions. For unclassified clostridia resembling *C. difficile,* genes encoding 16S rRNA were amplified and sequenced with the primers fD1 Mod and 533r by the method of Jalava *et al.* (1995) and compared to the GenBank database. The microbial composition and counts between different sampling days, different treatment groups and between corresponding jejunal and faecal samples were compared.

5. Identification, susceptibility testing and PFGE typing of coliforms

From each sample ten colonies or, if less, as many as possible of coliformic colonies (aerobic fermentative gram-negative rods) were isolated from Blood agar and CLED agar plates with optimal growth, and sub-cultured for further identification (Murray *et al.* 2003) and susceptibility testing (NCCLS 2002). The isolates were picked at random but all visibly different colonies were included to get a representative selection of the

coliform population. The resistant isolates may not present the most common morphologic phenotype nor differ by e.g. lactose fermentation profile (Levy *et al.* 1988). The species level identification of coliforms was performed with spot tests (catalase, indole, oxidase), an individual diagnostic tablet of β-glucuronidase (Rosco, Taastrup, Denmark) and with the Api 20E kit test (bioMérieux, Marcy l'Etoile, France).

For all isolated coliforms the resistance to several classes of antimicrobials including penicillins (ampicillin 10 µg), carbapenems (meropenem 10 µg), tetracyclines (tetracycline 30 µg), folate pathway inhibitors (trimethoprim 5 µg, trimethoprim/ sulfamethoxazole 24 µg), aminoglycosides (gentamicin 10 µg), quinolones (ciprofloxacin 5 µg), and cephems (cephalothin 30 µg, cefotaxime 30 µg) was tested (Oxoid, Hampshire, England). The susceptibility was determined using the disk diffusion method as recommended by the National Committee for Clinical Laboratory Standards (NCCLS 2002) on Müller Hinton agar inoculated with swabs dipped in bacterial suspension of 0.5 McFarland with 16-18 h incubation. *E. coli* ATCC 25922 was used as a control strain.

The selected *E. coli* isolates were typed using pulsed-field gel electrophoresis (PFGE) as described by Saari *et al.* (2001). The selected strains presented all different resistance patterns present in each dog at each time point. Bacterial suspension with A_{600} of 0.300 was done in TEN buffer and embedded in 1.8% agarose (InCert Agarose, BioWhittaker Molecular Applications, Inc., Denmark) to prevent mechanical damage to the bacterial chromosome, lysed with proteinase K (0.5 mg/ml) (Roche, Mannheim, Germany) in ES buffer overnight at +50°C, washed to remove the protein digestion products, and the DNA was digested at +37°C overnight with restriction endonuclease *Xba*I (New England Biolabs, Beverly, MA) that cuts DNA infrequently. The blocks were run in 1% agarose gel (Seakem Gold Agarose, BioWhittaker Molecular Applications, Inc., Denmark) in 0.5 x TBE buffer (6V/cm, 120°, 14°C, 21h, pulse times 5-40s) to resolve the restriction fragments into different patterns of discrete bands. The electrophoretic apparatus switches the direction of the current as programmed causing the fragments to reorientate between movements. As the swiftness of the reorientation is dependent on the size of the fragments this intensifies the separation of fragments with small difference in size. The

gels were stained with ethidium bromide (10µl EtBr / 200ml TBE, 30 min) and photographed under UV-light (Alpha Imager, Alpha Innotech, San Leandro, CA). The images were analysed using the Bionumerics version 3.0 cluster analysis with Dice similarity indices (1% position tolerance, UPGMA) (Applied Maths BVBA, Austin, Texas). The genetic relatedness of the strains based on PFGE profiles (fingerprint patterns) was interpreted as recommended by Tenover *et al.* (1995).

6. TGGE (temperature gradient gel electrophoresis)

TGGE was used to assess the bacterial profile and diversity with a molecular approach, based on the sequence variability of the V6 toV8 regions of the 16S rRNA. TGGE was performed as described by Muyzer *et al.* (1993) using primers P16S968GCF and P16S1401R. Briefly, 50 mg of faeces was homogenised and the bacterial DNA was extracted. PCR reaction was performed using Invitrogen's *Taq* DNA polymerase kit and PTC-200 PCR system (MJ-Research). A temperature gradient was established in polyacrylamide gel in parallel to an electric field. The gels were run (200 V, 60°C) in 1.5% (wt/vol) agarose gel using TGGE MAXI system (Biometra, Germany). The amplified DNA fragments migrate differently according to their size and sequence specific melting behaviour (thermal stability) to the point where the conformation of the double stranded DNA changes. As the sequences in TGGE are all the same length the fragments separate in sequence specific manner, and a profile reflecting the prominent bacterial content of the sample is achieved. Fragments containing more A-T pairs are more vulnerable to temperature than those containing C-G pairs. To prevent the double stranded DNA to denature into single stranded DNA, a C-G rich segment is added to one of the primers. The gels were silver stained with $AgNO_3$ by the method of Cairns and Murray (1994). The individually unique TGGE profiles obtained from the same individual on different sampling days were compared using similarity percentages and constructing dendrograms using GelCompar II software (Applied Maths, Belgium).

7. PCR for detecting TEM gene

The emergence of ampicillin resistance was evaluated also by performing quantitative real-time PCR of the TEM β-lactamase gene by quantifying the percentage of TEM

producing bacteria of the total number of bacteria as proportions of DNA in the faecal samples. The PCR was designed for V6-V8 region of the 16S rRNA gene using TemA, TemB, TemE and TemH primers and SYBR green (Molecular Probes, AA Leiden, Netherlands) as fluorogenic marker. The bacterial total count was set as 100% and the amount of Tem sequences was compared to the total bacterial DNA. SYBR Green is a higly sensitive (for low numbers of target DNA amplification product) fluorescent dye specific for double-stranded DNA. During quantitative PCR reaction, SYBR Green dye binds to the amplified double-stranded PCR products and emits a fluorescent signal. Detection of this signal enables direct quantitation of amplified DNA by comparing the fluorescence to known standards.

8. Measurement of ampicillin concentration

In addition to microbial analyses, the serum (quantification limit 1.0 μg /ml) and jejunal (0.5 μg /ml) ampicillin levels were monitored in samples collected 1 h after the first antibiotic administration of the day. The ampicillin analysis was performed using high performance liquid chromatography (HPLC) by modification of the method of Vree *et al.* (1978). HPLC analysis is based on the different migration rates of different compounds in particular chromatographic conditions. A characteristic peak at expected retention time is observed in a chromatograph for each compound in the injected sample. The quantification of the compound the peak presents is calculated from the area under the peak using standard calibration curve with known amounts of the compound.

The jejunal samples were frozen (-70°C) until analysis when they were thawed, mixed by vortex and centrifuged (4°C, 1800 g, 15 min). The tubes were placed on ice and the supernatant was transferred to a cooled glass beaker, diluted in cooled NaCl, filtered trough 0.22 μm filters and frozen (-70°C) until analysed by HPLC. The serum samples were centrifuged (1000g, 15 min) and frozen until analysed. A Hewlett Packard 1100 HPLC instrument with a diode-array detector was used as follows: Supelco Discovery RP Amide C16 column, 2 mobile phases at a flow-rate of 1.0 ml/min (96 % phosphate buffer, pH 6.0 and 4 % acetonitrile for quantitative determinations, 96 % ammoniumacetate, pH 5.2 and 4 % acetonitrile for spectral identification), detection at 229 nm for ampicillin and 296 nm for internal standard (benzylpenicillin procain).

9. Statistical analysis

The statistical analyses for culture-based methods were performed using the SPSS 11.5 (SPSS, Chicago, IL, USA) and Epi Info 6 Statcalc (CDC, Atlanta, GA USA) software for Windows. The pH values of the samples and number of isolates in the 2 sample types were compared by the paired samples t-test and frequencies of different bacteria by Statcalc single table tests. The differences between the bacterial counts were assessed by the Wilcoxon signed rank test (non parametric). A *P*-value <0.05 was considered statistically significant.

For the molecular methods the data analyses were performed with Statistical Analysis System (SAS) software (version 8.1, 2000; SAS Institute Inc.). The PROC MIXED program in SAS software was used to analyse the repeated measurements of the percent similarities of the faecal flora determined by TGGE. The nonparametric analysis of variance and ANOVA were used to compare the ampicillin concentrations and TEM gene levels. A *P*-value < 0.01 was considered statistically significant.

RESULTS

1. Jejunal versus faecal cultivable microbiota (I)

The microbial findings of jejunal chyme and corresponding faecal samples were compared. Jejunal fluid samples varied markedly in viscosity, texture, and color (mucous/running, reddish/brown/greenish). The odor of the fluid was mild and often slightly sweet. All samples were culture-positive: all growth in jejunal samples was recorded and identified, while from faecal samples only bacterial groups in dilutions $>10^{-5—6}$ could usually be isolated and identified. Because of relatively low bacterial counts, different colony types were technically easier to isolate from the jejunal than faecal samples. Significant differences between the two sample types were found both qualitatively and quantitatively. The total cultivable bacterial counts in the jejunum were outnumbered by the counts in faeces (10^{2-6} vs. 10^{8-11} cfu/g, respectively). Notably, bacterial counts in 9% (2/22) jejunal baseline samples reached 10^6 cfu/g and 27% (6/22) reached 10^5 cfu/g with varied dominating species. Anaerobes covered a significantly lower proportion of the total bacterial count in the jejunum compared to faeces (48% vs. 79%, respectively, P=0.004), and significantly less bacterial genera/species were found in the jejunum than in faeces (7 vs. 12, respectively, P<0.001).

The proportions of different cultivable bacteria differed significantly between the jejunal and faecal samples as presented in Figure 1 and 2. Altogether 25% of the bacteria found in the jejunum were not detected in the corresponding faecal sample, or 45% of the faecal findings in the jejunum. In addition, several microbes such as staphylococci, other catalase-producing gram-positive cocci, non-fermentative gram-negative rods and yeasts were more frequent in the jejunum than in faeces, and some bacteria (neisseria, micrococci), most likely of orophraryngeal origin, were detected only in the jejunum. Although gram-negative organisms had on average a higher prevalence in the faecal than jejunal samples (53% vs. 28%), the proportion of gram-negative organisms of the total count was higher in the jejunum than in faeces (28% vs 3%). The dominating cultivable species (those with highest counts) were the same in the corresponding faecal and jejunal sample in 5/22 (23%) dogs. In faeces, the dominating species were similar between

different dogs (anaerobic gram-positive cocci in 10 and streptococci in 5 dogs), whereas the dominating species isolated from jejunal chyme varied between the dogs (anaerobic gram-positive cocci in 4, staphylococci in 4, aerobic gram-positive rods in 4 dogs).

A group of unclassified clostridia ("*Clostridium hiranonis*- like" organisms) was found in just one jejunal sample but in 18/22 (82%) of faecal samples (P<0.001). The isolates resembled *C. difficile* biochemically whereas the 16S rRNA sequence analysis was 99% identical to *C. hiranonis* but the isolates were biochemically different from *C. hiranonis*. Among the *Bacteroides fragilis* group *B. fragilis* was the dominating species in the jejunum while in faeces it was just one species among others, *B. vulgatus* and *B. caccae* being at least as common. Out of the 166 faecal coliform isolates *E. coli* covered 98% while of the 9 coliform isolates from the jejunum 6 (67%) were *Citrobacter* spp. and 2 (22%) *E. coli*.

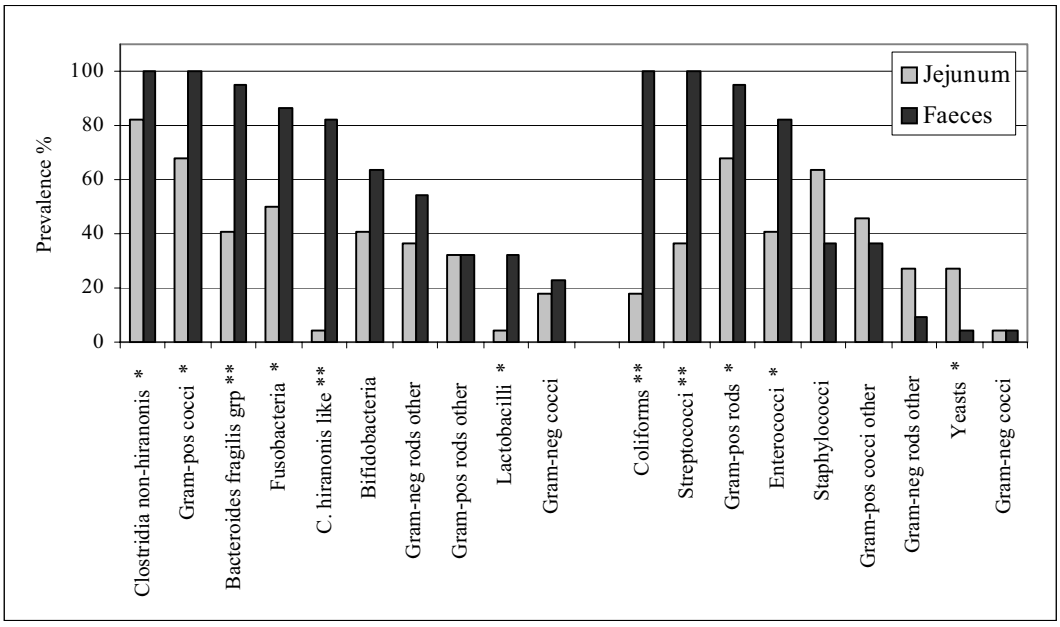


Figure 1. Prevalence of cultivable anaerobic and aerobic/facultative bacteria and yeasts in the jejunum and faeces at baseline (Study **I**). Epi Info 6 single table test:
* *P*<0.05, ***P*<0.001.

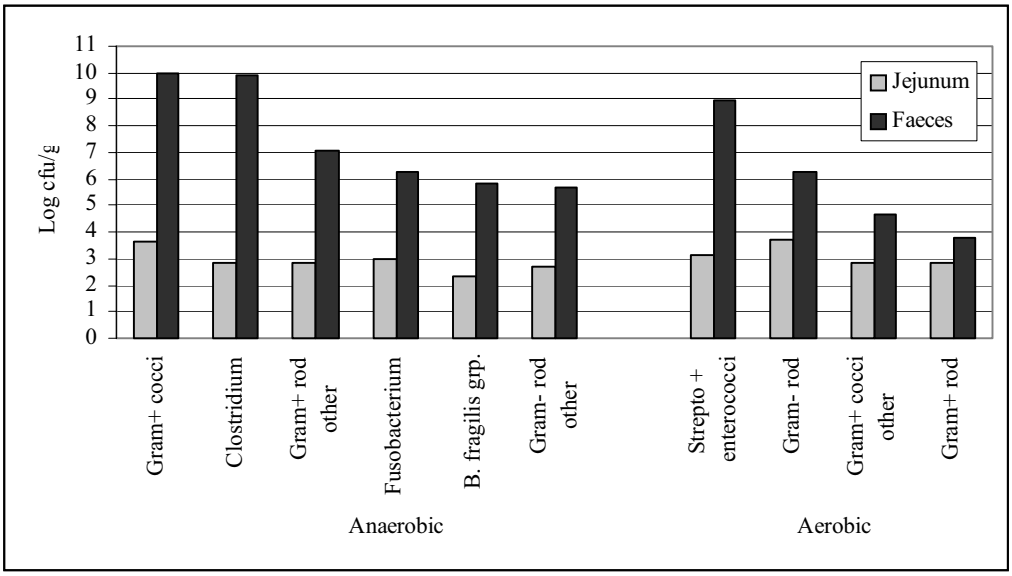


Figure 2. Median counts (log cfu/g) of different cultivable bacterial groups in the jejunum and faeces at baseline (Study **I**).

2. Stability of the cultivable intestinal microbiota (I)

Five consecutive samples from 7 of the dogs, collected during a month, were compared with each other. The predominant jejunal species varied between the dogs and between the consecutive samples while the faecal microbiota was more stable and more similar between the dogs. The findings in the consecutive samples were found repeatedly in 42% of the jejunal samples and in 60% of the faecal samples. In faecal samples the variation in time was biggest among subdominant species close to the detection limit while in jejunal samples all species varied. Several microbial groups or species such as streptococci, yeasts, gram-negative anaerobic and aerobic cocci, gram-positive anaerobic cocci, *C. perfringens* and staphylococci were never found in all consecutive jejunal samples of a dog. In faecal samples dominant species remained stable though their counts fluctuated.

3. Effects of ampicillin and prevention of them by TRBL (II, III)

The effects of ampicillin were followed in the group receiving ampicillin + placebo (Amp/Pla) (Table 1). Significant changes in the cultivable microbial composition and in the resistance rates were recorded in this treatment group, while oral β-lactamase preserved the faecal microbiota during the ampicillin treatment, monitored as significant

differences between the groups receiving ampicillin + placebo and ampicillin + TRBL (Amp/TRBL), and, on the other hand, no marked differences between Amp/TRBL and placebo + placebo (Placebo) groups. The effects with similar differences between treatment groups were found by all methods used: culture, susceptibility testing, TGGE and TEM-PCR.

Table 1. The effects of ampicillin in the Amp / Pla group.

Method	Effect detected in Amp/pla group
Bacterial culture	Altered bacterial profiles, decreased total counts
Susceptibility testing	Significant increase in ampicillin (multi)resistance
TGGE	Altered bacterial profiles (drop in similarity percentage)
TEM-PCR	Increased amount of TEM resistance gene
High performance liquid chromatography (HPLC)	High serum and jejunal ampicillin concentrations

3.1. Changes detected by culture (II, III)

During the intervention the total faecal count of cultivable aerobes decreased significantly in the Amp/Pla group (P=0.003) whereas in the Amp/TRBL group the total counts remained fairly stable. Although the decrease in the anaerobic total count in the Amp/Pla group was not significant compared to the baseline, the anaerobic total count was significantly lower than in the Amp/TRBL group during the treatment (P=0.041). Decreased counts were recorded for streptococci, enterococci, clostridia and anaerobic gram-positive cocci, while aerobic and anaerobic gram-negative rods increased their proportion of the total count.

3.2. Microbial shifts detected by TGGE (II)

During the treatment the overall percentage similarity of the faecal microbiota, determined by TGGE, dropped to a significantly lower level in the Amp/Pla group (38-53%) compared to the Amp/TRBL and Placebo groups (78-85%) (P<0.001).

3.3. Ampicillin concentrations (II)

β-Lactamase had no effect on the ampicillin concentrations in serum; the concentrations were similar in the groups receiving ampicillin and placebo or ampicillin and TRBL (mean 17.7 vs. 18.0 μg/ml, respectively). No ampicillin was detected in the Placebo group.

The ampicillin concentrations in the jejunum were significantly higher in the group receiving ampicillin and placebo than in the group receiving ampicillin and TRBL (mean 26.3 vs. 1.5 μg/ml, respectively, $P<0.0001$). In the latter group β-lactamase had degraded the intestinal antibiotic residue.

3.4. Change in TEM gene levels (II)

The TEM gene levels (determined as the mean percentage of TEM gene of the total amount of DNA) were very low or undetectable in all dogs at baseline. During the treatment the proportion of TEM gene reached a significantly higher level in the Amp/pla group (>0.15%) than in the other groups (<0.025%) ($P<0.001$).

4. Antimicrobial resistance in coliforms (II, III, IV)

Altogether 982 (860 faecal, 122 jejunal) coliform isolates were collected, identified and tested against 9 antimicrobials. *E. coli* covered 87% (106/122) of the jejunal and 99% (855/860) of the fecal isolates. A total of 22 different resistance patterns, with 12 patterns displaying ampicillin resistance, were identified. Each sample displayed 2-3 (ranging between 1-6) different patterns. The 5 most common patterns covered 94% of the faecal and 77% of the jejunal isolates.

4.1. Emergence of resistance in coliforms (II, III, IV)

An increase in ampicillin resistance (multi-resistance) was detected in all treatment groups but the resistance rate was significantly higher in the Amp/Pla group than in the

other groups (P<0.001) (Figure 3). Ampicillin-resistant isolates were frequently resistant also to trimethoprim, trimethoprim/sulfamethoxazole, tetracycline and occasionally to cephalotin. None of the isolates were resistant to ciprofloxacin, meropenem, or cefotaxime. The overall resistance rates in the 3 treatment groups are presented in Figures 4a-c. In the jejunal samples the pattern of increasing resistance was similar to faeces but, additionally, a significant increase was detected also in the number of jejunal coliforms available. During the treatment a total of 68 jejunal coliform isolates were obtained in the Amp/Pla group while in Amp/TRBL and Placebo groups there was no increase in the number of coliforms and only 16 and 15 isolates were obtained during the treatment, respectively.

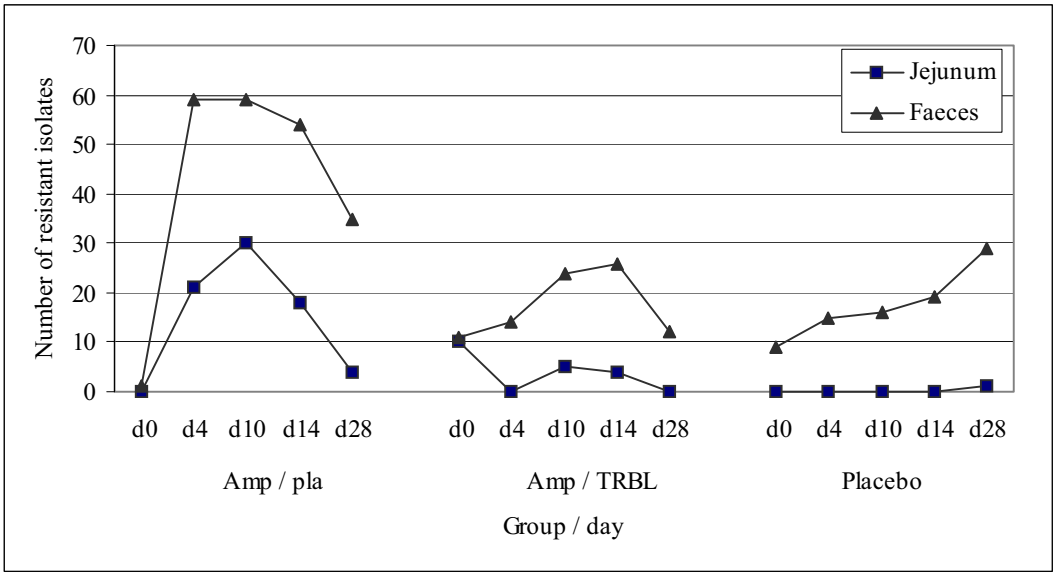


Figure 3. Number of ampicillin-resistant *E. coli* isolates from the jejunum and faeces in the treatment groups during the intervention.

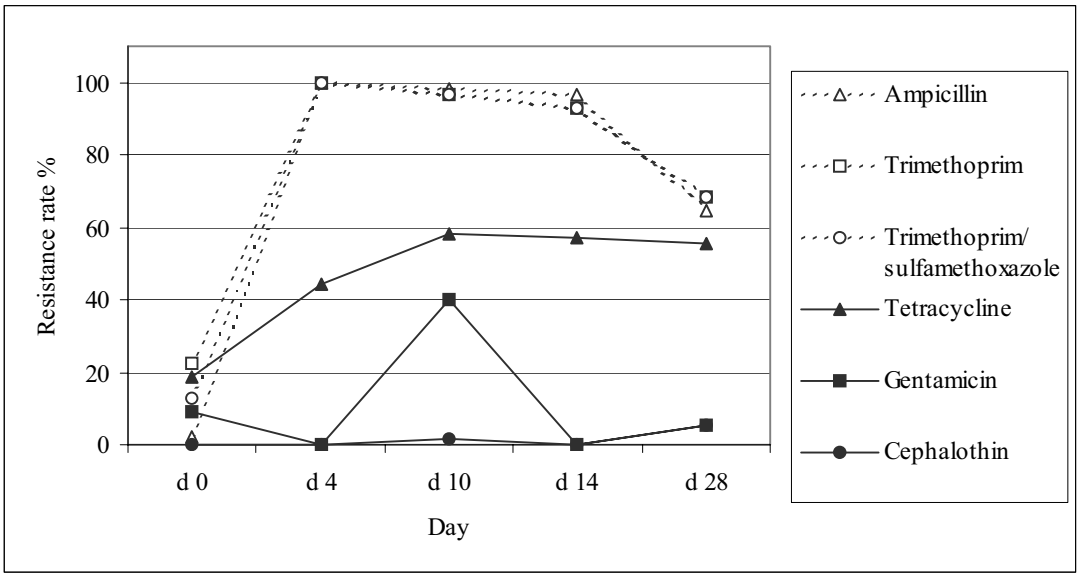


Figure 4a. Resistance rates of coliforms during the intervention in the 3 treatment groups: Amp / Pla group.

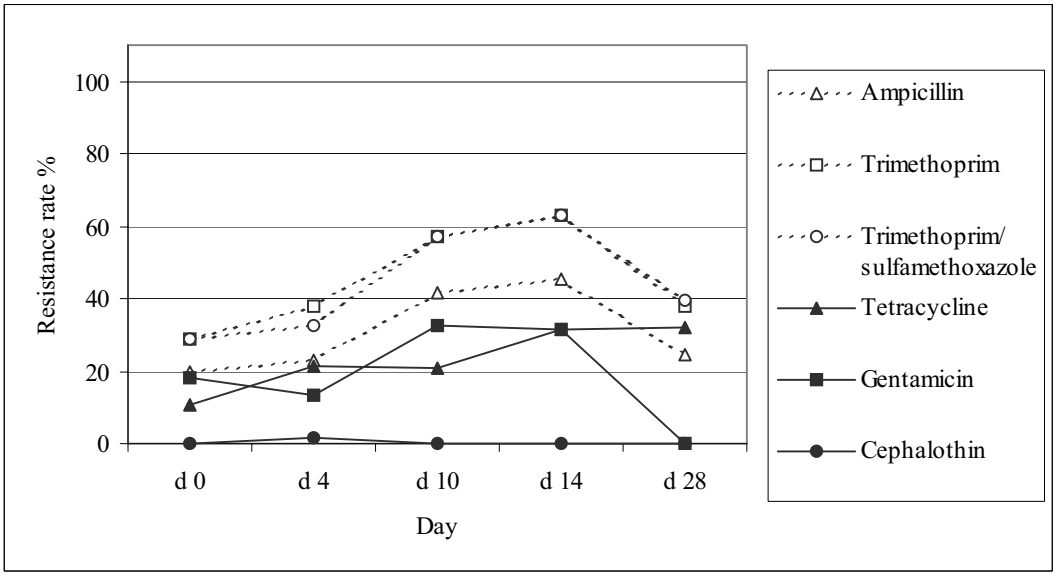


Figure 4b. Amp / TRBL group.

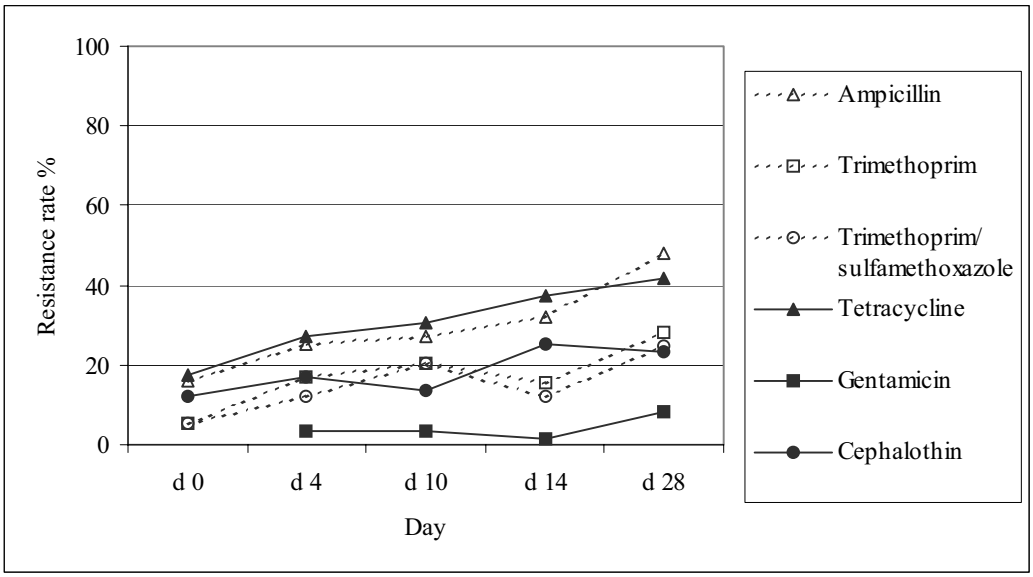


Figure 4c. Placebo group.

4.2. Assessing the relatedness of coliforms (IV)

The relatedness of coliform isolates was determined using the identification results and resistance patterns in combination with PFGE typing. Selected 402 (339 faecal, 63 jejunal) *E. coli* isolates were typed by PFGE and 25 different PFGE types were identified. Each dog harboured on average 5 (ranging between 2-8) different PFGE types during the 28 days' study period. The dogs shared several PFGE types. Ampicillin resistance was found in 6 different PFGE types in 9 different combinations of co-resistance (Table 2). None of the PFGE types was widely spread between the dogs in the different treatment groups but certain ampicillin-resistant PFGE types (F, G, E, A) were enriched in the Amp/Pla group where at least one new ampicillin-resistant PFGE type appeared in all dogs. In the Amp/TRBL group half of the dogs carried ampicillin resistant strains at baseline and during the treatment but among the other half no resistant strains were detected. In Placebo group new resistant strains appeared in 3 dogs during the treatment. Apart from the two strains in a single dog that seemed to acquire resistance during the Amp/pla treatment, the resistant strains enriched were most often selected from pre-existing resistant strains.

Resistance pattern	PFGE type	Isolated from group		
		Amp / Pla	Amp / TRBL	Placebo
Fully susceptible	A, B, C, D, H, I, J, K, M, P, Q, R, S, T, U, V, X, Z, AA, ff*	+	+	+
Amp, Tet, Tmp, Sxt	G, E, E_1	+	+	+
Amp, Gen, Tmp, Sxt	A, D, F, F_1, F_2	+	+	+
Amp, Cef, Tet, Tmp, Sxt	G	+	-	-
Amp, Cef, Tet, Tmp	ff*	-	-	+
Amp, Cef, Tet	ff*	-	-	+
Amp, Gen, Tet, Tmp, Sxt	A	+	-	-
Amp, Cep, Tmp	Q	-	+	-
Amp, Tet	G, ff*	-	+	+
Amp, Tmp, Sxt	G	-	+	
Tet, Tmp, Sxt	A, E, L, P	+	+	+
Tet	A, BB, Y	+	+	-
Gen, Tmp	F	+	-	-
Cef, Tet	BB	+	-	-
Tmp, Sxt	L	-	+	-
Tmp	A	-	+	+
Sxt	I	-	+	-
Gen	D, O	-	-	+
Cef	S	-	-	+

Table 2. Distribution of resistance patterns and PFGE types of the *E. coli* isolates. *ff, untypeable. Amp, ampicillin; Tet, tetracycline; Tmp, trimethoprim; Sxt, trimethoprim/ sulfamethoxazole; Gen, gentamicin; Cef, cephalothin.

DISCUSSION

Characterisation of jejunal and faecal microbiota by culture

The isolation and identification of all species present in a faecal sample is thought to be extremely demanding if not impossible. Regardless of the methodology chosen, only the predominant populations of the cultivable microbial groups can most often be monitored. Although the selectivity of currently available agar media is limited and requires further characterisation and identification methodology, our culture method proved to be sensitive enough to detect differences between the two sample types regardless of the restricted use of the species level identification.

In the present study the nature of the small intestinal microbiota, differing markedly from that of the colon, was characterised as fairly simple and variable, consisting of only few species at a time with fluctuating counts compared to the rich and stable faecal microbiota. All samples were frozen (-70°C) without delay and processed later in parallel, however, the effect of freezing on the microbes of jejunal samples with varying texture has not been fully assessed. The jejunal bacterial counts in some of these healthy beagles crossed the typical limit for small intestinal overgrowth (10^5 cfu/ml) (Corazza *et al.* 1990, Stotzer *et al.* 1998). The counts were not so high repeatedly but all jejunal samples grew bacteria confirming the unreliability of diagnosis of overgrowth based on a single culture. In addition, this finding questions the feasibility of such a definitive limit. On the other hand, the jejunal samples in the present study were obtained without anesthesia shortly after feeding and may have been therefore exceptionally productive yielding high bacterial counts including anaerobes. It cannot, however, be excluded that the permanent fistula may in some individuals have had an effect on the intestinal motility and bacterial counts.

The quantitative and qualitative relations within the cultivable microbiota were significantly different between the two sample types. Our observation is in agreement with previous findings reporting higher ratios of aerobes and differences in frequencies of gram-negative organisms in the upper human gut (jejunum, ileum and caecum) compared

to the colon and faeces (Gorbach *et al.* 1967, Justesen *et al.* 1984, Marteau *et al.* 2001). Oropharyngeal microbes have been reported to dominate in jejunal biopsies of humans (Sullivan *et al.* 2003), whereas *E. coli* and streptococci were the most common findings in the jejunum of 35 mongrel dogs (Thompson *et al.* 1998). Higher or comparable counts of fungi, staphylococci and lactobacilli have been reported in the human ileum than faeces (Gorbach *et al.* 1967). Similarly we found staphylococci, yeasts and also other catalase-producing cocci and non-fermentative gram-negative facultative rods more frequently in the canine jejunum than in faeces but less lactobacilli. Additionally, some species isolated from jejunal samples were undetected in faeces. Marteau *et al.* (2001) reported facultative bacteria to be as numerous as anaerobes in the human caecum. This seems to be true in the jejunum as well, as shown in the present stydy. *B. fragilis* proved to be the most frequent species of the *B. fragilis* group in the canine small intestine but not in faeces. In agreement with our observation, *B. fragilis* has been reported to be the most frequent isolate from mucosal surfaces of humans but not in colon contents (Namavar *et al.* 1989). In the present study the canine jejunal microbiota was also found to be less stable than the faecal microbiota. The canine duodenal microbiota monitored by DGGE profiles has been shown to differ more between individual dogs than between samples from the same dog (Suchodolski *et al*. 2004). The canine fecal bacterial DGGE profiles have individually unique and stable characteristic banding patterns (Simpson *et al*. 2002).

It is noteworthy that we found no *C. difficile* in contrast to some other animal studies (Marks *et al.* 2002, Buogo *et al.* 1995) but we detected instead a group of unclassified clostridia resembling *C. difficile* as part of the dominating faecal microbiota. In addition to strict anaerobes, facultative streptococci were among the dominating microbiota. Also, we found bifidobacteria more often than lactobacilli (in 64% vs. 32% of the dogs, respectively) while two recent studies on labrador and beagle dogs reported no bifidobacteria but numerous lactobacilli (Buddington 2003, Greetham *et al.* 2002). The lactic acid bacteria in fistulated dogs were recently characterised by Rinkinen *et al.* (2004) with *Streptococcus alactolyticus* dominating, followed by *Lactobacillus murinus* and *L. reuteri*.

Comparison of culture to molecular methods

Though molecular methods have brought new aspects to the phylogenetic and quantitative relations of different bacteria in the gut, culture-based and molecular-based profiling may not be contradictory and give often in general somewhat similar overview. Culture-based analyses may, however, give higher qualitative and quantitative fluctuations especially in the faecal microbiota compared to molecular methodology. Similar to our study, the vast fluctuation in canine small intestinal microbiota compared to faeces has been reported by both culture and DGGE reflecting genuine variation rather than weakness in sampling or analysis methodology (Willard *et al*. 1994, Delles *et al*. 1994, Harmoinen *et al*. 2001, Suchodolski *et al*. 2004). We found by culture in the canine faeces a relatively low mean proportion of *Bacteroides* (0.3%), a high proportion of anaerobic gram-positive cocci (30%) and clostridia (37%), a relatively high proportion of streptococci (19%) and relatively low proportion of bifidobacteria (4%) and enterobacteria (1%). Culture negativity or limited number of microbial findings may not always give an accurate picture of the number or consortia of microbes present. For example, cultures and microscopic examinations of oesophagal washes and biopsies have provided poor microbial yields but microbes in oesophageal biopsies detected by sequence based broad-range 16S rDNA are by a large majority (82%) cultivable bacterial species (Pajecki *et al.* 2002, Pei *et al.* 2004).

Ampicillin-induced changes

In the present study ampicillin was found to induce significant qualitative and quantitative changes and emergence of multi-resistant coliform strains among the canine intestinal microbiota. These phenomenona have been well documented for ampicillin and several other antimicrobials also in previous human studies (Nord 1986a, Black *et al.* 1991, Garau *et al.* 1999, Shanahan *et al.* 1994, Huovinen *et al.* 1995, Dradenovich *et al.* 2004). Among human faecal enterobacteria *E. coli* is the main carrier of resistance (Österblad *et al.* 2000). In the present study ampicillin resistance was most often combined to trimethoprim and trimethoprim/sulfamethoxazole resistance in canine strains. A similar combination of resistance occurs frequently, since transmissible

plasmids coding resistance to ampicillin and trimethoprim are common in human *E. coli*, and selective pressure for either is likely to select resistance to both drugs (Amyes 1989, Magee *et al.* 1999). As shown previously, the screening of coliform susceptibility is a feasible and sensitive method to follow the steps in the emergence of resistance (Corpet 1993). In the present study, to intensify the resistance screening, 10 isolates representing all colony morphotypes per sample were tested for resistance. Further, to overcome biases due to the culture method, molecular methods were used to follow the changes in resistance rates monitored as amounts of TEM gene. In addition, several samples during the treatment were analysed.

Resistance within coliform populations

PFGE typing revealed a considerable genetic heterogeneity within the ampicillin-resistant canine *E. coli* populations. Though digestion of DNA with more than one restriction enzyme could have increased the discriminatory power of the PFGE method and separated strains considered identical, the present analysis with one enzyme (*Xba*I) was successful in discriminating numerous genetically different clones with varying frequencies within a large number of related isolates in the three treatment groups. Dogs shared several clones but no single resistant strain was widely spread. Since the resistant strains were most often selected from the pre-existing microbiota or spread between dogs, and were genetically heterogeneous, several resistant types co-existed. The selection of resistant strains from the resident microbiota is thought to be more common than acquirance of a new resistance determinant in strains previously susceptible (Guyot *et al.* 1999, McDonald *et al.* 2001) as was the case also in the present study.

Heterogeneity, constant shifts between strains within *E. coli* populations and sharing of common types has been reported in humans, dogs and cattle (Levy *et al.* 1988, Avery *et al.* 2004, Akiba *et al.* 1999, Münnich & Lübke-Becker 2004, Dradenovich *et al.* 2004). The appeared resistant strains replaced the PFGE types present at baseline in the dogs receiving ampicillin without β-lactamase but not in the other two groups.

Prevention of ampicillin-induced changes by TRBL

Orally administered β-lactamase did not affect the ampicillin level in serum but degraded ampicillin in the intestine and prevented the ampicillin-induced changes in the intestinal microbiota. Colonisation resistance was reduced and multi-resistant coliforms colonised the jejunum only in dogs treated with ampicillin without β-lactamase but not in dogs treated with ampicillin with β-lactamase. The slight increase in resistance rates detected in the Amp/TRBL and Placebo groups could be due to the cross-contamination and spread of resistance determinants from the dogs in the Amp/pla group, since the dogs were not totally isolated from each other. Other possible sources of resistance determinants include food, environment, kennel facilities and personnel as reported also in other studies (Corpet 1987, Avery *et al.* 2004, de Graef 2004). Concurrent administration of antibiotic and β-lactamase could provide a new strategy to limit the emergence of resistance and spread of nosocomial pathogens, and reduce antibiotic-associated diarrhoea. In addition to ampicillin, β-lactamase has shown to be applicaple to inhibit piperacillin-induced changes (Stiefel *et al.* 2003).

Commensal microbes gradually overgrow the resistant strains when the selection pressure is absent (Trott *et al.* 2004). The restoration of the microbiota may, however, be slower than previously thought. The antibiotic-associated alterations in the microbiota can be detected for months or even up to 3 years after a single antibiotic course (Maxwell *et al.* 2002, Mangin *et al.* 1994, Sjölund *et al.* 2003). Antimicrobial resistance determinants are known to be transferable among the commensal microbiota (Gulay *et al.* 2000, Yates *et al.* 2004). In the present study, the microbiota had not recovered to the baseline configuration 2 weeks after the treatment. Although the bacterial counts and composition, TGGE profiles and TEM gene proportions resumed levels close to the baseline, the enriched or appeared ampicillin-resistant PFGE types in the Amp/pla and Placebo groups were still present and the proportion of resistant isolates was higher than at baseline.

Future aspects

The next phase in studying combined ampicillin and β-lactamase administration would be a study trial in humans. Further phases in developing means to prevent antimicrobial

resistance using β-lactamase could include development of new β-lactamases targeted to other β-lactams.

KEY FINDINGS AND CONCLUSIONS

To protect the indigenous microbiota in a safe and convenient manner the composition and functions of the microbiota should be well known and understood, and the desired changes, or their absense, as well as their magnitude and dimensions should be closely monitored with suitable methodology. The present study was carried out in order to develop the understanding of the jejunal microbial composition and stability, and to specify the dissimilarities between the jejunal and faecal microbiota. Another main focus of the study was to evaluate the ability of enzymatic inactivation of intestinal antimicrobial residues to inhibit antibiotic-induced adverse effects on the microbiota.

The key findings were:

I Significant microbial differences between the jejunal and faecal findings were recorded in regard to the bacterial counts, species composition, species variability, stability, relations of gram-negative to gram-positive organisms and relations of aerobic to anaerobic microbes.

II Ampicillin-induced changes among the intestinal microbiota were inhibited to a significant extent by simultaneous oral β-lactamase administration.

III Ampicillin treatment without simultaneous β-lactamase administration induced the emergence of multi-resistant strains displaying numerous combinations of co-resistance and significantly increased the colonisation of the jejunum with coliforms. The increase in resistance was markedly inhibited and the jejunal colonisation resistance was preserved when ampicillin treatment was combined to β-lactamase.

IV The emergence of resistance among intestinal coliforms was due to selection and/or dissemination of several genetically different strains rather than acquirance of resistance in previously susceptible strains or spread of single or few resistant strains between the dogs in different treatment groups.

Conclusions

The small intestinal microbiota has individually unique features in regard to counts, species composition and variability of the microbes, differing markedly from those of faeces. Orally administered β-lactamase was shown to have potential in clinical use by inhibiting the emergence of resistance and preserving the microbiota during antimicrobial therapy. β-Lactamase can be beneficial in inhibiting the emergence and enrichment of resistant strains and in maintaining the colonisation resistance in jejunum during ampicillin-induced selection pressure.

ACKNOWLEDGEMENTS

This study was carried out at the Anaerobe Reference Laboratory, National Public Health Institute, Helsinki, Finland. I thank Professor Pekka Puska and Professor Jussi Huttunen, the present and former Head of the Institute for providing excellent working facilities.

I wish to express my gratitude to my supervisor Professor Pentti Huovinen for supportive and instructive guidance, for clarifying the big pictures for me and also for kind-hearted care for my well being during the process. To my other supervisor Docent Eija Könönen I wish to express my warm thanks for her valuable advice and appreciation especially for her expertise and efficiency in preparing scientific papers.

This study was done in cooperation with Ipsat Therapies Ltd. and I am indebted to all the skilful and kind people there, especially Kai Lindevall, Lauri Jalkanen, Pertti Koski, Nina Wickstrand, Marion Carson and Tuula Heinonen.

I am deeply grateful to all the people involved in this study at the Department of Clinical Veterinary Sciences, University of Helsinki, especially to my co-authors Professor Elias Westermarck, Jaana Harmoinen, Matti Heikkilä and Rafael Frias. Special thanks go to Jaana for explicit and fruitful exchange of information and co-operation.

I wish to thank my co-authors Merja Rautio, Arja Kanervo-Nordström and Terhi Virtanen and colleagues Anne Bryk, Anne Rinta-Opas and Marja Piekkola at the KTL Anaerobe Reference Laboratory. This study was teamwork and could not have been done without your excellent and fluent collaboration. Merja is also acknowledged for her advise, empathy and common sense. I also thank Ani Sarpi, Mari Hyvönen, Nanna Väyrynen, Susan Nyfors and Mervi Latva-Aho from KTL for support and friendship. The warm, caring and cheerful atmosphere at the laboratory has turned colleagues to friends and made working enjoyable.

I also thank my co-authors Michel van der Rest, Päivi Rajala-Schultz and Curtis Donskey for collaboration.

The pre-reviewers of my thesis, Professor Charlotta Edlund and Docent Maria Saarela, are gratefully acknowledged for valuable suggestions to improve the thesis.

I am grateful to all my dear old friends, especially Russe, Sari, Kukka, Ahti, Teemu and Joge for enjoyable moments and reminding me that there is life outside science, and friends from student years, Kaisa Saloniemi and Shea Beasley, for listening and understanding the triumphs and troubles of this study.

My sincere thanks are due to my family for support and interest in my work, especially to my parents Aulikki and Pertti for encouraging finding my own strengths and showing that learning is a life long process. The warmest and most loving thanks are due to my life companion Tuomas. His caring and involved attitude and inventive mind have been vital for me during the years.

I owe my deepest gratitude to the late Professor Hannele “Haba” Jousimies-Somer, who taught me what science is about and showed how much fun it can be. Her knowledge, enthusiasm, conviction and support had a decisive effect on me to carry on doing research after she was gone.

Helsinki, May 2005

REFERENCES

Akiba M, Sameshima T, Nakazawa M. The shift of genetic subtypes of *Escherichia coli* O157:H7 isolates from cattle. Epidemiol Infect 1999;122:343-346.

Alander M, Korpela R, Saxelin M, Vilpponen-Salmela T, Mattila-Sandholm T, von Wright A. Recovery of *Lactobacillus rhamnosus* GG from human colonic biopsies. Lett Appl Microbiol 1997;24:361-364.

Alander M, Satokari R, Korpela R, Saxelin M, Vilpponen-Salmela T, Mattila-Sandholm T, von Wright A. Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG, after oral consumption. Appl Environ Microbiol 1999;65:351-354.

Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 1995;59:143-169.

Amyes SG. The success of plasmid-encoded resistance genes in clinical bacteria. An examination of plasmid-mediated ampicillin and trimethoprim resistance genes and their resistance mechanisms. J Med Microbiol 1989;28:73-83.

Avery SM, Liebana E, Hutchison ML, Buncic S. Pulsed field gel electrophoresis of related *Escherichia coli* O157 isolates associated with beef cattle and comparison with unrelated isolates from animals, meats and humans. Int J Food Microbiol 2004;92:161-169.

Barrett TJ, Lior H, Green JH, Khakhria R, Wells JG, Bell BP, Greene KD, Lewis J, Griffin PM. Laboratory investigation of a multistate food-borne outbreak of *Escherichia coli* O157:H7 by using pulsed-field gel electrophoresis and phage typing. J Clin Microbiol 1994;32:3013-3017.

Batt RM, Needham JR, Carter MW. Bacterial overgrowth associated with a naturally occurring enteropathy in the German shepherd dog. Res Vet Sci. 1983;35:42-46.

Belaaouaj A, Lapoumeroulie C, Canica MM, Vedel G, Nevot P, Krishnamoorthy R, Paul G. Nucleotide sequences of the genes coding for the TEM-like beta-lactamases IRT-1 and IRT-2 (formerly called TRI-1 and TRI-2). FEMS Microbiol Lett 1994;120:75-80.

Benno Y, Nakao H, Uchida K, Mitsuoka T. Impact of the advances in age on the gastrointestinal microflora of beagle dogs. J Vet Med Sci 1992a;54:703-706.

Benno Y, Nakao H, Uchida K, Mitsuoka T. Individual and seasonal variations in the composition of fecal microflora of beagle dogs. Bifidobact. Microfl 1992b;11:69-76.

Berg RD. The indigenous gastrointestinal microflora. Trends Microbiol 1996;4:430-435.

Berg RD. Bacterial translocation from the gastrointestinal tract. Adv Exp Med Biol 1999;473:11-30.

Bergogne-Berezin E. Treatment and prevention of antibiotic associated diarrhea. Int J Antimicrob Agents 2000;16:521-526.

Bhat P, Albert MJ, Rajan D, Ponniah J, Mathan V I, Baker SJ. Bacterial flora of the jejunum: a comparison of luminal aspirate and mucosal biopsy. J Med Microbiol 1980;13:247-256.

Biedenbach DJ, Moet GJ, Jones RN. Occurrence and antimicrobial resistance pattern comparisons among bloodstream infection isolates from the SENTRY Antimicrobial Surveillance Program (1997-2002). Diagn Microbiol Infect Dis 2004;50:59-69.

Black F, Einarsson K, Lidbeck A, Orrhage K, Nord CE. Effect of lactic acid producing bacteria on the human intestinal microflora during ampicillin treatment. Scand Infect Dis 1991;23:247-254.

Blazquez J, Morosini MI, Negri MC, Baquero F. Selection of naturally occurring extended-spectrum TEM beta-lactamase variants by fluctuating beta-lactam pressure. Antimicrob Agents Chemother 2000;44:2182-2184.

Blottiere HM, Buecher B, Galmiche JP, Cherbut C. Molecular analysis of the effect of short-chain fatty acids on intestinal cell proliferation. Proc Nutr Soc 2003;62:101-106.

Borriello P, Hudson M, Hill M. Investigation of the gastrointestinal bacterial flora. Clin Gastroenterol 1978;7:329-349.

Bouma JE, Lenski RE. Evolution of a bacteria/plasmid association. Nature 1988;335:351-352.

Brandtzaeg P, Halstensen TS, Kett K, Krajci P, Kvale D, Rognum TO, Scott H, Sollid LM. Immunobiology and immunopathology of human gut mucosa: humoral immunity and intraepithelial lymphocytes. Gastroenterology 1989;97:1562-1584.

Breeuwer P, Abee T. Assessment of viability of microorganisms employing fluorescence techniques. Int J Food Microbiol 2000;55:193-200.

Brinas L, Zarazaga M, Saenz Y, Ruiz-Larrea F, Torres C. Beta-lactamases in ampicillin-resistant *Escherichia coli* isolates from foods, humans, and healthy animals. Antimicrob Agents Chemother 2002;46:3156-3163.

Brock TD, Madigan MT, Martinko JM, Parker J. Biology of Microorganisms 10th ed. Prentice-Hall International, New Jersey, USA, 2003.

Brook I, Frazier EH. Significant recovery of nonsporulating anaerobic rods from clinical specimens. Clin Infect Dis 1993;16:476-480.

Brun T, Peduzzi J, Canica MM, Paul G, Nevot P, Barthelemy M, Labia R. Characterization and amino acid sequence of IRT-4, a novel TEM-type enzyme with a decreased susceptibility to beta-lactamase inhibitors. FEMS Microbiol Lett 1994;120:111-117.

Buddington RK. Postnatal changes in bacterial populations in the gastrointestinal tract of dogs. Am J Vet Res 2003;64:646-651.

von Buenau R, Jaekel L, Schubotz E, Schwarz S, Stroff T, Krueger M. *Escherichia coli* Strain Nissle 1917: Significant Reduction of Neonatal Calf Diarrhea. J Dairy Sci 2005;88:317-323.

Buogo C, Burnens AP, Perrin J, Nicolet J. Presence of *Campylobacter* spp., *Clostridium difficile*, *C. perfringens* and salmonellae in litters of puppies and in adult dogs in a shelter. Schweiz Arch Tierheilkd 1995;137:165-171.

Burman LG, Haeggman S, Kuistila M, Tullus K, Huovinen P. Epidemiology of plasmid-mediated beta-lactamases in enterobacteria Swedish neonatal wards and relation to antimicrobial therapy. Antimicrob Agents Chemother 1992;36:989-992.

Bush K, Jacoby GA, Medeiros AA. A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrob Agents Chemother 1995;39:1211-1233.

Bush K, Jacoby G. Nomenclature of TEM beta-lactamases. J Antimicrob Chemother 1997;39:1-3.

Bäckhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci USA 2004;101:15718-15723.

Carratala J, Fernandez-Sevilla A, Tubau F, Callis M, Gudiol F. Emergence of quinolone-resistant *Escherichia coli* bacteremia in neutropenic patients with cancer who have received prophylactic norfloxacin. Clin Infect Dis 1995;20:557-560; discussion 561-563.

Castagliuolo I, Riegler MF, Valenick L, LaMont JT, Pothoulakis C. *Saccharomyces boulardii* protease inhibits the effects of *Clostridium difficile* toxins A and B in human colonic mucosa. Infect Immun 1999;67:302-307.

Chachaty E, Depitre C, Mario N, Bourneix C, Saulnier P, Corthier G, Andremont A. Presence of *Clostridium difficile* and antibiotic and beta-lactamase activities in feces of volunteers treated with oral cefixime, oral cefpodoxime proxetil, or placebo. Antimicrob Agents Chemother 1992;36:2009-2013.

Chachaty E, Bourneix C, Renard S, Bonnay M, Andremont A. Shedding of *Clostridium difficile*, fecal beta-lactamase activity, and gastrointestinal symptoms in 51 volunteers treated with oral cefixime. Antimicrob Agents Chemother 1993;37:1432-1435.

Charteris WP, Kelly PM, Morelli L, Collins JK. Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations. Int J Food Microbiol 1997;35:1-27.

Cook RR. Antimicrobial resistance--use in veterinary and human medicine. J Antimicrob Chemother 1997;39:435.

Corazza GR, Menozzi MG, Strocchi A, Rasciti L, Vaira D, Lecchini R, Avanzini P, Chezzi C, Gasbarrini G. The diagnosis of small bowel bacterial overgrowth. Reliability of jejunal culture and inadequacy of breath hydrogen testing. Gastroenterol 1990;98:302-309.

Corpet DE. Antibiotic residues and drug resistance in human intestinal flora. Antimicrob Agents Chemother 1987;31:587-593.

Corpet DE. An evaluation of methods to assess the effect of antimicrobial residues on the human gut flora. Vet Microbiol 1993;35:199-212.

Cummings JH, Macfarlane GT. The control and consequences of bacterial fermentation in the human colon. J Appl Bacteriol 1991;70:443-459.

D'Agata EM. Rapidly rising prevalence of nosocomial multidrug-resistant, Gram-negative bacilli: a 9-year surveillance study. Infect Control Hosp Epidemiol 2004;25:842-846.

Davis CP, Cleven D, Balish E, Yale CE. Bacterial association in the gastrointestinal tract of beagle

dogs. Appl Environ Microbiol 1977;34:194-206.
Davis MA, Hancock DD, Besser TE, Call DR. Evaluation of pulsed-field gel electrophoresis as a tool for determining the degree of genetic relatedness between strains of *Escherichia coli* O157:H7. J Clin Microbiol 2003;41:1843-1849.
De Graef EM, Decostere A, Devriese LA, Haesebrouck F. Antibiotic resistance among fecal indicator bacteria from healthy individually owned and kennel dogs. Microb Drug Resist 2004;10:65-69.
Deitsch KW, Moxon ER, Wellems TE. Shared themes of antigenic variation and virulence in bacterial, protozoal, and fungal infections. Microbiol Mol Biol Rev 1997;61:281-293.
Delles EK, Willard MD, Simpson RB, Fossum TW, Slater M, Kolp D, Lees GE, Helman R, Reinhart G. Comparison of species and numbers of bacteria in concurrently cultured samples of proximal small intestinal fluid and endoscopically obtained duodenal mucosa in dogs with intestinal bacterial overgrowth. Am J Vet Res 1994;55:957-964.
Diekema DJ, Barr J, Boyken LD, Buschelman BJ, Jones RN, Pfaller MA, Herwaldt LA. A cluster of serious *Escherichia coli* infections in a neonatal intensive-care unit. Infect Control Hosp Epidemiol 1997;18:774-776.
Donaldson RM Jr. Normal bacterial populations of intewstine and their relation to intestinal function. New Engl J Med 1964;270:938-946, 994-1000, 1050-1056.
Doungudomdacha S, Rawlinson A, Walsh TF, Douglas CW. Effect of non-surgical periodontal treatment on clinical parameters and the numbers of *Porphyromonas gingivalis, Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* at adult periodontitis sites. J Clin Periodontol. 2001;28:437-445.
Drasar DR, Mill MJ. Human intestinal flora. Academic Press, London, 1975.
Drazenovich N, Ling GV, Foley J. Molecular investigation of *Escherichia coli* strains associated with apparently persistent urinary tract infection in dogs. J Vet Intern Med 2004;18:301-306.
Edlund C, Nord CE. Effect on the human normal microflora of oral antibiotics for treatment of urinary tract infections. J Antimicrob Chemother 2000;46(Suppl 1):41-48; discussion 63-65.
Eisenstein BI, Zaleznik DF. *Enterobacteriaceae*, p. 2294-2310. In: G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), Principles and practice of infectious diseases, 5th ed. Churchill Livingstone, Philadelphia, PA, USA, 2000.
El Oufir L, Flourie B, Bruley des Varannes S, Barry JL, Cloarec D, Bornet F, Galmiche JP. Relations between transit time, fermentation products, and hydrogen consuming flora in healthy humans. Gut 1996;38:870-877.
Falk PG, Hooper LV, Midtvedt T, Gordon JI. Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. Microbiol Mol Biol Rev 1998;62:1157-1170.
Farr BM. Prevention and control of nosocomial infections. Business briefing: global health care issue. 2002;3:37-41.
Favier CF, Vaughan EE, de Vos WM, Akkermans ADL. Molecular monitoring of succession of bacterial communities in human neonates. Appl Environ Microbiol 2002;68:219-226.
Felske A, Rheims H, Wolterink A, Stackebrandt E, Akkermans AD. Ribosome analysis reveals prominent activity of an uncultured member of the class Actinobacteria in grassland soils. Microbiology 1997;143:2983-2989.
Finegold SM, Sutter VL, Mathisen GE. Normal indigenous intestinal flora, p. 3-31. In D. J. Hentges (ed.), Human intestinal microbiota in health and disease. Academic Press, New York, USA, 1983.
Flynn DM, Weinstein RA, Nathan C, Gaston MA, Kabins SA. Patients' endogenous flora as the source of "nosocomial" Enterobacter in cardiac surgery. J Infect Dis 1987;156:363-368.
Franks AH, Harmsen HJ, Raangs GC, Jansen GJ, Schut F, Welling GW. Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. Appl Environ Microbiol 1998;64:3336-3345.
Garau J, Xercavins M, Rodriguez-Carballeira M, Gomez-Vera JR, Coll I, Vidal D, Llovet T, Ruiz-Bremon A. Emergence and dissemination of quinolone-resistant *Escherichia coli* in the community. Antimicrob Agents Chemother 1999;43:2736-2741.
Garcia L, Tercero JC, Legido B, Ramos JA, Alemany J, Sanz M. Rapid detection of *Actinobacillus actinomycetemcomitans, Prevotella intermedia* and *Porphyromona gingivalis* by multiplex PCR. J Periodontal Res 1998;33:59-64.
Gillian Hartley M, Hudson MJ, Swarbrick ET, Hill MJ, Gent AE, Hellier MD, Grace RH. 1992. The

rectal mucosa-associated microflora in patients with ulcerative colitis. J Med Microbiol 1992;36:96-103.
Goldin BR, Swenson L, Dwyer J, Sexton M, Gorbach SL. Effect of diet and *Lactobacillus acidophilus* supplements on human fecal bacterial enzymes. J Natl Cancer Inst 1980;64:255-261.
Gorbach SL, Plaut AG, Nahas L, Weinstein L, Spanknebel G, Levitan R. Studies of intestinal microflora. II. Microorganisms of the small intestine and their relations to oral and fecal flora. Gastroenterology 1967;53:856-867.
Gorbach SL, Nahas L, Lerner PI, Weinstein L. Effects of diet, age, and periodic sampling on numbers of faecal microorganisms in man. Gastroenterology 1967;53:845-855.
Gorbach SL. Bengt E. Gustafsson memorial lecture. Function of the normal human microflora. Scand J Infect Dis Suppl 1986;49:17-30.
Gordon HA, Pesti L. The gnotobiotic animal as a tool in the study of host microbial relationships. Bacteriol Rev 1971;35:390-429.
Gracey M, Suharjono, Sunoto. Use of a simple duodenal capsule to study upper intestinal microflora. Arch Dis Child 1977;52:74-76.
Gracey M. The contaminated small bowel syndrome, p. 495-513. In: Hentges DJ (ed), Human intestinal microflora in health and disease. Academic Press, New York, USA, 1983.
Greetham HL, Giffard C, Hutson RA, Collins MD, Gibson GR. Bacteriology of the Labrador gut: a cultural and genotypic approach. J Appl Microbiol 2002;93:640-646.
Grif K, Karch H, Schneider C, Daschner FD, Beutin L, Cheasty T, Smith H, Rowe B, Dierich MP, Allerberger F. Comparative study of five different techniques for epidemiological typing of *Escherichia coli* O157. Diagn Microbiol Infect Dis 1998;32:165-176.
Guardabassi L, Schwarz S, Lloyd DH. Pet animals as reservoirs of antimicrobial-resistant bacteria. J Antimicrob Chemother 2004;54:321-332.
Guarner F, Malagelada JR. Gut flora in health and disease. Lancet 2003;361:512-519.
Gulay Z, Bicmen M, Amyes SG, Yulug N. Beta-lactamase patterns and betalactam/clavulanic acid resistance in *Escherichia coli* isolated from fecal samples from healthy volunteers. J Chemother 2000;12:208-215.
Guyot A, Barrett SP, Threlfall EJ, Hampton MD, Cheasty T. Molecular epidemiology of multi-resistant *Escherichia coli*. J Hosp Infect 1999;43:39-48.
Hahm BK, Maldonado Y, Schreiber E, Bhunia AK, Nakatsu CH. Subtyping of foodborne and environmental isolates of Escherichia coli by multiplex-PCR, rep-PCR, PFGE, ribotyping and AFLP. J Microbiol Methods. 2003;53:387-399.
Hall RM. Mobile gene cassettes and integrons: moving antibiotic resistance genes in gram-negative bacteria. Ciba Found Symp. 1997;207:192-202; discussion 202-205.
Hamilton I, Worsley BW, Cobden I, Cooke EM, Shoesmith JG, Axon AT. Simultaneous culture of saliva and jejunal aspirate in the investigation of small bowel bacterial overgrowth. Gut 1982;23:847-853.
Harmoinen J, Mättö J, Rinkinen ML, Wilsson-Rahmberg M, Westermarck E. Permanent jejunal fistula: promising method for obtaining small intestinal chyme without disturbing intestinal function. Comp Med 2001;51:252-256.
Harmoinen J, Vaali K, Koski P, Syrjänen K, Laitinen O, Lindevall K, Westermarck E. Enzymic degradation of a beta-lactam antibiotic, ampicillin, in the gut: a novel treatment modality. J Antimicrob Chemother 2003;51:361-365.
Harmoinen J. A novel enzymatic therapy, targeted recombinant β-lactamase, in the prevention of antibiotic-induced adverse effects on gut microbiota. Academic dissertation. Faculty of Veterinary Medicine, University of Helsinki, 2004.
Hartemink R, Rombouts FM. Comparison of media for the detection of bifidobacteria, lactobacilli and total anaerobes from faecal samples. J Microbiol Methods 1999;36:181-192.
Hashimoto S, Igimi H, Uchida K, Satoh T, Benno Y, Takeuchi N. Effects of beta-lactam antibiotics on intestinal microflora and bile acid metabolism in rats. Lipids 1996;31:601-609.
Henquell C, Chanal C, Sirot D, Labia R, Sirot J. Molecular characterization of nine different types of mutants among 107 inhibitor-resistant TEM beta-lactamases from clinical isolates of *Escherichia coli*. Antimicrob Agents Chemother 1995;39:427-430.
Hentges DJ. The anaerobic microflora of the human body. Clin Infect Dis 1993;16(Suppl 4):S175-180.
Hill RC, Ellison GW, Burrows CF, Bauer JE, Carbia B. Ileal cannulation and associated complications

in dogs. Lab Anim Sci 1996;46:77-80.
Hirtzmann M, Reuter G. Klinische Erfahrungen mit einer neuen, automatisch gesteuterten Kapsel zur Gewinnung von Darminhalt und bakteriologische Untersuchungen des Inhalts hoherer Darmabschnitte. Klin Prax 1963;35:1408-1412.
Holdeman LV, Good IJ, Moore WE. Human fecal flora: variation in bacterial composition within individuals and a possible effect of emotional stress. Appl Environ Microbiol 1976;3:359-375.
Holloway K. Antimicrobial resistance: the facts. Essential Drug Monitor, WHO 2000;28-29:7-8.
Holzapfel WH, Haberer P, Snel J, Schillinger U, Huis in't Veld JH. Overview of gut flora and probiotics. Int J Food Microbiol 1998;41:85-101.
Hopkins MJ, Sharp R, Macfarlane GT. Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. Gut 2001;48:198-205.
Hopkins MJ, Macfarlane GT. Changes in predominant bacterial populations in human faeces with age and with *Clostridium difficile* infection. J Med Microbiol 2002;51:448-454.
Hooper LV, Gordon JI. Commensal host-bacterial relationships in the gut. Science 2001;292:1115-1118.
Hooper LV, Midtvedt T, Gordon JI. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr 2002;22:283-307.
Horie H, Kanazawa K, Okada M, Narushima S, Itoh K, Terada A. Effects of intestinal bacteria on the development of colonic neoplasm: an experimental study. Eur J Cancer Prev 1999;8:237-245.
Hull MW, Beck PL. *Clostridium difficile*-associated colitis. Can Fam Physician 2004;50:1536-1540, 1543-1545.
Huovinen S, Huovinen P, Jacoby GA. Detection of plasmid-mediated beta-lactamases with DNA probes. Antimicrob Agents Chemother 1988;32:175-179.
Huovinen P, Sundstrom L, Swedberg G, Skold O. Minireview. Trimethoprim and sulfonamide resistance. Antimicrob Agents Chemother 1995;39:279-289.
Huovinen P. Bacteriotherapy: the time has come. BMJ 2001;323:353-354.
Johansson M-L, Molin G, Jeppsson B, Nobaek S, Ahrné S, Bengmark S. Administration of different *Lactobacillus* strains in fermented oatmeal soup: *in vivo* colonization of human intestinal mucosa and effect on the indigenous flora. Appl Environ Microbiol 1993;59:15-20.
Jousimies-Somer H, Summanen P, Citron DM, Baron EJ, Wexler HM, Finegold SM. Wadsworth-KTL Anaerobic bacteriology manual 6th ed. Star Publishing, Belmont, CA, USA, 2002.
Justesen T, Nielsen OH, Jacobsen IE, Lave J, Rasmussen SN. The normal cultivable microflora in upper jejunal fluid in healthy adults. Scand J Gastroenterol 1984;19:279-282.
Kepner RL Jr, Pratt JR. Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. Microbiol Rev 1994;58:603-615.
Korycka-Dahl M, Richardson T, Bradley RL Jr. Use of microbial beta-lactamase to destroy penicillin added to milk. J Dairy Sci 1985;68:1910-1916.
Krause R, Schwab E, Bachhiesl D, Daxbock F, Wenisch C, Krejs GJ, Reisinger EC. Role of *Candida* in antibiotic-associated diarrhea. J Infect Dis 2001;184:1065-1069.
Kristinsson KG. Effect of antimicrobial use and other risk factors on antimicrobial resistance in pneumococci. Microb Drug Resist 1997;3:117-123.
Kruis W, Fric P, Pokrotnieks J, Lukas M, Fixa B, Kascak M, Kamm MA, Weismueller J, Beglinger C, Stolte M, Wolff C, Schulze J. Maintaining remission of ulcerative colitis with the probiotic *Escherichia coli* Nissle 1917 is as effective as with standard mesalazine. Gut 2004;53:1617-1623.
Kucers A, Bennett N McK. The use of antibiotics 4th ed. William Heinimann Medical Books, London, UK, 1987.
Kuwahara T, Yamashita A, Hirakawa H, Nakayama H, Toh H, Okada N, Kuhara S, Hattori M, Hayashi T, Ohnishi Y. Genomic analysis of *Bacteroides fragilis* reveals extensive DNA inversions regulating cell surface adaptation. Proc Natl Acad Sci USA 2004;101:14919-1424.
Lee A, Gordon J, Lee CJ, Dubos R. The mouse intestinal microflora with emphasis on the strict anaerobes. J Exp Med 1971;133:339-352.
Lenski RE. The cost of antibiotic resistance--from the perspective of a bacterium. Ciba Found Symp 1997;207:131-140; discussion 141-151.
Leonard F, Andremont A, Leclerq B, Labia R, Tancrede C. Use of beta-lactamase-producing anaerobes

to prevent ceftriaxone from degrading intestinal resistance to colonization. J Infect Dis 1989;160:274-280.
Levy SB, Marshall B, Schluederberg S, Rowse D, Davis J. High frequency of antimicrobial resistance in human fecal flora. Antimicrob Agents Chemother 1988;32:1801-1806.
Lin C, Raskin L, Stahl DA. Microbial community structure in gastrointestinal tracts of domestic animals: comparative analyses using rRNA-targeted oligonucleotide probes FEMS Microbiol Ecol 1997;22:281-294.
Livermore DM. beta-Lactamases in laboratory and clinical resistance. Clin Microbiol Rev 1995;8:557-584.
Livermore DM. Beta-lactamase-mediated resistance and opportunities for its control. J Antimicrob Chemother 1998;41(Suppl D):25-41.
Livermore DM. Bacterial resistance: origins, epidemiology, and impact. Clin Infect Dis 2003;36(Suppl 1):S11-23.
MacFaddin JF. Media for isolation-cultivation-identification-maintenance of medical bacteria, vol 1. Williams & Wilkins, Baltimore, MD, USA, 1985.
Macfarlane GT, Gibson GR, Cummings JH. Comparison of fermentation reactions in different regions of the human colon. J Appl Bacteriol 1992;72:57-64.
Macfarlane GT, Macfarlane S. Human colonic microbiota: ecology, physiology and metabolic potential of intestinal bacteria. Scand J Gastroenterol 1997;222(Suppl):3-9.
Mackie RI, Sghir A, Gaskins HR. Developmental microbial ecology of the neonatal gastrointestinal tract. Am J Clin Nutr 1999;69:1035S-1045S.
Mackowiak PA. The normal microbial flora. N Engl J Med 1982;307:83-93.
Macpherson A, Khoo UY, Forgacs I, Philpott-Howard J, Bjarnason I. Mucosal antibodies in inflammatory bowel disease are directed against intestinal bacteria. Gut 1996;38:365-375.
Macpherson AJ, Gatto D, Sainsbury E, Harriman GR, Hengartner H, Zinkernagel RM. A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science 2000;288:2222-2226.
Magee JT, Pritchard EL, Fitzgerald KA, Dunstan FD, Howard AJ. Antibiotic prescribing and antibiotic resistance in community practice: retrospective study, 1996-8. BMJ 1999;319:1239-1240.
Mangin I, Bourget N, Bouhnik Y, Bisetti N, Simonet JM, Decaris B. Identification of *Bifidobacterium* strains by rRNA gene restriction patterns. Appl Environ Microbiol 1994;60:1451-1458.
Marks SL, Kather EJ, Kass PH, Melli AC. Genotypic and phenotypic characterization of *Clostridium perfringens* and *Clostridium difficile* in diarrheic and healthy dogs. J Vet Intern Med 2002;16:533-540.
Marteau P, Rochart P, Dore J, Bera-Maillet C, Bernalier A, Corthier G. Comparative study of bacterial groups within the human cecal and fecal microbiota. Appl Environ Microbiol 2001;67:4939-4942.
Maslow JN, Whittam TS, Gilks CF, Wilson RA, Mulligan ME, Adams KS, Arbeit RD. Clonal relationships among bloodstream isolates of *Escherichia coli*. Infect Immun 1995;63:2409-2427.
Matsuki T, Watanabe K, Fujimoto J, Miyamoto Y, Takada T, Matsumoto K, Oyaizu H, Tanaka R. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl Environ Microbiol 2002;68:5445-5451.
Matthew M. Plasmid-mediated beta-lactamases of Gram-negative bacteria: properties and distribution. J Antimicrob Chemother 1979;5:349-358.
Maxwell PR, Rink E, Kumar D, Mendall MA. Antibiotics increase functional abdominal symptoms. Am J Gastroenterol 2002;97:104-108.
McCartney AL, Wenzhi W, Tannock GW. Molecular analysis of the composition of the bifidobacterial and lactobacillus microflora of humans. Appl Environ Microbiol 1996;62:4608-4613.
McCartney AL. Application of molecular biological methods for studying probiotics and the gut flora. Br J Nutr 2002;88(Suppl 1):S29-S37.
McDonald LC, Chen FJ, Lo HJ, Yin HC, Lu PL, Huang CH, Chen P, Lauderdale TL, Ho M. Emergence of reduced susceptibility and resistance to fluoroquinolones in *Escherichia coli* in Taiwan and contributions of distinct selective pressures. Antimicrob Agents Chemother 2001;45:3084-3091.
Medeiros AA. Evolution and dissemination of beta-lactamases accelerated by generations of beta-lactam antibiotics. Clin Infect Dis 1997;24(Suppl 1):S19-S45.

Midtvedt T. Monitoring the functional state of the microflora, p. 515–519. In: Hattori T, Ishida Y, Maruyama Y, Mortia RY, Uchida A (ed), Recent Advances in Microbial Ecology. Japan Sci Soc Press, Tokyo, Japan, 1989.
Mims C, Playfear J, Roitt I, Wakelin D, Williams R. Medical microbiology, 2nd ed. Mosby, London, UK, 1998.
Mitsuoka, T. Intestinal flora and aging. Nut Rev 1992;50:438-446.
Miyauchi S, Gopal E, Fei YJ, Ganapathy V. Functional identification of SLC5A8, a tumor suppressor down-regulated in colon cancer, as a Na(+)-coupled transporter for short-chain fatty acids. J Biol Chem 2004;279:13293-13296.
Moody FG, Haley-Russell D, Muncy DM. Intestinal transit and bacterial translocation in obstructive pancreatitis. Dig Dis Sci 1995;40:1798-1804.
Moore WE, Holdeman LV. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. Appl Microbiol 1974;27:961–979.
Moore WE, Moore LH. Intestinal floras of populations that have a high risk of colon cancer. Appl Environ Microbiol 1995;61:3202-3207.
Münnich A, Lübke-Becker A. *Escherichia coli* infections in newborn puppies-clinical and epidemiological investigations. Theriogenology 2004;62:562-575.
Murray PR, Baron EJ, Jorgensen JH, Pfaller MA, Yolken RH. Manual of clinical microbiology, 8th ed. ASM Press, Washington DC, USA, 2003.
Muyzer G, de Waal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 1993;59:695-700.
Namavar F, Theunissen EB, Verweij-Van Vught AM, Peerbooms PG, Bal M, Hoitsma HF, MacLaren DM. Epidemiology of the *Bacteroides fragilis* group in the colonic flora in 10 patients with colonic cancer. J Med Microbiol 1989;29:171-176.
National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing; twelfth informational supplement: Approved standard M2-A7- Disk diffusion, NCCLS, Wayne, PA, USA, 2000.
Nelson GM, George SE. Comparison of media for selection and enumeration of mouse fecal flora populations. J Microbiol Methods 1995;22:293-300.
Neuvonen PJ, Himberg J-J, Iisalo E, Mattila M.J, Ylitalo P. Kliininen farmakologia. Kandidaattikustannus Oy, Helsinki, Finland, 1994.
Nijsten R, London N, van den Bogaard A, Stobberingh E. *In-vivo* transfer of resistance plasmids in rat, human or pig-derived intestinal flora using a rat model. J Antimicrob Chemother 1995;36:975-985.
Nissen H, Dainty R. Comparison of the use of rRNA probes and conventional methods in identifying strains of *Lactobacillus sake* and *Lactobacillus curvatus* isolated from meat. Int J Food Microbiol 1995;25:311–315.
Nord CE, Kager L. The normal flora of the gastrointestinal tract. Neth J Med 1984;27:249-252.
Nord CE. Mechanisms of beta-lactam resistance in anaerobic bacteria. Rev Infect Dis 1986a;8(Suppl 5):S543-548.
Nord CE, Heimdahl A. Impact of orally administered antimicrobial agents on human oropharyngeal and colonic microflora. J Antimicrob Chemother 1986b;18(Suppl C):159-164.
Noverr MC, Noggle RM, Toews GB, Huffnagle GB. Role of antibiotics and fungal microbiota in driving pulmonary allergic responses. Infect Immun 2004;72:4996-5003.
Onoue M, Kado S, Sakaitani Y, Uchida K, Morotomi M. Specific species of intestinal bacteria influence the induction of aberrant crypt foci by 1,2-dimethylhydrazine in rats. Cancer Lett 1997;113:179-186.
Ora J. Hyvällä hygienialla miljoonasäästöt. Suomen lääkärilehti 2005;10:1135.
O'Sullivan DJ. Methods for analysis of the intestinal microflora. In Probiotics. A Critical Review, pp. 23–44. GW Tannock (ed). Horizon Scientific Press, Norfolk, UK, 1999.
Pajecki D, Zilberstein B, dos Santos MA, Ubriaco JA, Quintanilha AG, Cecconello I, Gama-Rodrigues J. Megaesophagus microbiota: a qualitative and quantitative analysis. J Gastrointest Surg 2002;6:723-729.
Pei Z, Bini EJ, Yang L, Zhou M, Francois F, Blaser MJ. Bacterial biota in the human distal esophagus. Proc Natl Acad Sci USA 2004;101(12):4250-4255.

Peltonen R, Ling WH, Hänninen O, Eerola E. An uncooked vegan diet shifts the profile of human fecal microflora: computerized analysis of direct stool sample gas-liquid chromatography profiles of bacterial cellular fatty acids. Appl Environ Microbiol 1992;58:3660-3666.

Phillips I, Casewell M, Cox T, De Groot B, Friis C, Jones R, Nightingale C, Preston R, Waddell J. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. J Antimicrob Chemother 2004;53:28-52.

Piddock LJ. Does the use of antimicrobial agents in veterinary medicine and animal husbandry select antibiotic-resistant bacteria that infect man and compromise antimicrobial chemotherapy? J Antimicrob Chemother 1996;38:1-3.

Plaut AG, Gorbach SL, Nahas L, Weinstein L, Spanknebel G, Levitan R. Intestinal microflora III. The microbial flora of human small intestinal mucosa and fluids. Gastroenterology 1967;53:868-873.

Poirel L, Mammeri H, Nordmann P. TEM-121, a novel complex mutant of TEM-type beta-lactamase from *Enterobacter aerogenes*. Antimicrob Agents Chemother 2004;48:4528-4531.

Poole K. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. J Mol Microbiol Biotechnol. 2001;3:255-264.

Poxton IR, Brown R, Sawyerr A, Ferguson A. Mucosa-associated bacterial flora of the human colon. J Med Microbiol 1997;46:85-91.

Poxton IR. *Clostridium difficile*. J Med Microbiol 2005;54:97-100.

Prins Jm, Kujper EJ, Mevissen MLCM, Speelman P, van Deventer SJH. Release of tumor necrosis factoe alpha and interleukin 6 during antibiotic killing of *Escherichia coli* in whole blood: influence of antibiotic class, antibiotic concentration, and presence of septic serum. Infect Imm 1985;63:2236-2242.

Rastegar LA, Gold LF, Borderon JC, Laugier J, Lafont J-P. Implantation and *in vivo* antagonistic effects of antibiotic susceptible *Escherichia coli* strains administered to premature newborns. Biol Neonate 1990;56:73-78.

Reddy BS, Rivenson A. Inhibitory effect of *Bifidobacterium longum* on colon, mammary, and liver carcinogenesis induced by 2-amino-3-methylimidazo[4,5-f]quinoline, a food mutagen. Cancer Res 1993;53:3914-3918.

Reguera JA, Baquero F, Perez-Diaz JC, Martinez JL. Factors determining resistance to beta-lactam combined with beta-lactamase inhibitors in *Escherichia coli*. J Antimicrob Chemother 1991;27:569-575.

Rembacken BJ, Snelling AM, Hawkey PM, Chalmers DM, Axon AT. Non-pathogenic *Escherichia coli* versus mesalazine for the treatment of ulcerative colitis: a randomised trial. Lancet 1999;354:635-639.

Rigottier-Gois L, Le Bourhis A-G, Gramet G, Rochet V, Doré J. Fluorescent hybridisation combined with flow cytometry and hybridisation of total RNA to analyse the composition of microbial communities in human faeces using 16S rRNA probes. FEMS Microbiology Ecology 2003;43:237-245.

Rinkinen ML, Koort JM, Ouwehand AC, Westermarck E, Björkroth KJ. *Streptococcus alactolyticus* is the dominating culturable lactic acid bacterium species in canine jejunum and feces of four fistulated dogs. FEMS Microbiol Lett 2004;230:35-39.

Rutgers HC, Batt RM, Proud FJ, Sorensen SH, Elwood CM, Petrie G, Matthewman LA, Forster-van Hijfte MA, Boswood A, Entwistle M, Fensome RH. Intestinal permeability and function in dogs with small intestinal bacterial overgrowth. J Small Anim Pract. 1996;37:428-434.

Sanz M, Lau L, Herrera D, Morillo JM, Silva A. Methods of detection of *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis* and *Tannerella forsythensis* in periodontal microbiology, with special emphasis on advanced molecular techniques: a review. J Clin Periodontol. 2004;31:1034-1047.

Satokari RM, Vaughan EE, Smidt H, Saarela M, Mättö J, de Vos WM. Molecular approaches for the detection and identification of bifidobacteria and lactobacilli in the human gastrointestinal tract. Syst Appl Microbiol 2003;26:572-584.

Savage DC, Dubos R, Schaedler RW. The gastrointestinal epithelium and its autochthonous bacterial flora. J Exp Med 1968;127:67–76.

Savage DC. Microbial ecology of the gastrointestinal tract. Annu Rev Microbiol 1977;31:107-133.

Schaberg DR, Culver DH, Gaynes RP. Major trends in the microbial etiology of nosocomial infection. Am J Med 1991;91:72S-75S.

Seppälä H, Klaukka T, Vuopio-Varkila J, Muotiala A, Helenius H, Lager K, Huovinen P. The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland. Finnish Study Group for Antimicrobial Resistance. N Engl J Med 1997;337:441-446.

Sghir A, Gramet G, Suau A, Rochet V, Pochart P, Dore J. Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. Appl Environ Microbiol 2000;66:2263-2266.

Shanahan PM, Thomson CJ, Amyes SG. Beta-lactam resistance in aerobic faecal flora from general practice patients in the UK. Eur J Clin Microbiol Infect Dis 1994;13:760-763.

Sharma R, Sharma CL, Kapoor B. Antibacterial resistance: Current problems and possible solutions. Indian J Med Sci. 2005;59:120-129.

Shiner M, Waters TE, Gray JDA. Culture studies of the gastrointestinal tract with a newly devised capsule. Gastroenterology 1963;45:625-632.

Shoemaker NB, Vlamakis H, Hayes K, Salyers AA. Evidence for extensive resistance gene transfer among *Bacteroides* spp. and among *Bacteroides* and other genera in the human colon. Appl Environ Microbiol 2001;67:561-568.

Simon GL, Gorbach SL. Intestinal flora in health and disease. Gastroenterology 1984;86:174-193.

Simon GL, Gorbach SL. The human intestinal microflora. Dig Dis Sci 1986;31(Suppl):147-162.

Singer RS, Sischo WM, Carpenter TE. Exploration of biases that affect the interpretation of restriction fragment patterns produced by pulsed-field gel electrophoresis. J Clin Microbiol 2004;42:5502-5511.

Singh J, Rivenson A, Tomita M, Shimamura S, Ishibashi N, Reddy BS. *Bifidobacterium longum*, a lactic acid-producing intestinal bacterium inhibits colon cancer and modulates the intermediate biomarkers of colon carcinogenesis. Carcinogenesis 1997;18:833-841.

Simpson JM, Martineau B, Jones WE, Ballam JM, Mackie RI. Characterization of fecal bacterial populations in canines: effects of age, breed and dietary fiber. Microb Ecol. 2002;44:186-197.

Sirot D, Recule C, Chaibi EB, Bret L, Croize J, Chanal-Claris C, Labia R, Sirot J. A complex mutant of TEM-1 beta-lactamase with mutations encountered in both IRT-4 and extended-spectrum TEM-15, produced by an *Escherichia coli* clinical isolate. Antimicrob Agents Chemother 1997;41:1322-1325.

Sjölund M, Wreiber K, Andersson DI, Blaser MJ, Engstrand L. Long-term persistence of resistant *Enterococcus* species after antibiotics to eradicate *Helicobacter pylori*. Ann Intern Med 2003;139:483-487.

Smith HW. Observations on the flora of the alimentary tract of animals and factors affecting its composition. J Path Bact 1965;89:95-122.

Smith EA, Macfarlane GT. Enumeration of human colonic bacteria producing phenolic and indolic compounds: effects of pH, carbohydrate availability and retention time on dissimilatory aromatic amino acid metabolism. J Appl Bacteriol 1996;81:288-302.

Sneath PHA, Mair NS, Sharpe ME, Holt JG. Bergey's manual of systematic bacteriology, vol 2. Williams & Wilkins, Baltimore, MD, USA, 1986.

Stackebrandt E, Liesack W, Goebel BM. Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. FASEB J 1993;7:232-236.

Stappenbeck TS, Hooper LV, Gordon JI. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. Proc Natl Acad Sci USA 2002;99:15451-1545.

Stark PL, Lee A, Parsonage BD. Colonization of the large bowel by *Clostridium difficile* in healthy infants: quantitative study. Infect Immun. 1982;35:895-899.

Stark C, Edlund C, Hedberg M, Nord CE. Induction of beta-lactamase production in the anaerobic microflora by cefoxitin. Clin Infect Dis 1995;20(Suppl 2):S350-S351.

Stevens CE, Hume ID. Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. Physiol Rev. 1998;78:393-427.

Stiefel U, Pultz NJ, Harmoinen J, Koski P, Lindevall K, Helfand MS, Donskey CJ. Oral administration of beta-lactamase preserves colonization resistance of piperacillin-treated mice. J Infect Dis 2003;188:1605-1609.

Strachan DP. Hay fever, hygiene, and household size. BMJ 1989;299:1259-1260.

Stotzer PO, A Brandberg, and AF Kilander. Diagnosis of small intestinal bacterial overgrowth in

clinical praxis: a comparison of the culture of small bowel aspirate, duodenal biopsies and gastric aspirate. Hepatogastroenterol 1998;45:1018-1022.
Suau A, Bonnet R, Sutren M, Godon JJ, Gibson G, Collins MD, Doré J. Direct rDNA community analysis reveals a myriad of novel bacterial lineages within the human gut. Appl Environ Microbiol 1999;65:4799-4807.
Suchodolski JS, Ruaux CG, Steiner JM, Fetz K, Williams DA. Application of molecular fingerprinting for qualitative assessment of small-intestinal bacterial diversity in dogs. J Clin Microbiol. 2004;42:4702-8.
Sullivan Å, Edlund C, Nord CE. Effect of antimicrobial agents on the ecological balance of human microflora. Lancet Infect Dis 2001;1:101-114.
Sullivan Å, Törnblom H, Lindberg G, Hammarlund B, Palmgren A-C, Einarsson C, Nord CE. The microflora of the small bowel in health and disease. Anaerobe 2003;9:11-14.
Sunde M, Sorum H. Characterization of integrons in *Escherichia coli* of the normal intestinal flora of swine. Microb Drug Resist 1999;5:279-287.
Suomen eläinlääkkeet. pp 76. RT-Print Oy, Pieksämäki, Finland, 2003.
Sutter VR, Citron DM, Edelstein MAC, Finegold SM. Wadsworth Anaerobic bacteriology manual 4th ed. Star Publishing, Belmont, CA, USA, 1985.
Swidsinski A, Ladhoff A, Pernthaler A, Swidsinski S, Loening-Baucke V, Ortner M, Weber J, Hoffmann U, Schreiber S, Dietel M, Lochs H. Mucosal flora in inflammatory bowel disease. Gastroenterology 2002;122:44-54.
Tanaka K, Ishikawa H. Role of intestinal bacterial flora in oral tolerance induction. Histol Histopathol 2004;19:907-914.
Tannock GW. Analysis of the intestinal microflora: a renaissance. Antonie Van Leeuwenhoek 1999a;76:265-278.
Tannock GW. The normal microbiota: an introduction, p. 1-23. In: Tannock GW (ed). Probiotics: a critical review. Horizon Scientific Press, Wymondham, UK, 1999b.
Tannock GW. The bowel microflora: an important source of urinary tract pathogens. World J Urol. 1999c;17:339-344.
Tannock GW. Molecular assessment of intestinal microflora. Am J Clin Nutr 2001;73(2 Suppl):410S-414S.
Tannock GW, Munro K, Bibiloni R, Simon MA, Hargreaves P, Gopal P, Harmsen H, Welling G. Impact of consumption of oligosaccharide-containing biscuits on the fecal microbiota of humans. Appl Environ Microbiol 2004;70:2129-2136.
Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 1995;33:2233-2239.
Thomson CJ, Tait S, Jesudason MV, Amyes SG. Dissemination of the TEM-I beta-lactamase gene into disparate plasmids in faecal strains of *Escherichia coli* isolated in Vellore, south India. J Infect 1993;27:47-50.
Thompson JS, Quigley EM, Adrian TE. Qualitative changes in enteric flora and short-chain fatty acids after intestinal resection. Dig Dis Sci. 1998;43:624-631.
Toivanen P, Vaahtovuo J, Eerola E. Influence of major histocompatibility complex on bacterial composition of fecal flora. Infect Immun 2001;69:2372-2377.
Topping DL, Clifton PM. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol Rev 2001;81:1031-1064.
Tortorano AM, Peman J, Bernhardt H, Klingspor L, Kibbler CC, Faure O, Biraghi E, Canton E, Zimmermann K, Seaton S, Grillot R; ECMM Working Group on Candidaemia. Epidemiology of candidaemia in Europe: results of 28-month European Confederation of Medical Mycology (ECMM) hospital-based surveillance study. Eur J Clin Microbiol Infect Dis 2004;23:317-322.
Trott DJ, Filippich LJ, Bensink JC, Downs MT, McKenzie SE, Townsend KM, Moss SM, Chin JJ. Canine model for investigating the impact of oral enrofloxacin on commensal coliforms and colonization with multidrug-resistant *Escherichia coli*. J Med Microbiol 2004;53:439-443.
Van de Merwe JP, Stegeman JH, Hazenberg MP. The resident faecal flora is determined by genetic characteristics of the host. Implications for Crohn's disease? Antonie Leewenhoek 1993;49:119-124.
Van Dijk JE. Morhology of the gut barrier. Eur J Compar Gastroenterol 1997;2:23-27.

Veringa EM, van der Waaij D. Biological inactivation by faeces of antimicrobial drugs applicable in selective decontamination of the digestive tract. J Antimicrob Chemother 1984;14:605-612.
Vollaard EJ, Clasener HA. Colonization resistance. Antimicrob Agents Chemother 1994;38:409-414.
Vree TB, Hekster YA, Baars AM, Van der Kleijn E. Rapid determination of amoxycillin (clamoxyl) and ampicillin (penbritin) in body fluids of many by means of high-performance liquid chromatography. J Chromatogr 1978;145:496-501.
de Vries-Hospers H, Jansen G, Tonk R, Oenema D, van der Waaij D. The *in vitro* inactivation of thirteen beta-lactam antibiotics by other mechanisms than adsorption to faecal substance. Infection 1993;21:127-130.
van der Waaij D, JM Berghuis-de Vries, JEC Lekkerkerk-van der Wees. Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. J Hyg 1971;69:405-411.
van der Waaij D, de Vries-Hospers HG, Welling GW. The influence of antibiotics on gut colonization. J Antimicrob Chemother 1986;18(Suppl C):155-C158.
van der Waaij D, Nord CE. Development and persistence of multi-resistance to antibiotics in bacteria; an analysis and a new approach to this urgent problem. Int J Antimicrob Agents 2000;16:191-197.
Wachtershauser A, Stein J. Rationale for the luminal provision of butyrate in intestinal diseases. Eur J Nutr 2000;39:164-171.
Waldeck F. Funktionen des Magen-Darm-Kanals. In: Schmidt RF, Thews G. (ed). Physiologie des Menschen, 24 Aufl. Springer, Berlin, Germany, 1990.
Wang XD, Guo WD, Wang Q, Andersson R, Ekblad E, Soltesz V, Bengmark S. The association between enteric bacterial overgrowth and gastrointestinal motility after subtotal liver resection or portal vein obstruction in rats. Eur J Surg 1994;160:153-160.
Wang RF, Cao WW, Cerniglia CE. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl Environ Microbiol 1996;62:1242-1247.
Welling GW, Groen G. Inactivation of amoxycillin, clavulanate and the combination by faecal preparations from volunteers. J Antimicrob Chemother 1990;25:301-302.
Wang X, Heazlewood SP, Krause DO, Florin TH. Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. J Appl Microbiol 2003;95:508-520.
Whittam TS, Wolfe ML, Wachsmuth IK, Orskov F, Orskov I, Wilson RA. Clonal relationship among *Escherichia coli* strains that cause hemorrhagic colitis and infantile diarrhea. Infect Immun 1993;61:1619-1629.
Willard MD, Simpson RB, Fossum TW, Cohen ND, Delles EK, Kolp DL, Carey DP, Reinhart GA. Characterization of naturally developing small intestinal bacterial overgrowth in 16 German shepherd dogs. J Am Vet Med Assoc 1994;204:1201-1206.
Wilson KH, Blitchington RB. Human colonic biota studied by ribosomal DNA sequence analysis. Appl Environ Microbiol 1996;7:2273-2278.
Wilson KH, Ikeda JS, Blitchington RB. Phylogenetic placement of community members of human colonic biota. Clin Infect Dis 1997;25(Suppl 2):S114-S116.
Wilson KH, Wilson WJ, Radosevich JL, DeSantis TZ, Viswanathan VS, Kuczmarski TA, Andersen GL. High-density microarray of small-subunit ribosomal DNA probes. Appl Environ Microbiol 2002;68:2535-2541.
Wilsson-Rahmberg M, Jonsson O. Method for long-term intestinal access in the dog. Lab Anim 1997;31:231-240.
Wold AE. The hygiene hypothesis revised: is the rising frequency of allergy due to changes in the intestinal flora? Allergy 1998;53(46 Suppl):20-25.
Wostmann BS, Larkin C, Moriarty A, Bruckner-Kardoss E. Dietary intake, energy metabolism, and excretory losses of adult male germfree Wistar rats. Lab Anim Sci 1983;33:46–50.
Xu J, Gordon JI. Inaugural Article: Honor thy symbionts. Proc Natl Acad Sci USA 2003;100:10452-10459.
Yates CM, Pearce MC, Woolhouse ME, Amyes SG. High frequency transfer and horizontal spread of apramycin resistance in calf faecal *Escherichia coli*. J Antimicrob Chemother 2004;54:534-537.
Yi JH, Ni RY, Luo DD, Li SL. Intestinal flora translocation and overgrowth in upper gastrointestinal tract induced by hepatic failure. World J Gastroenterol 1999;5:327-329.
Zinkevich V, Beech IB. Screening of sulfate-reducing bacteria in colonoscopy samples from healthy and colitic human gut mucosa. FEMS Microbiol Ecol 2000;34:147-155.

Zoetendal EG, Akkermans AD, De Vos WM. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Appl Environ Microbiol 1998;64:3854-3859.

Zoetendal EG, Akkermans AD, Akkermans-van Vliet WM, de Visser J, de Vos WM. The host genotype affects the bacterial community in the human gastrointestinal tract. Microb Ecol Health Dis 2001;13:129–134.

Zoetendal EG, von Wright A, Vilpponen-Salmela T, Ben-Amor K, Akkermans AD, de Vos WM. Mucosa-associated bacteria in the human gastrointestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. Appl Environ Microbiol 2002;68:3401-3407.

Zoetendal EG, Collier CT, Koike S, Mackie RI, Gaskins HR. Molecular ecological analysis of the gastrointestinal microbiota: a review. J Nutr 2004;134:465-472.

Österblad M, Hakanen A, Manninen R, Leistevuo T, Peltonen R, Meurman O, Huovinen P, Kotilainen P. A between-species comparison of antimicrobial resistance in enterobacteria in fecal flora. Antimicrob Agents Chemother 2000;44:1479-1484.