SARI TUOMISTO

Intestinal Bacteria

Post-mortem changes, migration and association with alcoholic liver cirrhosis

■

ACADEMIC DISSERTATION
To be presented, with the permission of
the Board of the School of Medicine of the University of Tampere,
for public discussion in the Small Auditorium of Building B,
School of Medicine of the University of Tampere,
Medisiinarinkatu 3, Tampere, on November 1st, 2014, at 12 o'clock.

UNIVERSITY OF TAMPERE

SARI TUOMISTO

Intestinal Bacteria

Post-mortem changes, migration and association with alcoholic liver cirrhosis

Acta Universitatis Tamperensis 1977
Tampere University Press
Tampere 2014

TAMPEREEN
YLIOPISTO

ACADEMIC DISSERTATION
University of Tampere, School of Medicine
Tampere Graduate Program in Biomedicine and Biotechnology (TGPBB)
Finland

Supervised by
Professor Pekka J Karhunen
University of Tampere
Finland
Docent Tanja Pessi
University of Tampere
Finland

Reviewed by
Professor Airi Palva
University of Helsinki
Finland
Professor Helena Isoniemi
University of Helsinki
Finland

The originality of this thesis has been checked using the Turnitin OriginalityCheck service in accordance with the quality management system of the University of Tampere.

Distributor:
kirjamyynti@juvenes.fi
http://granum.uta.fi

Cover design by
Mikko Reinikka

Layout by
Sirpa Randell

Acta Universitatis Tamperensis 1977
ISBN 978-951-44-9579-3 (print)
ISSN-L 1455-1616
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 1464
ISBN 978-951-44-9580-9 (pdf)
ISSN 1456-954X
http://tampub.uta.fi

Suomen Yliopistopaino Oy – Juvenes Print
Tampere 2014

441 729
Painotuote

Bad Digestion is the Root of All Evil
Hippocrates (400 B.C)

To my family

TABLE OF CONTENTS

ABSTRACT

The intestinal microbiota, that has been estimated to consist of around 10^{14} bacteria, is known to affect the well-being of the host, and has been linked to various diseases such as alcoholic liver cirrhosis. Alcohol has been hypothesised to alter intestinal bacterial populations and enhance bacterial migration from the intestine into the liver. These bacteria and their residuals could then promote chronic inflammation that could eventually lead to fibrosis and cirrhosis.

After death, bacteria start to migrate into the organs as a part of normal putrefaction. Bacterial migration, *i.e.*, translocation from the intestinal lumen into the blood and visceral organs, occurs also normally during life. The time sequence of post-mortem changes in intestinal bacterial populations and their migration into organs has not been extensively studied. Therefore, it is not known whether results obtained from post-mortem samples reflect conditions during life.

The aim of this doctoral thesis was to examine time-dependent changes in the intestinal microbiota after death and the translocation of these bacteria into tissues. Finally, we used these results to evaluate the role of intestinal bacteria in alcoholic liver cirrhosis.

Post-mortem changes in major intestinal bacterial groups (*Bacteroides* spp., *Bifidobacterium* spp., *Clostridium coccoides*, *Clostridium leptum* group, *Enterobactericeae*, *Lactobacillus* spp.) and *Streptococcus* spp. were evaluated in 61 rectal and ceacal samples obtained at autopsy and in 7 healthy volunteers using quantitative real-time PCR (RT-qPCR, Study I). Bacterial migration after death into the liver, mesenteric lymph nodes, pericardium, portal vein and peripheral blood was examined in a time-dependent manner with RT-qPCR and conventional bacterial culturing in 33 post-mortem samples. RT-qPCR was used to study the following bacterial groups: *Bacteroides* spp., *Bifidobacterium* spp., *Clostridium leptum* group, *Clostridium coccoides* group, *Enterobacter* spp., *Staphylococcus* spp. and *Streptococcus* spp. (Study II). The composition of selected intestinal bacteria (*Bacteroides* spp., *Bifidobacterium* spp., *Clostridium leptum* group, *Enterobactericeae* and *Lactobacillus* spp.) was investigated in 42 rectal samples obtained at autopsy (13 cirrhotics, 15 alcoholics, and 14 controls) and in faecal samples from 7 healthy volunteers (Study III). Intestinal bacterial translocation into the liver (n=42) and its association with cirrhosis and ascites (n=12) was also studied with RT-qPCR and CD14 immunohistochemistry.

Our results show that the relative amounts of *Bacteroides* spp., *Bifidobacterium* spp., *Clostridium* spp., *Streptococcus* spp., *Enterobactericeae* and *Lactobacillus* spp. remained stable in the rectum for up to 5 days after death (Study I). The proportions of the major intestinal bacteria populations in rectal autopsy samples were comparable to faecal samples from living persons. In contrast, post-mortem caecal samples proved to be microbiologically unreliable, as significant time-dependent post-mortem differences were observed in bacterial populations of *Bacteroides* spp. ($p=0.014$) and *Lactobacillus* spp. ($p=0.024$, Study I). Bacterial culturing and RT-qPCR analyses revealed the presence of bacteria in peripheral blood soon after death as a result of post-mortem bacterial migration. Outside the intestinal tract, the most reliable post-mortem microbiological sampling sites at up to 5 days after death were the pericardial fluid and the liver (Study II). Based on the results of Studies I and II, cases over 5 days post-mortem were excluded from Study III, and RT-qPCR measurements of *Bacteroides* spp., *Bifidobacterium* spp., *Clostridium leptum* group, *Enterobactericeae* and *Lactobacillus* spp. were selected for the samples. Alcoholic liver cirrhotics harboured 27 times more DNA of *Enterobactericeae* in their faeces than living controls ($p=0.011$). Genomes of this bacterial species were also found in the liver and ascites samples from cirrhotics (Study III). The total amount of bacterial DNA was associated with the level of CD14 expression ($p=0.045$). Furthermore, the CD14 expression percentage was statistically significantly higher in cirrhotic livers than in autopsy controls ($p=0.004$, Study III).

In conclusion, post-mortem samples from the rectum and liver can be used in basic research if taken within 5 days after death. Alcoholic cirrhotics harboured more gram-negative *Enterobactericeae* in their faeces and had higher amounts of *Enterobactericeae* in the liver. This suggests the possible involvement of intestinal bacteria in the development of alcoholic liver cirrhosis.

TIIVISTELMÄ

Suoliston mikrobioston, jonka on arvioitu koostuvan noin 10^{14} bakteerista, tiedetään vaikuttavan isäntänsä hyvinvointiin ja se on myös liitetty erilaisiin sairauksiin kuten alkoholimaksakirroosiin. On oletettu, että alkoholi muuttaa suoliston bakteeriston koostumusta ja lisää bakteerien kulkeutumista suolistosta maksaan. Nämä bakteerit ja niiden jäännökset voisivat sitten aiheuttaa maksassa kroonisen tulehdustilan, joka voi lopulta johtaa fibroosiin ja kirroosiin.

Kuoleman jälkeen bakteerit alkavat siirtyä elimiin osana luonnollista mätänemisprosessia. Translokaatiota eli bakteerien siirtymistä suolen ontelosta vereen ja sisäelimiin tapahtuu normaalisti myös elämän aikana. Kuoleman jälkeisiä suoliston bakteeriryhmien muutoksia ja näiden bakteerien kulkeutumista elimiin on tutkittu vain vähän. Tämän vuoksi ei tiedetä, voidaanko kuoleman jälkeen otetuilla näytteillä tutkia elämänaikaisia tapahtumia.

Tämän väitöskirjatyön tarkoituksena on selvittää miten suoliston bakteeristo kuoleman jälkeen muuttuu ajan mukaan sekä miten nämä bakteerit kulkeutuvat kudoksiin. Sovelsimme näitä saatuja tuloksia suolistobakteerien roolin määrittämisessä alkoholimaksakirroosissa.

Kuoleman jälkeisiä muutoksia suoliston merkittävissä bakteeri populaatioissa (*Bacteroides* spp., *Bifidobacterium* spp., *Clostridium coccoides*, *Clostridium leptum* ryhmä, *Enterobactericeae*, *Lactobacillus* spp.) ja *Streptococcus* spp. määritettiin 61 ruumiinavauksesta kerätyissä peräsuoli- ja umpisuolinäytteissä sekä 7 terveen vapaaehtoisen ulostenäytteissä kvantitatiivisella, reaaliaikaisella PCR:llä (RT-qPCR, Osatyö I). Bakteerien kulkeutumista kuoleman jälkeisen ajan-funktiona maksaan, suolilieve imusolmukkeeseen, sydänpussinesteeseen, porttilaskimoon ja ääreisvereen tutkittiin RT-qPCR:llä ja soluviljelyllä 33 ruumiinavauksen näytteissä. RT-qPCR:llä tutkittiin bakteroideksien, bifidobakteerien, klostridien, enterobakteerien, stafylokokkien ja streptokokkien suhteellisia määriä (Osatyö II). Valikoitujen suoliston bakteeriryhmien koostumusta (*Bacteroides* spp., *Bifidobacterium* spp., *Clostridium leptum* ryhmä, *Enterobactericeae* ja *Lactobacillus* spp.) tutkittiin 42 vainajan peräsuolinäytteissä (alkoholimaksakirrootikkoja 13, alkoholisteja 15, kontrolleja 14) ja 7 terveen vapaaehtoisen ulosteissa (Osatyö III). Suoliston bakteerien kulkeutumista maksaan (n=42) ja näin osallistumista alkoholimaksakirroosiin syntyyn ja

kulkeutumista askitesnesteeseen (n=12) tutkittiin myös RT-qPCR:llä ja CD14 immunohistokemialla.

Tuloksemme osoittavat, että bakteroideksien, bifidobakteerien, klostridien, streptokokkien, *Enterobactericeae*-ryhmän ja laktobasillien suhteelliset määrät pysyivät vakaina 5 päivään kuoleman jälkeen (Osatyö I). Ruumiinavausten peräsuolinäytteet olivat verrattavissa elävien ihmisten ulostenäytteisiin. Kuoleman jälkeen otetut umpisuolinäytteet osoittautuivat epäluotettaviksi, koska bakteroideksien (p=0.014) ja laktobasillien (p=0.014) määrät muuttuivat merkitsevästi ajan funktiona (Osatyö I). Viljely ja RT-qPCR tunnistivat bakteereita ääreisveressä heti kuoleman jälkeen osoituksena bakteereiden nopeasta kulkeutumisesta. Parhaat mikrobiologiset kuolemanjälkeiset näytteenottopaikat olivat sydänpussineste ja maksa aina 5 päivään saakka kuoleman jälkeen (Osatyö II). Osatöiden I ja II tulosten perusteella, ruumiinavaukset, jotka olivat vanhempia, kuin 5 päivää jätettiin pois Osatyö III:sta, sekä tutkittaviksi bakteeriryhmiksi valittiin *Bacteroides* spp., *Bifidobacterium* spp., *Clostridium leptum* ryhmä, *Enterobactericeae* ja *Lactobacillus* spp.. Alkoholimaksakirrootikoilla oli 27-kertaisesti enemmän *Enterobactericeae*-ryhmän bakteerien DNA:ta ulostenäytteissään, kuin elävillä kontrolleilla (p=0.011). Kirrootikoilla *Enterobactericeae*-ryhmää löydettiin myös maksasta ja askitesnesteestä. Maksan kokonaisbakteeri-DNA:n määrä oli yhteneväinen maksan CD14 ilmentymisprosentin kanssa (p=0.045) sekä lisäksi CD14 ilmentymisprosentti oli kirrootikoilla merkitsevästi isompi (p=0.004), kuin kontrolleilla (Osatyö III).

Johtopäätöksenä voidaan sanoa, että kuoleman jälkeen otettuja näytteitä peräsuolesta ja maksasta voidaan käyttää perustutkimuksessa, jos ne otetaan 5 päivän kuluessa kuolemasta. Alkoholisti kirrootikoilla oli enemmän gram-negatiivisia *Enterobactericeae*-heimon lajeja ulosteessaan, kuin kontrolleilla. Tämän ryhmän bakteereja löydettiin enemmän myös kirrootikkojen maksa-näytteistä. Näiden tulosten perusteella voidaan arvella, että suoliston bakteereilla voi olla rooli alkoholimaksakirroosin synnyssä.

LIST OF ORIGINAL COMMUNICATIONS

This dissertation is based on the subsequent articles, which are referred to in the text by their Roman numerals (I–III).

I Tuomisto S, Karhunen PJ and Pessi T (2013): Time-dependent post mortem changes in the composition of intestinal bacteria using real-time quantitative PCR. Gut Pathogens 5, 35.

II Tuomisto S, Karhunen PJ, Vuento R, Aittoniemi J and Pessi T (2013): Evaluation of post mortem bacterial migration using culturing and real-time quantitative PCR. Journal of Forensic Sciences 58, 910-6.

III Tuomisto S, Pessi T, Collin P, Vuento R, Aittoniemi J and Karhunen PJ (2014): Changes in gut bacterial populations and their translocation into liver and ascites in alcoholic liver cirrhotics. BMC Gastroenterology 14, 40.

ABBREVIATIONS

ANOVA	Analysis of Variance
ADP	Adenosine Diphosphate
ALC	Alcoholic Liver Cirrhosis
ALD	Alcoholic Liver Disease
ATP	Adenosine Triphosphate
BMI	Body Mass Index
CD14	Cluster of Differentiation 14
CYP2E1	Cytochrome P450 2E1
FRET	Fluorescence Resonance Energy Transfer
GALT	Gut-Associated Lymphoid Tissue
GI	Gastrointestinal
HMP	Human Microbiome Project
IL	Interleukin
IRAK	Interleukin-Receptor-Associated Kinase
IκB	Inhibitor of NF-κB
IKKα/ß/μ	IκB Kinase
LBP	Lipopolysaccharide Binding Protein
LPS	Lipopolysaccharide, Endotoxin
LSD	Fisher's Least Significant Difference Test
MD-2	Myeloid Differentiation Protein 2
MLN	Mesenteric Lymph Node
MyD88	Myeloid Differentiation Primary Response Protein 88
NF-κB	Nuclear Factor-κB
Ns	Non-significant
PM	Post-mortem
rDNA	Ribosomal Deoxyribonucleic Acid
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
SCFA	Short Chain Fatty Acid

SOCS-1	Suppressor of Cytokine Signaling 1
Spp.	Species
TGF-β1	Transforming Growth Factor β1
TIRAP	TIR-Domain-Containing Adaptor Protein
TLR	Toll-Like Receptor
TNF-α	Tumour Necrosis Factor α
TRAF6	Tumour Necrosis Factor Receptor Associated Factor 6

INTRODUCTION

The human gastrointestinal (GI) tract can be divided into upper and lower segments. The upper GI tract consists of the oesophagus, stomach, and duodenum; the lower GI tract includes the jejunum, ileum, caecum, colon and rectum. The main tasks of the GI tract are food digestion and nutrient intake from the lumen into epithelial cells for host to use, the absorption of water and the removal of waste material from the body (Lindsay 1995). The normal composition of the human intestinal microbiota has not yet been totally described, mainly due to the richness of species and considerable inter- and intra-individual differences between persons (Eckburg et al. 2005, Ley et al. 2008, Turnbaugh et al. 2009). The complexity of the microbial intestinal habitat varies throughout the intestines, both longitudinally along the intestine and in the lumen compared to the mucosa (Leser and Molbak, 2009). Aerotolerant and acid-resistant bacteria are abundant in the upper GI tract, whereas strictly anaerobic bacteria are present in the lower GI tract. The density and diversity of bacteria also increases from the stomach to the colon, where transit of the intestinal contents is slower and bacteria can colonise more intensely.

It is currently known that intestinal bacteria play an important role in host normal well-being (Grenham et al. 2011). This interaction is very complicated and traditionally thought to be commensal (*i.e.*, beneficial for one, whereas the other remains unaffected) or nowadays sometimes even mutualistic (*i.e.*, beneficial for both). However, in some circumstances, when these resident bacteria opportunistically migrate through the intestinal mucosa, they can cause various harmful effects. It has been suggested that intestinal bacteria may play a role in the pathogenesis of alcoholic liver cirrhosis. Alcohol abuse can change the normal intestinal microbiota (Hauge et al. 1997). Due to bacterial overgrowth and metabolism, intestinal bacteria or their cell components may migrate into the liver, where they can induce chronic inflammation, fibrosis and eventually cirrhosis.

Traditionally, it has been believed that during life, bacteria are only present on mucosal surfaces; inner organs, such as the blood and liver, have been considered bacteria-free, *i.e.*, sterile. After death, epithelial integrity and the host's natural defences against bacteria vanish, allowing bacterial migration through epithelia into the blood and surrounding tissues. Thus, the degradation of the body starts as a part of normal putrefaction.

After death, it is easy to obtain samples that would be unethical to collect from living individuals, *e.g.*, simultaneous biopsies of the liver and intestine. Using post-mortem

samples, different disease mechanisms can be studied more intensively. Studies with post-mortem tissue and blood samples have been conducted (Morris et al. 2006, Tsokos and Puschel 2001, Weber et al. 2010), but post-mortem intestinal composition of bacteria and studies of the usability of these samples for research purposes have not been performed almost at all.

This dissertation focuses on intestinal bacteria and their migration in alcoholic liver cirrhosis as well as on the usability and reliability of post-mortem samples obtained from the liver, pericardium, blood and mesenteric lymph nodes. The studies were performed in autopsy cases as well as living subjects and provide novel information about the usability and reliability of post-mortem bacteriological samples. In the first part, the usability of post-mortem intestinal samples for basic microbiological research was analysed. In the second part, a time-dependent investigation of post-mortem bacterial migration into different tissues was performed. In the third study, we aimed to investigate the association between changes in major intestinal bacterial populations and alcoholic liver cirrhosis.

REVIEW OF THE LITERATURE

1 Intestinal Microbiota

1.1 Overview of Intestinal Microbiota

The intestine can be separated into the small and large intestines. The small intestine consists of the duodenum, jejunum and ileum, whereas the large intestine includes the caecum, colon and rectum. The intestinal tract's main tasks are food digestion, intake of nutrients from the lumen into epithelial cells for host to use, the absorption of water and the removal of waste material from the body. Food digestion occurs mainly in the small intestine. The primary task of the large intestine is to salvage energy from carbohydrates not digested in the upper gut and to absorb water, sodium and any useful nutrients left in from the digested material (Lindsay 1995).

Since the days of Louis Pasteur and Ilya Mechnikov, microbiologists have emphasised the importance of understanding the effects of the intestinal microbiota on human health and well-being (Pai and Kang 2008). The intestinal microbiota is the largest and the most diverse of all the human microbiotas, which also include the microbiotas, *e.g.*, of the vagina, skin and mouth. The symbiotic interactions between the intestinal microbiota and human host are very complicated, and many kinds of different interactions exist. Mainly, the nature of these separate interactions remains still unknown. The intestinal bacteria-host relationship has traditionally for decades been described as a commensal (beneficial for one partner but meaningless for the other), rather than a mutualistic (beneficial for both) relationship. In other words, one member benefits (the host), whereas the other remains mainly unaffected (the bacterial residents) (Ley et al. 2008). Recently, intestinal bacteria have been recognized more as beneficial, changing the interaction for more as mutualistic (Garrett et al. 2010). Diet, host morphology and host phylogeny have affected the composition of the vertebrate gut microbiota; the human gut bacterial microbiota is therefore typical of an omnivorous primate (Ley et al. 2008). The intestinal microbiota alone counts for 3–5 million genes, 100 times more than in humans (Gill et al. 2006), belonging to approximately 1000 prevalent bacterial species, whose functions are only now being explored (Qin et al. 2010).

The intestinal microbiota begins to evolve already at birth, depending on the method of delivery (vaginal birth or Caesarean section) (Jakobsson et al. 2013). The composition of the microbiota also depends on other factors, *e.g.*, diet (Wu et al. 2011), gender (Markle

et al. 2013), habitat and residential area (Yatsunenko et al. 2012), age (Mitsuoka 1992), stress, antimicrobial medications (Angelakis et al. 2013) and genetics (Knights et al. 2013, Turnbaugh et al. 2009). Nutrition, host defences and disease all affect one another (Kelly 2010).

Projects such as the Human Microbiome Project (HMP) (Blaser 2010, NIH HMP Working Group et al. 2009) and MetaHIT (Qin et al. 2010) have already put much effort on characterising the human intestinal microbiota. Since 2007, the HMP has assembled a key reference data set of microbial genomes; however, information regarding the distributions and functions of these organisms is still lacking, and the effects of microbiome diversity on both health and disease have yet to be defined (Lewis et al. 2012, Proctor 2011).

1.2 Diversity of Intestinal Bacteria

The adult human GI tract contains all three domains of life (bacteria, archaea, and eukarya), and it has the highest cell density of any ecosystem (Whitman et al. 1998). Intestinal bacteria are taxonomically classified via the traditional biological nomenclature of phylum–class–order–family–genus–species–strains. Although 55 phyla of bacteria and 13 phyla of archaea have been described living on earth to date, only 9 bacterial phyla have been described in the intestine (Ley et al. 2006), of which 5 are rare (Backhed et al. 2005). These taxa include *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, *Verrucomicrobia* and *Cyanobacteria* (Backhed et al. 2005, Ley et al. 2006), of which *Bacteroidetes* and *Firmicutes* dominate, together accounting for approximately 85% of all sequences in the intestinal tract (Durban et al. 2011). In addition to *Bacteroidetes* and *Firmicutes*, the two most abundant bacterial phyla in the intestinal microbiota are *Actinobacteria* and *Proteobacteria* (Arumugam et al. 2011, NIH HMP Working Group et al. 2009).

Bacterial phylotypes in human GI tract mainly from faeacal samples have been attempted to be characterized. The count of bacterial phylotypes (defined as organisms whose rRNA sequences are 97% homology) is rising constantly but the final richess still remains unknown. Eckburg et al. identified 395 different bacterial phylotypes (Eckburg et al. 2005) whereas Claesson et al. more than 12 000 (Claesson et al. 2009). One complicating factor for evaluating bacterial diversity is that all the bacterial profiles are not shared with all individuals; even though all share a common bacterial core. Arumugan et al. divided intestinal bacterial populations into 3 different enterotypes (robust clusters) (Arumugan et al. 2011). Each of these enterotypes is identifiable by the variation in the levels of 1 of 3 bacterial genera: *Bacteroides*, *Prevotella* and *Ruminococcus* (Arumugan et al. 2011).

At the species level, the most commonly used estimate of the total amount of bacterial species is approximately 500 (Steinhoff et al. 2005, O'Hara et al. 2006), but the number has

been reported to vary between 300 (Guarner and Malagelada 2003) and 1000 (Sears 2005). It is thought that only 30–40 species account for 99% of the intestinal microbiota (Savage 2001). Major intestinal species are *Bacteroides* spp., *Bifidobacterium* spp. and clostridia (Delgado et al. 2004, Moore and Holdeman 1974a, Salyers 1984, Sears 2005). *Bacteroides* spp. alone make up approximately 30% of all intestinal bacteria (Salyers 1984, Sears 2005), *Bifidobacterium* spp. account for 1–5%, and *Lactobacillus* spp. comprise less than 1–2% of bacteria (Franks et al. 1998, Sghir et al. 2000). Other bacteria, such as enterobacteria, exist in minor numbers (Delgado et al. 2004). The amount of *Enterobactericeae* species is approximately 0.1% of all intestinal bacteria (Eckburg et al. 2005).

The major species in the small intestine, including the duodenum, jejunum and ileum, are aerotolerant species such as lactobacilli (Saito et al. 2004). In the large intestine, *i.e.*, the colon, the bacterial composition depends on the location: the first part of colon, the caecum, differs slightly from the rest of the colon. Marteau et al. (Marteau et al. 2001) demonstrated that the number of facultative anaerobes that tolerate small concentrations of oxygen, *e.g.*, *Escherichia* spp. and *Enterobacter* spp., are similar in caecal and faecal samples. However, obligatory anaerobes including *Bacteroides* spp., *Clostridium leptum*, and *Clostridium coccoides* are less common in the caecum than in the faeces. Compared to faeces, smaller numbers of bifidobacteria have been detected in the caecum (Marteau et al. 2001). The most abundant species in the colon are *Bacteroides* spp., *Bifidobacterium* spp. and clostridia bacteria belonging to the *Clostridium coccoides* (cluster XIVa) and *Clostridium leptum* groups (cluster IV) (Lay et al. 2005, Orrhage and Nord 2000). Bacterial cell density increases along the intestine (Table 1).

Table 1. Bacterial cell densities. Density levels are presented as bacteria per gram of content.

Site	Cell Density
STOMACH	10^1–10^4
SMALL INTESTINE	
Duodenum	10^4–10^5
Jejunum	10^5–10^7
Ileum	10^7–10^8
LARGE INTESTINE	
Caecum	10^{10}–10^{11}
Colon	10^{10}–10^{12}
Rectum	10^{11}–10^{12}

In addition to the longitudinal heterogenity of intestinal bacteria, differences in bacterial composition between the lumen and intestinal mucosa have been observed. For example, *Bacteroides* spp., *Bifidobacterium* spp., *Streptococcus* spp., *Enterococcus* spp., *Clostridium* spp., *Lactobacillus* spp. and members of *Enterobactericeae* can be found in feces, whereas

only *Clostridium* spp., *Lactobacillus* spp. and *Enterococcus* can be detected in in the mucosal layer and epithelial crypts (Swidsinsky et al. 2005).

1.3 Functions of Intestinal Bacteria

The intestinal microbiota plays a significant role in human well-being and health, due to its strong interactions with host. The host-microbe interaction occurs at the large surface of the intestinal epithelium, which measures approximately 400 m^2 (Lindsay 1995). The bacterial content of the intestine is only one cell layer apart from the host's interns (Lindsay 1995). The epithelium is the first line of defence; it regenerates itself constantly, and it actively discriminates between commensal bacteria and possible pathogens. This function is called a barrier effect. The intestinal barrier function is modified by the gut microbiota (Hooper and Gordon 2001, Kauet al. 2011) and *vice versa*. The microbiota significantly influences many structural, metabolic or protective host physiological and immunological processes.

The main beneficial functions of intestinal bacteria include: protection from invading pathogens (known as colonising resistance), supporting energy metabolism by digesting carbohydrates and proteins that the host cannot digest on its own, modulation of the function and structure of the immune system, vitamin (vitamin K and some B-vitamins) biosynthesis and mediation of the breakdown of dietary carcinogens (Backhed et al. 2004, Canny and McCormick 2008, Garrett et al. 2010, Hooper and Gordon 2001, Pai and Kang 2008).

1.3.1 Physical Functions of Intestinal Bacteria

Resident bacteria, as a part of the physical barrier, compete with pathogens for available nutrients in ecological niches, thereby maintaining the collective microenvironment (Canny and McCormick 2008) and so inhibiting the growth of other bacteria. Intestinal bacteria in the mucus can physically prevent the attachment of pathogenic bacteria and inhibit their entry into the epithelium (Canny and McCormick 2008). The intestinal bacteria produce acids that lower the pH of the gut wall, making it undesirable for harmful bacteria. Colonisation resistance, *i.e.*, preventing new microorganisms from surviving and multiplying in the gut lumen, thereby protecting the host against pathogens, is maintained in different ways. Anaerobic rods such as *Bacteroides* spp. and *Bifidobacteria* spp. maintain colonisation resistance and thus are considered beneficial (Backhed et al. 2005, Hooper et al. 2001), whereas *Staphylococcus* spp. are thought to be pathogenic for the gut barrier (Sekirov et al. 2010, Shimizu et al. 2006). Studies in germ-free mice have shown that

intestinal bacteria induce the proliferation of epithelial cells and increase the total intestinal surface area (Heyman et al. 1986).

Just as some nutrients enhance bacterial growth in the gut, other antibacterial metabolites produced by intestinal bacteria are known to be protective against invading pathogens (Pai and Kang 2008). Certain antimicrobial substances, such as bacteriocins and lactic acid, are produced by commensal bacteria, *e.g.*, *Lactobacillus* spp. (Sekirov et al. 2010). An antimicrobial peptide, thuricin, produced by *Bacillus thuringiensis*, has recently been shown to inhibit a small spectrum of *Clostridium difficile* species (Rea et al. 2010). *Bifidobacterium* inhibits translocation of the enterohaemorrhagic toxins produced by *Escherichia coli* from the intestine into the blood through the production of acetate (Fukuda et al. 2011); *Bacillus subtilis* activates cytoprotective heat shock proteins, thus protecting the host from oxidative injury to the intestinal epithelium and preserving barrier function (Fujiya et al. 2007).

Total or partial digestion of nutrients is one of the physical functions of the gut microbiota. Indigestible plant-derived polysaccharides, including cellulose, pectin, hemicellulose, and resistant starches, are digested into short chain fatty acids (SCFAs) by intestinal bacteria (Backhed et al. 2005). The main SCFAs produced are acetic acid, propionic acid and butyric acid (Vinolo et al. 2011). These provide a source of energy and nutrients for the host and are used by different organs (Pai and Kang 2008). SCFAs also have a role in controlling intestinal epithelial cell differentiation and proliferation. They also help to absorb essential dietary minerals (Grenham et al. 2011, Guarner and Malagelada 2003). SCFAs are also produced by intestinal bacteria themselves and are known to function as growth-inhibiting factors for many microorganisms, such as yeast and certain strains of *E. coli*. Beside carbohydrates, other substrates for microbial digestion include proteins, lipids, nucleic acids, dietary carcinogens and other polymers. It has been shown that certain intestinal strains of lactobacilli reinforce epithelial function and protein degradation (Pessi et al. 1998) as well as neutralise toxins (El-Nezami et al. 2000). It has been estimated that 100 g of such material is being degraded per day by bacteria (Cummings and Macfarlane 1997). Addition to this, intestinal microbiota can also digest up to 1 kg per day of substances produced by the host, such as epithelial cells, mucins or enzymes. By digesting substrates, the microbiota plays an important part in the host's energy balance by influencing the efficiency of the calorie harvest from digested food and how this harvested energy is used and stored (Turnbaugh et al. 2009). SCFAs also enhance lipogenesis in the liver (Rolandelli et al. 1989), eventually increasing fat uptake and storage in the host's body. It has been shown that a host needs a 30% increase in energy intake to maintain body weight in the absence of intestinal bacteria (Wostmann et al. 1983).

Intestinal microbiota influences peristalsis, the periodic movement of the intestine, by contributing to the development and maintenance of sensory and motor functions, including propulsive activity (Pai and Kang 2008). Other physical influences exerted by the

intestinal microbiota include enteric nerve regulation, promotion of angiogenesis (Holmes et al. 2011) and regulation of the growth and differentiation of intestinal epithelial cells (Morris et al. 2007, Pai and Kang 2008). Biological substances such as SCFAs, toxins and dietary carcinogens influence peristalsis.

Moreover, the gut microbiota also plays a major role in the breakdown of dietary carcinogens called heterocyclic amines, which are produced by cooking meat or fish at high temperatures (Azcarate-Peril et al. 2011, Humblot et al. 2007, O'Hara & Shanahan 2006), and in the biosynthesis of some vitamins. Members of the *Bacteroides*, *Eubacterium*, and *Propionibacterium* genera have a beneficial effect on the host by synthesising certain vitamins that the host cannot produce, *i.e.*, the B vitamins folate, B12, and biotin, as well as vitamin K (Canny and McCormick 2008).

1.3.2 Immune System Functions of Intestinal Bacteria

The intestinal microbiota influences the development and maturation of the immune system, beginning shortly after birth (Bouskra et al. 2008, Taschuk and Griebel 2012). It is known that germ-free mice have fewer B (plasma) cells, smaller Peyer's patches, fewer intraepithelial lymphocytes, and improperly functioning antimicrobial and immunoglobulin A (IgA) secretion (Round and Mazmanian 2009), all of which can be corrected with recolonisation by intestinal bacteria (Chung et al. 2012). Once the host immune system has developed and tolerance between bacteria and the host has evolved, the host regulates bacterial composition in the gut, and possibly harmful bacteria are recognised in a co-operative way. The discrimination between normal intestinal bacteria and possible pathogens is usually mediated by toll-like receptors (TLRs), which are pattern-recognition receptors situated in the intestinal epithelium. These receptors recognise pathogens by their different pathogen-associated molecular patterns (Sansonetti 2008), triggering the inflammatory cascade. Intestinal bacteria also have immunomodulatory activity, reflected in their influence on inflammatory mediators and immune regulatory cells (Pessi et al. 1998, Pessi et al. 2000, Pessi et al. 2001, Strauch et al. 2005). Signals from resident intestinal microbiota are required for normal immune development and maintenance of the epithelium and associated immune cells throughout life. Substances secreted by these bacteria can be stimulatory or inhibitory to the immune system (Fukuda et al. 2011, Mazmanian et al. 2005, Sokol et al. 2008).

Five types of differentiated cell lineages contribute to barrier function: goblet cells, Paneth cells, micro-fold (M) cells, enteroendocrine cells and absorptive enterocytes (Garrett et al. 2010). A thick mucus layer, produced by goblet cells, inhibits bacterial attachment into the epithelium (Davies et al. 1998). Paneth cells actively sense the microbiota, produce antimicrobial substances and activate TLRs. Enteroendocrine cells that work as important

sensors of luminal bacteria and absorptive enterocytes can produce antimicrobial substances (Garrett et al. 2010). Both cell types are involved in immunoglobulin transport though the epithelium and the incorporation of the secretory A component into IgA. M cells are important in pathogen handling and initiation of antibody production (Debard et al. 2001).

IgAs are important in maintaining the balance between gut microbiota and immune system of the host. IgA prevents aerobic bacteria from adhering to the enterocytes. These antibodies can be divided into two categories: IgA1 and IgA2; IgA2 is dominant in mucosal surfaces (Mestecky et al. 1999). IgAs are produced in the lamina propria (a highly vascular layer of connective tissue under the basement membrane lining a layer of intestinal epithelium), where they form complexes with immunoglobulin receptors expressed on epithelial cells. They are then transported across the epithelial cell into the apical surfaces near to the intestinal lumen (Wershil and Furuta 2008). IgA is then disengaged from the cell receptor, and it passes into the lumen. Secretory IgA prevents possible pathogens from penetrating the intestinal epithelium by neutralising toxins and infectious organisms, as well as by opsonisation (Mestecky et al. 1999). Secretory IgA also maintains gut homeostasis by regulating the composition of the intestinal microbiome and dampening local inflammation produced by bacteria (Fagarasan and Honjo 2004). It has been shown that the intestinal microbiota increases the production of duodenal IgA-plasmocytes (Moreau et al. 1978) and the number of enteroendocrine cells in the jejunal and colonic epithelium (Sharma and Schumacher 1995, Sharma et al. 1995), indicating enhancement of the production of secretory IgA and mucus.

Intestinal homeostasis is maintained by the intestinal microbiota; although the intestinal microbiota usually has many beneficial effects, homeostasis can sometimes be disrupted, and intestinal diseases can develop. Most intestinal bacteria are commensals or mutualistic; however, after translocation through the mucosa or under specific conditions, they can opportunistically cause pathogenic outcomes. Invading pathogens have developed different ways to enhance survival in the dense intestinal bacterial community, where the level of nutrients is modulated by the resident microbiota. Invading pathogens, like members of the normal microbiota, must compete for nutrients; some organisms, *e.g.*, *Vibrio cholerae*, *Campylobacter jejuni* and enterohaemorrhagic *E. coli*, have therefore developed their own strategies to utilise the unique metabolites found within the intestines to compete and induce virulence (Nakanishi et al. 2009, Schild et al. 2007, Velayudhan et al. 2004). To obtain beneficial molecules for their existence, some pathogens even interact directly with the host. Infection with *Salmonella typhimurium* generates a respiratory electron acceptor tetrathionate that provides a growth advantage for the bacterium (Winter et al. 2010, Winter and Baumler 2011). Some species of gut flora, such as some members of the genus *Bacteroides*, are able to change their surface receptors to mimic those of host cells and so escape the host's immune response (Guarner and Malagelada 2003, Steinhoff 2005).

Changes in the composition of the intestinal microbiota have also been shown to affect organs outside the GI tract. It has been suggested that gut microbiota may play a role in the development of diverse and complex diseases, *e.g.*, alcoholic liver cirrhosis (Chen et al. 2011) and atherosclerosis (Rosenfeld and Campbell 2011), due to abnormal interactions between commensal bacteria and the immune system (Rioux et al. 2005). Autism, diabetes, obesity and allergies are also thought to be caused by imbalances in intestinal microbiota populations (Willing et al. 2011). Autism has particularly been associated with the presence of *Clostridium* spp. (Finegold et al. 2002). The intestinal microbiota has also been associated with the development of obesity. In obesity, the correlation between weight and the *Bacteroidetes-Firmicutes* ratio has been shown to be altered (Turnbaugh et al. 2006), with a decrease in the amount of *Bacteroidetes* and an increase in *Firmicutes* (Ley et al. 2005). In obesity, the microbial population in the intestine harbours a notably greater number of genes coding for enzymes that take part in the degradation of polysaccharides than in normal-weight individuals (Turnbaugh et al. 2006). Although much is known about bacterial involvement in the development of different diseases, further studies are needed to understand and fully clarify the associations between the intestinal microbiota and illnesses.

1.4 Methods for Studying Intestinal Bacteria

The normal composition of the human intestinal microbiota has not yet been totally resolved, mainly due to the large number of species present and significant inter- and intra-individual differences (Eckburg et al. 2005, Ley et al. 2008, Turnbaugh et al. 2009). The methodological limitations of conventional bacterial culture have also hindered efforts to describe the spectrum of intestinal bacteria. Traditionally, the composition of the human intestinal microbiota has been determined by conventional culturing. It is estimated that only 40–30% of bacteria living in the human intestinal tract can be cultured with current methods (Hayashi et al. 2002). As most intestinal bacteria being anaerobes, they won't grow on normal plates (Eckburg et al. 2005, Rinttilä et al. 2004). Further, identification of bacteria with, *e.g.*, gram staining, biochemical tests, specific media or antibiotics, can only be done below the genus level. However, as it is relatively inexpensive, widely available and allows quantification of bacterial populations, culturing has provided a good indication of ecosystem complexity, if it is carried out by skilled and experienced microbiologists (Furrie 2006). As a method, culturing is a relatively slow and results are widely affected by media and growth conditions. Traditional culturing may also not provide any information regarding the microbe of interest if the bacteria in the samples have been exposed to unfavourable or pernicious conditions that disrupt or end growth. Thus, the reliability of culturing has been questioned due to the possibility of false negative results (Alain and Querellou 2009).

New methods have been discovered to provide quicker and more accurate identification of intestinal bacteria, mainly for research purposes. Culture-independent polymerase chain reaction-based methods, such as amplification of bacterial RNA or DNA sequencies (Furet et al. 2009) are widely used. A major advantage of DNA-based detection methods is that microorganisms do not need to be cultured before analysis, resulting in a more accurate picture of microbial diversity and population composition. Other widely used molecular microbiological methods for studying intestinal bacteria are sequencing-based methods such as pyrosequencing (Claesson et al. 2009), shotgun sequencing (Sanger sequencing) (Eisen 2007) and microbial metagenomics (Manichanh et al. 2006, National Research Council (US) Committee on Metagenomics: Challenges and Functional Applications, 2007, Schloss and Handelsman 2005), which are capable of producing a huge amount of sequence data in a relatively short time. Metagenomics can identify the different species of bacteria that are present in a sample, but not the relative amounts of each species (Furrie 2006).

1.4.1 Real-Time Quantitative PCR

Real-time quantitative PCR (RT-qPCR), a technology used to quantify DNA sequences amplified by PCR, has revolutionised human intestinal bacteria reseach, because both PCR chemistry and fluorescent probe detection of the desired microbe can be performed in the same reaction at the same time. This saves both money and time. As the nucleic acid amplification and detection reactions are carried out in the same tube, the risk of contamination of subsequent analyses is reduced (Espy et al. 2006). The three most commonly used technologies for nucleic acid detection in the microbiological RT-qPCR reaction are molecular beacons, fluorescence resonance energy transfer (FRET) hybridisation and 5′ Nuclease (TaqMan) probes, which are based on the transfer of light energy between two different dye molecules, known as fluorescence resonance energy transfer (Espy et al. 2006). FRET hybridisation consists of two probes, whereas in molecular beacons and TaqMan probes, two dye molecules are attached to one probe. All these probes are collectively known as FRET probes, even though the term specifically refers to the FRET hybridisation method. The first dye is fluorescent, and the second is a quencher or another fluorescent dye that is able to absorb that fluorescent light from the first dye and to re-emit it at a different wavelength (Espy et al. 2006). The first fluorescent probe made for RT-qPCR was the 5′ nuclease probe that is generally known as that TaqMan probe. The TaqMan probe is a short DNA oligonucleotide that contains a fluorescent dye at the 5′ end of the molecule and a quencher dye at the 3′ end. As the probe binds to a complementary DNA strand during the PCR reaction, the 5′ end is cleaved by Taq polymerase activity, separating the quencher from the fluorescent dye. The separated 5′ dye can be detected as an

emitted light signal. The cleaved fluorescent dye accumulates after each PCR temperature step and can be measured at any time, unlike molecular beacons and FRET hybridisation, where fluorescence can be measured only in the hybridisation step (Espy et al. 2006).

There are two different methods for performing RT-qPCR quantification of the target: absolute quantification and relative quantification (Page and Stromberg 2011). With absolute quantitative RT-PCR, the amount of the desired target can be calculated from a standard curve, which is generated by adding diluted standard series into each run. These standards have a known amount of the target nucleic acids. Using the known DNA concentration in each standard sample, the instrument's software creates a standard curve in a fluorescence plot that can be used to determine the copy number of the target sequence (Espy et al. 2006, Hyndman and Mitsuhashi 2003). The amount of the measured molecule can be calculated using the equation y = slope log (X) + intercept (Yoshida et al. 2003). Using the relative calculation method, the relative amounts of the desired molecule compared to a reference value can be calculated (Suzuki et al. 2005, Tichopad et al. 2010).

Primers for RT-qPCR must be unique to the DNA sequence of the desired microbe. The primers must be efficient and specific in identifying the target complementary sequence in the specimen of interest, *e.g.*, faeces, tissue or blood (Hyndman and Mitsuhashi, 2003). Usually, the primers are constructed into the bacterial 16S ribosomal DNA (rDNA) sequence, as this sequence is universally distributed between different species and is reliable for inferring phylogenetic relationships (Eckburg et al. 2005, Woese and Fox 1977). Prokaryotic ribosomes are constructed of two parts, the 50S (large) subunit and the 30S (small) subunit. The small subunit includes the 16S RNA unit and 21 proteins (Stanier et al. 1990). Additionally, part of the 16S rDNA is highly conserved and thus can be used for primer binding sites. The gene sequences also contain hypervariable regions that are species-specific and therefore can be used to identify different bacteria (Pereira et al. 2010). Certain bacterial species, like oral viridans streptococci, are highly homologous. Because their genetic similarity is more than 97%, the identification of those species using the sequence analysis of the 16S–23S rDNA intergenic spacer is highly recommended (Chen et al. 2004, Chen et al. 2005).

There are publicly available databases of bacterial 16S rDNA sequences, such as the National Centre for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/), EzTaxon-e (http://eztaxon-e.ezbiocloud.net/) and the Ribosomal Database Project (http://rdp.cme.msu.edu/probematch/search.jsp).

2 Post-Mortem Bacteriology

2.1 Bacteria in Life and after Death

Bacteria are present in every ecosystem in the earth. They are able to colonise all mucosal surfaces, the gut, as well as the skin of the human body. It has been estimated that the number of microbes in life in our bodies together make up ten times the number of human cells (Ley et al. 2006), representing a gene set 100 times greater than ours (Qin et al. 2010). Many of these unique genes are products of a long process of co-evolution of the host and the resident bacteria. Successive genes have been selected based on advantages that are provided for the well-being of the host, not just for the bacterium itself (Willing et al. 2011). Normally, during life, there is homeostasis between resident bacteria and the host. However, inner organs, like the blood, brain, heart, liver and spleen are considered to be sterile.

After death, all the defensive immune mechanisms in the human body that are operational during life cease, and resident bacteria start to degrade the body, as a part of the normal cycle of life. Tissue barriers and immune processes that destroy bacteria naturally vanish, and bacteria start to migrate through epithelial surfaces into blood and surrounding tissues. Decomposition is very important in the cycling of nutrients and organic matter in ecosystems and is the base for food webs. Although micro-organisms play a key role in normal decomposition, little is known about the processes that occur in a cadaver during degradation. It is known that several factors, such as moisture and humidity (Carter et al. 2010), temperature (Barton et al. 2013), type of tissue (Dickson et al. 2011) and pH (Haslam and Tibbett, 2009) influence microbial populations and their functions during the decomposition process.

The presence of pathogenic bacteria in post-mortem tissue samples can be used as an indication of lifetime infection, referring to the presence of such bacteria during the individual's life (Krous et al. 2004) and can be used in determining the possible cause of death. Apart from the use of post-mortem bacteriological results for diagnosing an infection during life, this procedure has been applied to a broad spectrum of specific forensic questions, *e.g.*, determination of the post-mortem interval (Pechal et al. 2014, Tsokos and Puschel 2001). Post-mortem bacteriology is also a valuable tool for studying the role of bacteria in causing different diseases during life. After death, autopsy allows samples to be obtained simultaneously from different tissues (*e.g.*, intestine and liver) that generally cannot be obtained from living individuals because of ethical and practical reasons.

2.1.1 Post-Mortem Intestinal Microbiota

Little research has been conducted regarding the composition of post-mortem intestinal microbiota and time-dependent changes in intestinal flora after death. Only one study, in three elderly women, has examined intestinal bacterial populations after death (Hayashi et al. 2005). The results of this study showed that the jejunal and ileal microbiota consist of mostly facultative anaerobes or aerobes belonging to streptococci, lactobacilli, *Gammaproteobacteria*, *Enterococcus* spp. and *Bacteroides* spp.. Hayashi et al. also demonstrated that the caecal microbiota was more complex than that found in the jejunal and ileal regions. The *C. coccoides* group, the *C. leptum* subgroup and the *Bacteroides* group were detected in the caecum, whereas colonic microbiota consisted of more complex microbial communities belonging to the *C. coccoides* and *C. leptum* groups, *Bacteroides* spp., *Gammaproteobacteria*, *Bifidobacterium* spp., streptococci and lactobacilli (Hayashi et al. 2005). Thus, it is unknown whether post-mortem intestinal samples are usable for research of the possible involvement of the intestinal microbiota in the pathogenesis of different diseases during life.

After death, the body's temperature drops from approximately 37°C to that of the environment, which is usually close to 20°C (room temperature), but depends on the circumstances of death. In a mortuary, the temperature is kept at +4°C. Most human intestinal bacteria are temperature-dependent thermophilic bacteria, meaning that they require a normal body temperature to survive and multiply. After death, interactions between the body and intestinal bacteria stop due to a lack of oxygen, a consequent imbalance in nutrient levels, and a shift from communities dominated during first decomposition phases by aerobic bacteria (*Staphylococcus* spp. and *Enterobacteriaceae*) to those dominated by anaerobic bacteria (*Clostridium* spp. and *Bacteroides* spp.) (Hyde et al. 2013). Bacteria conquer these difficulties with strategies that differ depending on the capabilities of the species. For example, the facultative anaerobic *Enterobactericeae* may be more sensitive to oxygen levels than obligatory anaerobes *Bacteroides* spp. and *Clostridium* spp. (Hyde et al. 2013). The diversity of bacterial phyla decreases over the course of decomposition (Pechal et al. 2014).

During life, the major site of bacterial fermentation is the caecum. The principal sources of carbon and energy for intestinal bacteria are indigestible starches and other carbohydrates (Macfarlane and Macfarlane 1997). Over 99% of intestinal bacteria are anaerobes, but in the caecum, aerobic bacteria reach high densities (Sears 2005). After death, available oxygen is depleted, turning aerobic into anaerobic fermentation, with conversion of adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and lactic acid (Butzbach 2010). This type of fermentation rapidly acidifies the intracellular environment. As a result of cell autolysis, intestinal pH decreases, and numerous enzymes lose their activity (Vass et

al. 2002). These phenomena interfere with intestinal bacterial growth and population diversity (Butzbach 2010) in an unknown way.

2.2 Microbiological Reliability of Post-Mortem Samples

The reliability of post-mortem samples for clinical purposes and determining the cause of death has been studied (Lobmaier et al. 2009, Roberts 1998, Tsokos and Puschel 2001, Weber et al. 2010). However, debate continues regarding the optimal sampling site and the length of the time period after death during which samples can be taken. It has been proposed that samples for post-mortem microbiology should be taken from at least two different sites to improve reliability (Tsokos and Puschel 2001). Thus, sampling from two sites is currently an essential part of standard procedures when there is a suspicion of possible involvement of bacteria in death (Tsokos and Puschel 2001). This protocol is used in cases of sudden unexpected death in infancy (Bajanowski et al. 2007).

The best possible site for taking the samples is also controversial. Different sampling sites have been proposed, including cerebrospinal fluid, blood, spleen, lung, and liver (Lobmaier et al. 2009, Roberts 1998, Tsokos and Puschel 2001, Weber et al. 2010). Only few studies have concentrated on the effect of post-mortem time on bacterial migration after death (Morris et al. 2006, Weber et al. 2010, Wilson et al. 1993).

2.2.1 Origins of Bacteria in Post-Mortem Samples

During life, the immune system normally clears bacteria from the bloodstream in a rapid and efficient fashion, regardless of whether the bacteria originate from a focal infection or from the intestine. Occasionally, bacteria escape immune surveillance, and life-threatening events such as septicaemia can occur (Morris et al. 2007).

The possible origins of bacteria in post-mortem samples include all mucosal and epithelial surfaces of the body, *e.g.*, the gut, oral cavity and respiratory tract. Four different mechanisms are responsible for the presence of bacteria in post-mortem samples, including agonal spread, contamination, post-mortem translocation, and being genuine positive (Morris et al. 2006). In agonal spread, bacterial invasion into tissues or blood happens during the death struggle or during resuscitation, when circulation is artificially maintained. Mucosal integrity decreases, allowing bacteria to more easily migrate into tissues. This most likely generates mixed bacterial growth (Morris et al. 2006). Sample contamination from outside sources is a major problem in life and cannot be completely avoided after death. Sample contamination can be caused by insufficient sampling techniques. Post-mortem translocation, *i.e.*, bacterial migration, is a part of normal decomposition in

which bacteria migrate from the mucosal surfaces into the blood and body tissues (Morris et al. 2006, Morris et al. 2007), probably causing increased multimicrobial growth and the loss of sterility (Wilson et al. 1993). In genuinely positive cases, bacteria were already present in life and can be found after death. During life, bacterial translocation is defined as the passage of viable bacteria from the GI tract through the mucosal epithelium to extraintestinal sites (Berg and Garlington 1979). Genuine positive results will consist of pure growth of a recognised pathogen, whereas mixed bacterial findings indicate post-mortem contamination or growth.

2.2.2 Effect of Post-Mortem Time

Another unanswered question in post-mortem microbiology is that of how long after death samples can be taken. In 1964, it was demonstrated that 40% of blood samples are positive for bacteria within 18 hours after death (Carpenter and Wilkins 1964), but the time needed for bacteria to reach visceral organs is unknown. Only a few post-mortem microbiological investigations have studied the effects of time since death on bacterial translocation (Morris et al. 2006, Weber et al. 2010), and the results of these studies are controversial.

It has been thought that bacterial translocation through gut mucosal surfaces into the blood and into visceral organs increases according to the length of time that has elapsed since death (Wilson et al. 1993). However, a retrospective study by Weber et al. suggested that a longer post-mortem interval is not associated with an increased frequency of bacterial positive results or with an increased rate of polymicrobial findings (Weber et al. 2010). Additionally, Morris et al. proposed that the post-mortem interval has only a small effect on the bacterial isolation rate and that bacterial translocation is not a problem if the body has been stored properly at +4°C soon after death (Morris et al. 2006). Clearly, more investigations into bacterial behaviour after death are needed.

3 Effect of Alcohol on the Intestinal Tract

3.1 Alcohol

Ethyl alcohol, or ethanol (CH_3CH_2OH), is an acyclic organic compound in which a functional hydroxyl group (-OH) is linked to a carbon molecule. Ethanol is the psychoactive ingredient in alcoholic beverages and can cause severe intoxication when consumed in excess. In nature, alcohol is usually fermented from carbohydrates by yeast. The earliest evidence of alcohol use is present in 9,000-year-old pottery from China, and the first scientific findings regarding ethanol date from the 9th century.

Sex, ethnicity, weight, nutritional status, and the dose and quality of alcoholic beverages all influence the sensitivity of tissues to alcohol (Alatalo et al. 2008, Bode and Bode 2003). In Finland in 2013, the total consumption of alcoholic beverages in a year was 11.6 liters per every 15 years old or older Finnish citizen presented as 100% alcohol (THL 2013). In Finland, the weekly limits defining at-risk alcohol use are 280 g (24 portions/week, 7 portions in a single use) for men and 190 g (16 portions/week, 5 portions in a single use) for women. According to these limits, up to 15% of the adult Finnish population can be considered to be at-risk users.

In 2012, 1518 men and 442 women died due to alcohol in Finland (SVT 2012a and b). Of all these deaths, 20% (375 persons) died because of alcoholic intoxication (76% men) and the rest (1585) were due to diseases, mainly liver (1099) and heart diseases, induced by alcohol abuse (SVT 2012c).

3.2 Alcohol and the Intestinal Microbiota

Documented changes occur in intestinal bacterial populations in response to ethanol. Bacterial overgrowth has been observed along almost the entire GI tract (Hauge et al. 1997, Yan et al. 2011). The occurrence of intestinal bacterial overgrowth in alcoholics has been found to be three times higher than in subjects who do not abuse alcohol (Bode et al. 1993). Significant decreases in amounts of probiotics, *i.e.*, beneficial bacteria for the host, such as *Lactobacillus* spp., *Pediococcus* spp., *Leuconostoc* spp. and *Lactococcus* spp. (Hartmann et al. 2013, Yan et al. 2011) have been observed. Mutlu and colleagues showed that ingested alcohol changes intestinal bacterial population toward more harmful species by decreasing *Bacteroidetes* and increasing *Proteobacteria* populations (Mutlu et al. 2012). The altered intestinal microbiota is able to produce ethanol and metabolise it into acetaldehyde, which in turn can further increase intestinal permeability.

3.3 Alcohol and the Gut Epithelium

The absorption of alcohol occurs by diffusion through the mucosa of the whole GI tract. The diffusion rate depends on mucosal permeability, blood flow and the concentration gradient across the mucosa (Bode 1980). Most of this absorption happens in the duodenum, jejunum and ileum. Alcohol concentration levels in the ileum are approximately the same as in the blood. Thus, most of the epithelial damage is expected to occur at those sites. In ethanol-fed animals, a large increase in villus height has been observed in the jejunum. In addition, ethanol alters the villus surface area and mucosal surface area (Keelan et al. 1985). Furthermore, alcohol has an effect on absorption of nutrients, vitamins, water, and

sodium, as well as the immune functions of the intestine. Alcohol and its metabolites can cause mucosal damage (*i.e.*, duodenal erosion and bleeding) and intestinal bacterial overgrowth (Bode and Bode 2003). Chronic alcohol consumption significantly disrupts both peripheral and mucosal immune homeostasis, possibly by changing microRNA expression in intestinal immune cells and reducing the amount of colonic T-helper cells 1 and 17 in a dose-dependent manner (Asquith et al. 2013). Alcohol and its metabolites have an effect on intestinal permeability and cause leakage of intestinal cell junctions (Basuroy et al. 2005). An association between increased intestinal permeability to macromolecules and chronic alcohol use was first shown in the 1970s (Bungert 1973, Worthington et al. 1978).

Alcohol is metabolised into acetaldehyde in an oxidative process by alcohol dehydrogenase and cytochrome P450 2E1 (CYP2E1), and further into acetate by the mitochondrial acetaldehyde dehydrogenase, which occurs mainly in the liver (Elamin et al. 2013). Alcohol metabolism and acetaldehyde formation also occur in intestinal epithelial cells (Seitz et al. 1996, Seitz and Becker 2007) and in the intestinal lumen, mediated by the alcohol dehydrogenases of the microbiota (Jokelainen et al. 1996, Nosova et al. 1996). Extrahepatic ethanol metabolism makes up approximately 40% of total ethanol elimination in patients with liver cirrhosis (Utne and Winkler 1980). One major extrahepatic site for microbial oxidation of alcohol is thought to be the colon (Tillonen et al. 1999). Colonic bacteria, such as *Escherichia coli*, can oxidize alcohol in a microaerobic environment using their alcohol dehydrogenases (Salaspuro et al. 1999). Alcohol metabolism is a slow process, and when alcohol is abused constantly for prolonged periods, acetaldehyde can build up in the liver, activating collagen synthesis in the hepatic stellate cells (Wang et al. 2006). Acetaldehyde accumulation can also be observed in the intestinal lumen as well as in the epithelium. Acetaldehyde, a highly unstable molecule that quickly forms potentially harmful adducts with other molecules, is the first and most toxic metabolite of ethanol metabolism. These metabolites can stimulate the redox state, hepatic steatosis, the production of reactive oxygen species and lipid peroxidation in the liver. In turn, these processes increase the production of other reactive aldehydes that can alter normal liver functions, induce cell death and promote liver inflammation (Schaffert et al. 2009).

Acetaldehyde causes leakiness of the intestinal epithelium by affecting close contacts between the plasma membranes of adjacent cells, known as tight junctions. The loss of protein–protein interactions is associated with acetaldehyde-induced barrier dysfunction. Tight junctions regulate epithelial integrity and cell-cell adhesion, as well as transportation through the extra-cellular matrix (Basuroy et al. 2005). Acetaldehyde has a direct effect on gut epithelium barrier by disrupting tight junctions and adherens junctions by increasing tyrosine phosphorylation of proteins, such as occludin, E-cadherin and beta-catenin, present in them. As a result, levels of the tight junction-associated proteins occludin, zonula occludens-1, E-cadherin and beta-catenin decrease in tight junctions (Basuroy et al. 2005).

Ethanol exposure has been shown to reduce the distribution of phosphorylated occludin in the activated intestinal epithelial through Toll-like receptor 4 (TLR-4) (Li et al. 2013). Furthermore, acetaldehyde is known to activate the transcription factor Snail in intestinal epithelial cells, which is involved in transport from the epithelium to the mesenchyma. The activation of Snail suppresses the expression of the tight junction and adherens junction proteins claudins and occludins, thereby weakening these junctions (Ikenouchi et al. 2003). Ethanol's activation of Snail and nitric oxide synthase also disrupts intestinal epithelial tight junctions (Forsyth et al. 2011). A decrease in the amount of proteins in the junction loosens the connections of adjacent cells. Looser connections allow a greater variety of molecules to cross the epithelium. This may enhance the translocation of bacteria and their products from the intestines into the liver.

Alcohol use has been associated with increased plasma levels of bacterial endotoxins (Adachi et al. 1995, Nanji et al. 1993, Tamai et al. 2000). The increase in plasma endotoxin levels may be due to reduced reticuloendothelial phagocytosis in monocytes and leakage of the intestinal epithelium (Bode et al. 1987) due to alcohol metabolites (Basuroy et al. 2005). The elevated endotoxin levels may promote chronic inflammation through the cluster-of-differentiation 14 (CD14) receptor, further increasing leakage through the intestinal wall (Bode et al. 1987).

4 Association between Alcoholic Liver Cirrhosis and Gut Bacteria

4.1 Alcoholic Liver Cirrhosis (ALC)

The liver is the largest organ. Its main tasks are the breakdown of red blood cells, storage of glycogen and blood, synthesis of proteins, *e.g.*, coagulation factors and vitamins, hormone production, detoxification of drugs and toxins and production of digestives like bile (Lindsay 1995). The normal liver is constructed of lobes that are further separated by connective tissue into lobuli. There is a central vein at the centre of each lobulus. In hepatic tissue, hepatocytes, the main liver cells, are organised into single-cell layers that are separated on both sides by sinusoids (small blood capillaries). The portal vein and hepatic artery are located between the lobuli. Blood from the portal vein and hepatic artery flows through the sinusoids into the central vein. The sinusoidal epithelium is non-discontinuous, with holes both in the intracellular epithelium and in the cells, permitting removal of substances from the blood. The epithelial macrophages in the liver are called Kupffer cells. The space of Disse lies between the sinusoids and hepatocytes and is where the exchange of molecules between blood and hepatocytes occurs. The hepatic stellate cells are in the space of Disse (Hall and Arnold 1995).

There are three major forms of alcoholic liver disease (ALD): alcoholic fatty liver, alcoholic steatohepatitis and alcoholic liver cirrhosis (ALC). Chronic alcohol consumption may lead to alteration of the normal structure of hepatic tissue. If alcohol consumption continues, it leads almost invariably to the development of fatty liver, which is characterised by the accumulation of triglycerides in hepatocytes, possibly leading to fibrosis and cirrhosis (Hall and Arnold 1995). In 1978, the World Health Organization defined cirrhosis as a process that is characterised by fibrosis and the conversion of the normal liver structures into structurally abnormal nodules (Anthony et al. 1977). ALC is the irreversible end stage of ALD and is characterised by the conversion of normal hepatic tissue into collagen due to fibrinogenesis. The space of Disse is also occupied by extracellular matrix. Thus, the linkage between hepatocytes and portal blood sharply declines, and liver injury occurs (Hall and Arnold 1995). Hepatitis is an inflammation of the liver caused by toxic substances such as alcohol, infections, or viruses (Hall and Arnold 1995)

ALC is a major cause of mortality worldwide (Purohit et al. 2009). In Western countries, including Finland, more than 80% of liver cirrhosis cases are caused by excessive ethanol consumption. However, only a minority (approximately 10–20%) of alcoholics develop liver cirrhosis, regardless of the duration or amount of drinking, suggesting the possible involvement of genetic and other risk factors (Färkkilä 2013).

Ascites, or the pathologic accumulation of fluid in the peritoneum, is common complication of cirrhosis and is observed in approximately of 10% of cirrhosis patients (Gordon 2012). In cirrhotic patients, bacteria can translocate into the ascitic fluid and cause spontaneous bacterial peritonitis, which has a high mortality rate (Hoefs et al. 1982). The frequency of spontaneous bacterial peritonitis has been estimated at 7% in cirrhotics with ascites and is thought to happen due to bacterial overgrowth in the intestine and the translocation of these bacteria into the ascitic fluid (Khan et al. 2009). Llovet and colleagues showed that the occurrence of bacterial translocation to mesenteric lymph nodes is approximately 40% in cirrhotic rats with ascites and approximately 80% in animals with spontaneous bacterial peritonitis (Llovet et al. 1994). In community-acquired spontaneous bacterial peritonitis, gram-negative bacteria (such as *Enterobacter* spp.) that originate from the gut are usually isolated, whereas in hospitalised cirrhosis patients, nosocomial infections with gram-positive bacteria (like *Staphylococcus* spp.) predominate (Hoefs et al. 1982).

4.2 Current Hypothesis of ALC

It is clear that alcohol consumption alone is not sufficient for the development of ALC. Other possible contributing factors for developing alcoholic liver injury are genetic, *i.e.*, gender, patatin-like phospholipase domain-containing protein-3 and endotoxin receptor polymorphisms; and environmental factors, *i.e.*, duration and amount of drinking,

diet, smoking and overweight as well as the amount of acetaldehyde (Färkkilä 2013). Acetahdehyde is a metabolite of alcohol degradation that is highly toxic for hepatocytes and increases cellular oxidative stress and epithelial leakiness (Färkkilä 2013).

Current understanding of the pathogenesis of cirrhosis strongly suggests the involvement of gut bacteria with alcoholic metabolites and genetic vulnerability. The populations of intestinal bacteria are changing toward more gram-negative majority due to chronic alcohol usage (Hauge et al. 1997). Alcohol also causes leakage in the intestinal wall due to its effects on intercellular tight junctions (Basuroy et al. 2005). This allows bacteria and their components to translocate from the intestine into the liver, causing activation of CD14 endotoxin receptor in the hepatic Kupffer cells. Together with the activity of alcohol's metabolites, this can lead to an increase in the levels of pro-inflammatory cytokines and chemokines, resulting in a positive feedback loop that further enhances liver inflammation, infiltration of inflammatory cells and fibrosis, ultimately resulting in liver damage. Intestinal sterilisation has been proven to prevent alcohol-induced liver injury in the rat (Adachi et al. 1995), supporting the idea that intestinal bacteria are involved in cirrhosis via the endotoxin-mediated activation of Kupffer cells. Continuous bacterial translocation from the gut is not sufficient on its own for the development of cirrhosis but can induce the condition in combination with other factors.

It has been hypothesised that the translocation of bacteria can induce cirrhosis through TLR and CD14 activation. This activation starts a cascade in which pro-inflammatory cytokines (Heumann and Roger 2002) are released, possibly inducing cirrhosis. A correlation between ALC and the genetics of the bacterial recognition receptor CD14 has been described (Campos et al. 2005, Jarvelainen et al. 2001, LeVan et al. 2001). This further supports the current hypothesis that intestinal bacteria and their endotoxins, including lipopolysaccarides (LPS), are involved in the development of inflammation (Yan and Schnabl 2012) that leads to fibrosis and cirrhosis in genetically susceptible individuals (Jarvelainen et al. 2001).

Further support for the theory of bacterial involvement in the development of ALC is provided by the fact that septicaemia is a 20-fold more common cause of death in cirrhotics than in the general population (Vilstrup 2003). Increasing evidence suggests that alterations in the intestinal microbiota may play a significant role in the development of ALC (Yan and Schnabl 2012).

4.3 ALC and Intestinal Bacteria

Faecal microbial diversity is known to be lower in cirrhosis patients (Chen et al. 2011) than in normal individuals. Bacterial overgrowth in the small intestine has been demonstrated in one-third of patients with cirrhosis (Pande et al. 2009). Intestinal bacterial overgrowth

can predispose patients to bacterial infections and other major complications; it also plays a role in the pathogenesis of chronic liver disorders (Steffen et al. 1988). The main cause of bacterial overgrowth is believed to be decreased intestinal motility (Wiest and Garcia-Tsao 2005) and achylia. Other causes of imbalance in the intestinal microbiota of cirrhotic hosts include: decreased gastric acidity and pancreato-biliary secretions, malnutrition, reduced IgA secretion (allowing bacteria to attach to colonocytes) and portal hypertensive enteropathy (or colopathy) (Lata et al. 2011). Other possible factors influencing the development of cirrhosis include alcohol-induced injury to the intestinal microvilli and increased adhesion of bacteria to the epithelium due to decreased bacterial immune clearance (Chesta et al. 1993). Congestion and edema of the intestinal mucosa can result in decreased local resistance to invading pathogens. An increased pH in the intestinal lumen, which is due to lower bile acid concentrations and a decrease in the amount of ammonia- and hydrogen-sulphide-producing bacteria, affects the growth of intestinal microflora and diminishes the secretion of mucus, lysozymes, acids and alkali, resulting in a potentially favourable environment for bacterial growth (Chen et al. 2011, Zhao et al. 2004).

The amounts of *Lachnospiraceae* (Chen et al. 2011), *Bacteroides* spp. and *Bifidobacterium* spp. have been reported to be decreased in the faeces of cirrhotic patients, whereas the amounts of *Enterobacter* spp., bacilli and clostridia appear to be increased (Zhao et al. 2004), as shown in Table 2.

Table 2. Changes in intestinal microbiota in alcoholic liver disease according to studies performed by other groups. Ns=non-significant change.

Method	Decrease	Increase	Place	Reference
Culture		Aerobic bacteria, *E. coli*, *Proteus* spp.	Caecum, rat	(Guarner et al. 1997)
Culture		*Enterobacter* spp. (ns), *Enterococcus* spp. (ns)	Caecum, ileum, rat	(Chiva et al. 2002)
Culture		*E. coli*, *Staphylococcus* spp.	Faeces, human	(Liu et al. 2004)
Culture	*Bifidobacterium*, *Lactobacillus* (ns)	*Enterobacter* spp., *Clostridium* spp., *Enterococcus* spp., yeast (ns)	Faeces, human	(Zhao et al. 2004)
Culture	*Lactobacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp.	*Enterobacteriaceae*	Ileal faecal samples from distal small intestine, rat	(Zhang et al. 2010)
Pyrosequencing RT-qPCR	*Bacteroidetes*, *Lachnospiraceae*	*Proteobacteria*, *Fusobacteria*, *Gammaproteobacteria*, Bacillus, *Streptococcaceae*, *Clostridium* cluster XI, *Enterococcus faecalis*	Faeces, human	(Chen et al. 2011)
Culture	*Clostridium leptum* group, *Clostridium coccoides* group		Caecum, ileum, mouse	(Gomez-Hurtado et al. 2011)
RT-qPCR	Lactic acid bacteria (*Lactobacillus*, *Weissella Pediococcus*, *Leuconostoc*) *Bifidobacterium*, *Clostridium*, *Feacalibacterium prausnitzii*	*Enterobacteriaceae*, *Enterococcus feacalis*	Faeces, human	(Lu et al. 2011)
RT-qPCR	Firmicutes (*Lactococcus* spp., *Pediococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp.)	*Verrucomicrobia*, *Bacteroidetes* (*Bacteroidales*, *Bacteroides Porphyromonadaceae*)	Caecum, mouse	(Yan et al. 2011)
Multitag pyrosequencing	Ruminococcaceae, Lachnospiraceae	*Enterobacteriaceae*, *Alcaligeneceae*, *Fusobacteriaceae*	Faeces, human	(Bajaj et al. 2012)
Multitag pyrosequencing	*Lachonospiraceae*, *Ruminococcus* spp., *Blautia* spp.	*Enterobacteriaceae*	Faeces, human	(Kakiyama et al. 2013)
RT-qPCR	*Bifidobacterium* spp. (ns)	*Enterobacteriaceae*, *Enterococcus* spp.	Faeces, human	(Liu et al. 2012)
Multitag pyrose-quencing	*Lachnospiraceae*, *Ruminococcaceae*, *Clostridialies* XIV	*Enterobacteriaceae Enterococcaea*	Faeces, human	(Bajaj et al. 2014)

Pathological bacterial translocation has been termed the "Achilles heel" of liver disease (Akira et al. 2001), playing an important role in the pathogenesis and complications of cirrhosis. Gram-negative bacteria, such as *Escherichia coli*, *Proteus* spp. and *Enterobacter* spp., have been shown to translocate more efficiently than gram-positive bacteria (Steffen et al. 1988). Bacterial translocation has also been recognised as a crucial event in the non-

decompensated and non-cirrhotic stages of liver disease and is particularly relevant to the progression of alcoholic liver disease (Benten and Wiest 2012). Compared to the tissues of the stomach and small intestine, larger numbers of bacteria have been demonstrated in the caecum and colon. This suggests that most bacterial translocation occurs in the caecum or colon (Wells and Erlandsen 1991). The permeability of the large intestine increases in cirrhotic patients (Pijls et al. 2014). The failure of anti-bacterial defense mechanisms, such as reduced opsonic activity due to decreased synthesis of complement in the liver, disturbed function of macrophage Fc gamma receptors, and a reduction in the phagocytic and killing capacity of neutrophils (Gomez et al. 1994, Hassner et al. 1981, Rajkovic and Williams 1986) to efficiently clear these translocating bacteria, can lead to cirrhosis (Wiest and Garcia-Tsao 2005).

4.4 Bacterial Recognition by Kupffer Cells

Seventy percent of the blood supply of the liver is provided by the portal vein, which is a direct venous outflow of the intestine (Son, Kremer and Hines, 2010); the liver is therefore continuously exposed to bacteria and bacterial components originating from the gut. Animal studies have shown that living and dead bacteria can escape from the intestine and be found in the liver (White et al. 2006). The liver is enriched with innate immune cells (*e.g.*, Kupffer cells, natural killer cells) and hepatic stellate cells (Suh and Jeong 2011).

Bacteria and their residuals are cleared from portal blood coming from intestines mainly together by liver sinusoidal endothelial cells lining the blood vessels and Kupffer cells. Kupffer cells are liver macrophages that are able to engulf and kill bacteria, cancer cells and viruses; however, their major task is to recruit other immune cells to the inflammatory cascade (Beutler 2004, Kim et al. 2011). Kupffer cells express CD14 endotoxin receptors on their surfaces; when stimulated by bacterial lipopolysaccharide (LPS), which is a component of gram-negative bacteria, they produce pro-inflammatory and fibrosis-promoting cytokines as well as reactive oxygen species, which can cause liver injury (Thurman 1998). Levels of LPS, also known as bacterial endotoxin, are increased in the portal and/or systemic circulation in several types of chronic liver disease (Ilan 2012). The amount of CD14 receptors in the Kupffer cell membrane is adjusted according to the amount of endotoxin present in plasma (Landmann et al. 1996, Lukkari et al. 1999). Sinusoidal endothelial cells are the first line of defense (Knolle and Limmer 2003), expressing CD14 and TLR-4 receptors (Hayashi et al. 2006). During activation, endothelial cells greatly increase normal pro-inflammatory signals. When the normal activity of endothelial cells is impaired due to chronic ethanol consumption, LPS and bacteria can access the bloodstream (Bjarnason et al. 1984, Bode et al. 1987, Schenker and Bay 1995). Translocated LPS is an activator for the CD14 and TLR-4 subreceptors that are found in hepatic Kupffer cells.

Bacterial LPS is detected in hepatic Kupffer cells by a trimeric membrane system (Figure 1) consisting of three components: CD14, TLR-4 and myeloid differentiation protein 2 (MD-2) (Lloyd and Kubes 2006). The CD14 receptor works with the other components and is involved in innate immune reactions, controlling the immune system's level of response. It was originally referred as an endotoxin-binding receptor (Lloyd and Kubes 2006) but is currently thought to be a common cellular pathway for the recognition of several bacterial components. Even though LPS is the major virulence factor in gram-negative bacteria, CD14 can also recognise other bacterial components, including the following: peptidoglycan, which is a cell wall component of all bacteria (Stanier at al. 1990); lipoteichoic acid, found in gram-positive bacteria (Stanier at al. 1990); lipoarabimannan, derived from *Myobacteria* (Leist et al. 1988, Zhang et al. 1993); the rhamnose-glucose polymers of *Streptococcus* spp. (Soell et al. 1995); chitin (Jahr et al. 1997); and the amphiphilic molecules of *Staphylococcus aureus* (Kusunoki and Wright 1996). Membrane-bound CD14 is an acute-phase, glycosylphosphatidylinositol-anchored, 55-kDa membrane glycoprotein (Bas et al. 2004). It can be found in a soluble form as well as a membrane-bound form. Soluble CD14 enables cells that do not have membranic CD14, *e.g.*, endothelial cells (Haziot et al. 1993, Pugin et al. 1993), smooth muscle cells (Loppnow et al. 1995) and epithelial cells (Pugin et al. 1993), to respond to LPS. The CD14 response can be initiated by concentrations of LPS as small as 1 ng/ml (Lloyd and Kubes 2006). In addition to LPS, lipid A, heat-killed *Escherichia coli*, lipoteichoic acid and the cell wall component of *Staphylococcus aureus* have shown to regulate the amount of CD14 (Landmann et al. 1996). CD14 lacks an intracellular component, so it needs a partner for transmitting the response into the nucleus.

CD14 functions together with TLR-4 to detect LPS, whereas, other members of the TLR family mediate the recognition of other microbial ligands, *i.e.*, of such as those derived from gram-positive bacteria, with their extracellular leucine-rich domain called the Toll domain (Heumann and Roger 2002). Ten members of the TLR family have been identified in humans, several of which appear to recognise specific microbial products, including LPS, lipoteichoic acid, lipoproteins, bacterial CpG DNA, and peptidoglycan. TLR-4 is the principal LPS receptor. TLR-2 is responsible for the recognition of gram-positive bacteria, mycobacterial species, bacterial lipoproteins, and lipoteichoic acid; TLR-3 responds to double-stranded viral RNA. TLR-5 responds to flagellin and flagellated bacteria. TLR-6, in cooperation with TLR-2, responds to gram-positive bacteria, yeast particles, peptidoglycan, modulin, and Pam2Cys mycoplasma lipoproteins (Lien and Ingalls 2002). TLR-7 and TLR-8 mediate the cellular stimulation induced by small antiviral compounds (Heine and Lien 2003), and TLR-9 mediates responses to CpG bacterial DNA (Lien and Ingalls 2002). TLRs can work together to recognise bacterial lipopeptides; for example, TLR-2/TLR-6 and TLR-2/TLR-1 recognise complexes on bacterial cell membranes (Uematsu and Akira 2006). TLR signalling can occur through a number of different pathways, many of which

lead to the translocation of nuclear factor κB (NF-κB) into the nucleus and thereby the formation of pro-inflammatory cytokines (Heine and Lien 2003). TLRs bind to conserved bacterial structures and discriminate between self and non-self, which is a basic concept of innate immunity (Hoffmann et al. 1999).

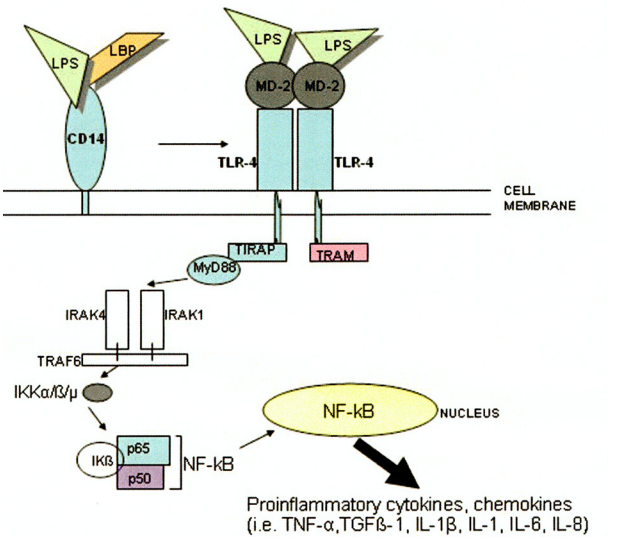

Figure 1. Bacterial recognition by CD14 in the Kupffer cell. When LPS, with lipopolysaccharide binding protein (LBP), attaches to the CD14 receptor, it approaches TRL-4, activating homodimerisation of the TLR-4 and MD-2 molecules and activating the MyD88-dependent pathway in the cytosol. This leads to the nuclear translocation of the nuclear factor κB (NF-kB). As a result, pro-inflammatory cytokines and chemokines are released. LPS= lipopolysaccharide, MD-2= myeloid differentiation protein 2, TLR-4= toll-like receptor 4, TIRAP=TIR-domain-containing adaptor protein, IRAK1=interleukin-1-receptor associated kinase, IRAK4= interleukin-4-receptor associated kinase, TRAF6=tumor necrosis factor receptor-associated factor 6, MyD88=myeloid differentiation primary response protein 88, IKKα/β/μ=IκB kinase, IκB=inhibitor of NF-κB, NF-κB=nuclear factor-κB, TNF-α=tumor necrosis factor α, IL-1β=interleukin 1β, TGFβ-1 = transforming growth factor β1 (Figure by Sari Tuomisto).

The activation of the CD14 cascade by LPS leads to the production of pro-inflammatory molecules (cytokines and chemokines) such as tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), IL-1, IL-6, IL-8 and transforming growth factor β1 (TGFβ-1). Furthermore, produced TGFβ-1 activates hepatic stellate cells (also known as liver lipocytes), decreases cell regeneration and inhibits matrix degradation by inhibiting metalloproteinases. As hepatic stellate cells are activated from their quiescent, non-fibrotic state, they start to proliferate and begin fibrinogenesis. During fibrinogenesis, extracellular matrix is produced in hepatic tissue, leading to the excess production of collagen that is characteristic of hepatic fibrosis (Milani et al. 1990). A correlation between increased hepatic stellate cell proliferation and increased production of extracellular matrix has been observed (Zhang et al. 2004).

The LPS signalling sensitivity of Kupffer cells is adjusted by controlling the amounts of CD14 receptors, lipopolysaccharide binding protein (LBP) and other factors involved in the cascade. However, the actual number of Kupffer cells does not increase. CD14 expression in the membrane is up-regulated by the plasma concentrations of rough and smooth LPS (Landmann et al. 1996). A direct correlation has been shown between TLR-4 and LPS sensitivity (Kalis et al. 2003). Chronic alcohol abuse increases the sensitivity of Kupffer cells to LPS (Schaffert et al. 2009). Consequently, the Kupffer cells increase the production of pro-inflammatory cytokines, thereby increasing cellular expression of LBP and CD14 (Lukkari et al. 1999). Alcohol consumption is often also associated with increased plasma concentrations of LPS (Bode et al. 1987). The positive feedback loop that is generated by enhanced LPS concentration and recognition and response hypersensitivity is amplified further by increasing cytokine production in the nucleus. These cytokines further increase the amounts of CD14, LBP and TLR-4 molecules in the cell. As a result, the cell needs smaller amounts of LPS to promote a response and so becomes hypersensitive. Subsequently, increased concentrations of TGFß-1 and TNF-α further promote hypersensitivity, fibrosis, inflammation, necrosis, apoptosis and liver damage, possibly even leading to endotoxic shock.

AIMS OF THE STUDY

The aims of the present study were as follows:

1. To study bacterial ratios and their changes in intestinal samples after death in order to evaluate the usability of post-mortem intestinal samples in basic bacteriological research.

2. To study post-mortem bacterial translocation from the gut into the blood, liver, portal vein, mesenteric lymph nodes and pericardium.

3. To study the possible involvement of intestinal bacteria in the development of alcoholic liver cirrhosis.

SUBJECTS AND METHODS

1 Samples and Study Subjects

1.1 Samples

1.1.1 Autopsy Samples

All autopsy samples for the present study were collected at the Department of Forensic Medicine at the University of Tampere. The bodies were not cooled during transportation to the mortuary but were kept refrigerated in the mortuary at around +4°C. The criteria for selecting cases for the series among routine autopsies were as follows: out-of-hospital death, male sex, age over 18 years, time interval between death and storage of the body in the mortuary of less than 24 hours, intact middle torso and bowel, no reported use of antibiotics within the two weeks before death, no signs of bacterial infections or drug addiction and no visible wounds or necrosis. The hospital records and police reports were available for each of the autopsied cases.

Blood, mesenteric lymph node (MLN), pericardium and liver samples were taken aseptically with sterile disposable instruments during routine autopsies. Autopsy samples were put in sterile Petri dishes and stored at -80°C until further processing.

Samples of the bowel contents were taken aseptically from autopsy cases with sterile disposable instruments from the rectum and caecum. All faecal samples were frozen at -20°C immediately after sampling and were stored within 2 hours at -80°C until further processing.

1.1.2 Clinical Samples

Ascitic fluid samples were drawn aseptically at the bedside from 12 male volunteers with alcoholic liver cirrhosis during their hospital stay in the Tampere University Hospital at the Department of Gastroenterology and Alimentary Tract Surgery. All these patients had a diagnosis of alcohol-related cirrhosis; the diagnosis was based on drinking history and clinical findings. Bacterial cultures, polymorphonuclear cell counts and albumin levels were obtained using standard methods by the accredited labroratory, FimLab Ltd.

Seven healthy male volunteers gave faecal samples after defecation. Samples were frozen first at -20°C and then within 2 hours transferred to -80°C.

1.2 Subjects in the Study

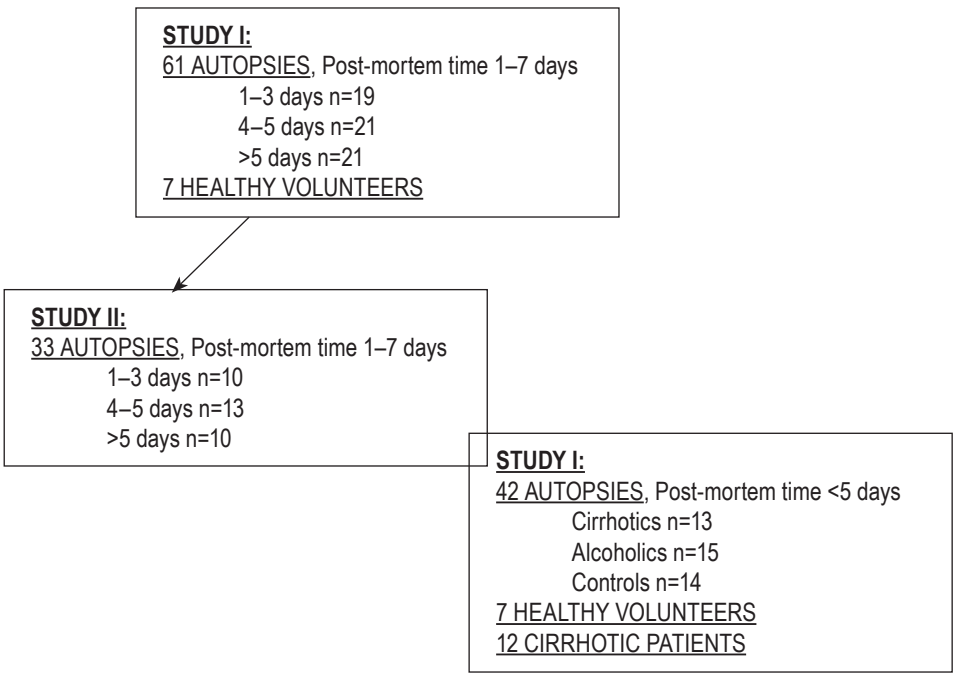

Figure 2. Overview of all study subjects used in this thesis.

1.2.1 Study I

Study I is a prospective autopsy series of 61 male cases (mean age 57 years, range 18–86 years) and 7 male volunteers (47 years, range 26–57). Written consent was obtained from all the volunteers. Of the autopsy cases, 32 (52%) died of heart disease, 15 (25%) died of other non-cardiac disease, and 14 (23%) suffered violent death (suicide, accident, poisoning). The autopsy cases were divided into groups based on time since death: 1–3 days, 4–5 days and >5 days. Details regarding the study groups are provided in Table 3.

Table 3. Details of the groups of Study I, divided by post mortem time. PM mean= Mean time since death of the collected cases, BMI= Body mass index.

	Volunteers	Autopsy Cases			P-value
		1–3 days	4–5 days	>5 days	
N	7	19	21	21	
Age, mean (range)	45 (26–57)	55 (18–79)	58 (20-86)	61 (28–76)	0.373
BMI, mean (range)	27.1 (20.8–37.2)	29.3 (20.4–42.1)	28.4 (18.4–43.6)	30.7 (21.1–50.3)	0.543
PM time, mean		2.3	4.5	6.5	
Cause of death					
Heart disease (%)		7 (37%)	10 (48%)	15 (71%)	0.079
Other disease (%)		4 (21%)	9 (43%)	2 (10%)	0.086
Non-natural death[1] (%)		8 (42%)	2 (10%)	4 (19%)	0.096

[1] Suicide, accident, poisoning

1.2.2 Study II

The autopsy series in Study II includes the first 33 consecutive cases of the 61 autopsy cases used in Study I. These cases were collected from 2009 to 2010. The time elapsed since death was less than 8 days in all cases. Samples were collected using aseptic techniques from the liver, pericardial fluid, portal venous blood, as well as the right cardiac ventricle (n=20, control blood), jugular vein (n=11, control blood) or femoral vein (n=2, control blood), and mesenteric lymph nodes (MLN).

Of these cases, 16 (49%) died of heart disease, 12 (36%) died of other non-cardiac diseases, and 5 (15%) suffered violent death (suicide, accident, poisoning). The cause of death was assessed on a routine basis, without knowledge of the results of bacteriological analyses.

The mean age of subjects in Study II was 56.7 years (range 20-86 years), and the average time elapsed since death was 2.4 days (range 1–7 days). On the basis of the time elapsed since death, the samples were divided into 3 groups: 1–3 days, 4–5 days and 5–7 days. See details in Table 4.

Table 4. Details of the of the study groups in Study II. PM mean= Mean time since death, BMI= Body mass index.

	Autopsy Cases		
	1–3 days	**4–5 days**	**>5 days**
N	10	13	10
Age, mean (range)	51.8 (34–77)	53.5 (20–86)	65.8 (51–82)
BMI, mean (range)	28.9 (20.4–42.1)	27.7 (18.4–43.6)	28.7 (21.1–38.9)
PM time, mean	2.4	4.6	6.6
Cause of death			
Heart disease (%)	3 (30%)	6 (46%)	7 (70%)
Other disease (%)	3 (30%)	6 (46%)	2 (20%)
Non-natural death[1] (%)	4 (40%)	1 (8%)	1 (10%)

[1] Suicide, accident, poisoning

1.2.3 Study III

Study III was an autopsy series of 42 males who had been included in the 61-patient autopsy series of Study I. Of these cases, 13 had alcoholic cirrhosis, 15 were alcoholics without liver cirrhosis, and 14 were non-alcoholic men who served as a control series (Table 5). The time elapsed since death in all cases was 5 days or less. In this study, there were also 7 healthy male volunteers and 12 male volunteers who had alcoholic liver cirrhosis and ascites. Cirrhosis was confirmed with liver histology.

Table 5. Details of the of the study groups in Study III. PM= mean time since death, BMI= Body mass index.

	Cirrhosis Patients	**Volunteers**	**Autopsy Cases**			**P-value**
			Cirrhotics	**Alcoholics**	**Non-alcoholic Controls**	
N	12	7	13	15	14	
Age, mean (range)	58 (39–73)	45 (26–57)	56 (39-77)	54 (34–77)	58 (18–86)	0.383
BMI, mean (range)	28.1 (19.7–39.2)	27.1(20.8–37.2)	29.1 (19.6–41.9)	30.1 (20.4–42.1)	27.4 (18.4–43.6)	0.573
PM time, mean			3.6	3.3	3.8	0.537
Cause of death						
Heart disease (%)			5 (38.5%)	3 (20.0%)	10 (71.4%)	0.007
Other disease (%)			5 (38.5%)	8 (53.3%)	1 (7.1%)	0.017
Non-natural death[1] (%)			3 (23.1%)	4 (26.7%)	3 (21.4%)	0.885

[1] Suicide, accident, poisoning

Of these 42 male autopsies, one died from alcoholic liver cirrhosis and two died of alcohol intoxication. In 17 cases, alcohol was a contributory factor for death. Data on alcohol use

was obtained from the hospital records of the autopsy cases. Cases were defined as alcoholics based on comments in the hospital or police reports and/or the presence of alcoholism-related microscopic findings. Positive post-mortem alcohol test at the time of autopsy was also used as a definition. Eleven of the 15 alcoholics had mentions of alcoholism in their reports, 12 had a positive alcohol test, and 6 also had alcoholism-related microscopic findings (fatty liver, cerebellar atrophy or chronic pancreatitis).

Healthy volunteers did not report drinking more than 3 drinks per week (36 g of alcohol), but cirrhosis patients reported excessive alcohol consumption. Neither healthy volunteers nor cirrhosis patients were reported to have taken antibiotics for 2 weeks prior to sampling. Antibiotic use was not reported in any of the autopsy cases.

2 Methods

2.1 DNA Extraction

2.1.1 Stool Samples

In Study I and III, the faecal samples were weighed to 150 mg (wet weight). Bacterial DNA was extracted from the faecal samples using a commercial DNA extraction kit (Zymo Fecal DNA Kit, Zymo Research Corporation, Irvine, California, USA) according to the instructions provided. Endogen-free, *i.e.*, bacterial residual-free, water (Thermo Fisher Scientific Inc., Waltham, USA) was extracted simultaneously for use as a negative control. DNA was stored at -20°C for further study.

2.1.2 Tissue, Blood and Ascites Samples

DNA from liver, blood, MLN, pericardial fluid and ascitic fluid was extracted using the Zymo Bacterial/Fungal DNA Kit (Zymo Bacterial/Fungal DNA Kit, Zymo Research Corporation, Irvine, California, USA). DNA was extracted from 200 µl of blood, pericardial fluid and ascitic fluid and from 200 mg of liver and mesenteric lymph node tissue. For each extraction patch, endogen-free water (Thermo Fisher Scientific Inc., Waltham, USA) was extracted for a negative control. DNA was stored at -20°C.

2.2 Bacterial Culturing

After being obtained at autopsy, samples were immediately transported at room temperature to the accredited laboratory in the Department of Microbiology, FimLab ltd (J.A, R.V), for culturing and bacteriological assessment. Bacterial cultures of autopsy samples were performed using standard procedures. Blood samples were cultured with BacT/ALERT-blood culturing bottles (bioMérieux SA, Marcy l'Etoile, France) and tissue samples with blood-, chocolate- and fastidious anaerobe agar-plates as well as with fastidious anaerobe-broth. Samples were also gram-stained. Identification of bacteria at the species level was performed using standard biochemical tests.

2.3 Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

2.3.1 Designing and Selection of Primers and Probes Used

Primers and probes used in this study are presented in Table 6. The target sequence in bacteria for primers and probes was the bacterial 16S ribosomal RNA gene. *Enterobacteriacaea*, *Lactobacillus* spp., *Streptococcus* spp. mainly *Str. mitis*-group and *Staphylococcus aureus* and *S. epidermidis* primers and probes were designed and confirmed using BLAST, from the National Centre for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/) and the Ribosomal Database Project (http://rdp.cme.msu.edu/probematch/search.jsp). Specificity and cross-reactivity of the designed primers and probes were tested using bacterial cultures from clinical samples (*Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus mitis*, *Streptococcus sanguinis*, *Streptococcus anginosus*) and reference bacteria from the ATCC collection (*Streptococcus mitis* ATCC 49456, *Streptococcus sanguinis* ATCC 10556, *Streptococcus anginosus* ATCC 33397). Amplification primers and probes designed by others were synthesised according to published sequences.

In Study II, the results from two different *Clostridium* measurements were marked as *Clostridium* spp., *S. aureus* and *S. epidermidis* were marked as *Staphylococcus* spp., and *Streptococcus* were marked as *Streptococcus* spp..

Table 6. Primers and probes used in the study.

Primer and probe	Sequence (5'-3')	Reference
***Bacteroides* spp.**		(Brunk et al. 2002)
Forward	TGGTAGTCCACACAGTAAACGATGA	
Reverse	CGTACTCCCCAGGTGGAATACTT	
Probe	GTTTGCCATATACAGTAAGCGGCCAAGCG	
***Bifidobacterium* spp.**		(Furet et al. 2009)
Forward	CGGGTGAGTAATGCGTGACC	
Reverse	TGATAGGACGCGACCCCA	
Probe	CTCCTGGAAACGGGTG	
***Clostridium leptum* group**		(Furet et al. 2009)
Forward	CCTTCCGTGCCGSAGTTA	
Reverse	GAATTAAACCACATACTCCACTGCTT	
Probe	CACAATAAGTAATCCACC	
***Clostridium coccoides* group**		(Furet et al. 2009)
Forward	GACGCCGCGTGAAGGA	
Reverse	AGCCCCAGCCTTTCACATC	
Probe	CGGTACCTGACTAAGAAG	
Enterobactericeae		This study
Forward	GCGGTAGCACAGAGAGCTT	
Reverse	GGCAGTTTCCCAGACATTACTCA	
Probe	CCGCCGCTCGTCACC	
***Enterobacter* spp.**		(Sen and Asher 2001)
Forward	GCGGTAGCACAGAGAGCTT	
Reverse	GGCAGTTTCCCAGACATTACTCA	
Probe	AGCAAGCTCTCTGTGCTACCGCTCGA	
***Lactobacillus* spp.**		This study
Forward	GCTAGGTGTTGGAGGGTTTCC	
Reverse	CCAGGCGGAATGCTTAATGC	
Probe	TCAGTGCCGCAGCTAA	
***Streptococcus* spp. mainly *Str. mitis*-group**		This study
Forward	CCAGCAGCCGCGGTAATA	
Reverse	CCTGCGCTCGCTTTACG	
Probe	ACGCTCGGGACCTACG	
Staphylococcus aureus* and *S. epidermidis		This study
Forward	GCGTTTTTCACGTGGAATATC	
Reverse	AATCCAAAACACAAACAAAGACAAGGT	
Probe	ACGTGCCATATTAATTTAC	
Universal		(Yang et al. 2002)
Forward	TGGAGCATGTGGTTTAATTCGA	
Reverse	TGCGGGACTTAACCCAACA	
Probe	CACGAGCTGACGACA[A/G]CCATGCA	

2.3.2 PCR Runs for Faecal and Ascitic Fluid Samples

Assays on stool and ascitic fluid samples were performed with the AbiPrism 7000 HT Sequence Detection System (Taqman™, AppliedBiosystems, California, USA), using a reaction volume of 20 µl in a 96-well reaction plate, standard Taqman conditions, and

specific bacterial primers and probes. Faecal samples were diluted 1:100, and 1 µl of diluted DNA was added into the amplification reaction for the detection system. Bacterial DNA from ascitic fluid was used as undiluted. The MasterMix was prepared using Taqman™ Environmental MasterMix (Taqman™, AppliedBiosystems, California, USA) with final concentrations of 1000 nM of each primer and 250 nM of each fluorescence-labelled probe. Samples were performed as duplicates. Endogen and DNA-free water (Thermo Fisher Scientific Inc., Waltham, USA) was used a negative control in each PCR run.

2.3.3 PCR Runs for Liver, MLN, Blood and Pericardial Fluid

RT-qPCR assays for liver, MLN, blood and pericardial fluid were performed with bacterial primers and probes for *Bacteroides* spp., *Bifidobacterium* spp., *Clostridium leptum*, *Clostridium coccoides*, *Enterobacter* spp., *Staphylococcus aureus* and *S. epidermidis,* and *Streptococcus* spp. mainly *Str. mitis*-group. Amplification was performed under standard conditions using specific Taqman™ allele hybridisation and the AbiPrism 7900 HT Sequence Detection System (Taqman®, AppliedBiosystems, Carlsbad, California, USA). A reaction volume of five microlitres was used with the Taqman™ Environmental MasterMix (Taqman™, AppliedBiosystems, California, USA). At final concentrations, 1000 nM of each primer and 250 nM of each probe were added. One microlitre of undiluted DNA was inserted into the reaction. All amplifications and detections were carried out as duplicates or quadruples (depending on test runs) in a MicroAmp optical 384-well reaction plate (AppliedBiosystems, Carlsbad, California, USA) with optical caps (Sarsted, Nümbrecht, Germany). Extracted water (Thermo Fisher Scientific Inc., Waltham, USA) was used as a negative control.

2.3.4 Bacterial DNA Measurements

To determine the relative amount of bacterial DNA content in samples compared to human DNA, the human housekeeping gene RNase P was measured (TaqMan® RNase P Detection Reagents Kit, AppliedBiosystems, Carlsbad, California, USA). TaqMan™ Universal PCR Mastermix was used in a reaction of 20 µl. Real-time PCR was performed according to the manufacturer's specifications, using the AbiPrism 7000 HT Sequence Detection System (Taqman®, AppliedBiosystems, Carlsbad, California, USA).

Total amounts of bacteria were evaluated in rectal and caecal samples obtained at autopsy and from healthy volunteers in Study I and in the liver samples in Study II, using two types of standard curves. Tenfold dilution series of between 33 ng/ml and 0.00033 ng/ml from *E. coli* genomic DNA (ATCC 35401-5) and between 10^9 and 10^5 colony

forming units (CFU) per millilitre from *E. coli* (ATCC 25922) were used. Calculations were performed using the equation y= slope log (X) + intercept (Yoshida et al. 2003) with Universal primers and probe values.

2.3.5 Calculation of the Relative Amount of Bacterial DNA

Data from the RT-qPCR assays were analysed using SDS 2.2 software for tissue and blood samples and with SDS 2.1 software (Applied Biosystems California, USA) for stool and ascitic fluid samples. These programs calculate ΔRn using the equation Rn(+)−Rn(−). Rn(+) is the emission intensity of the reporter divided by the emission intensity of the quencher at any given time, whereas Rn(−) is the value of Rn(+) prior to PCR amplification. Thus, ΔRn indicates the magnitude of the signal generated. The critical threshold cycle (Ct) is the cycle at which a statistically significant increase in ΔRn is first detected and at which the fluorescence becomes detectable above background. Ct is inversely proportional to the logarithm of the initial number of template molecules, meaning the initial amount of sample DNA. The comparative Ct method (ΔΔCt, $\Delta Ct_{sample} - \Delta Ct_{\text{reference sample}}$), (Suzuki et al. 2005) for calculations of relative amounts of bacteria was used (with simplification) for all parts of this study, but the reference varied. The differences of the Ct values between the candidate bacteria and the universal bacteria measurement (ΔCt) for each sample were calculated; the comparative Ct (ΔΔCt) values for each sample and reference samples were also calculated (Tichopad et al. 2010).

To determine the relative amounts of bacteria in rectal autopsy samples in Study I, the corresponding mean value from the 7 healthy volunteers was used as a reference. To determine the relative amounts of bacteria in caecal autopsy samples, the rectal sample was used as an internal reference. To contrast the bacterial composition of the caecal and rectal autopsy samples, intra-individual differences in bacterial populations between these two sites were calculated, using the rectal sample as an internal reference for the caecal sample.

In Study II, the value obtained from a healthy volunteer's blood sample, calculated separately for each bacterial group in each run, was used as a reference for determining the presence and relative amounts of bacterial DNA.

In Study III, the mean values obtained from healthy male volunteers' stool samples were calculated and used as a reference to define the relative amounts of bacteria in the rectum of autopsy cases with alcoholic liver cirrhosis, alcoholics and controls. For ascitic fluid and liver samples, blood sample from healthy individual was used as a reference.

2.3.6 Determination of Bacterial Positivity

In Study II, the detection limits of Ct values in tissue and blood samples and in Study III for ascitic fluid and liver samples were between 15 and 35 for *Bacteroides* spp., *Bifidobacterium* spp., *Enterobacter* spp., *Clostridium leptum*, *S. aureus* and *S. epidermidis* and 15–40 for *Clostridium coccoides* and *Streptococcus* spp. mainly *Str. mitis*-group. Liver, MLN, blood, pericardial fluid and ascitic fluid samples were considered to be positive for bacterial DNA if the n-fold value was more than two times higher than the reference value. The cumulative bacterial negativity, *i.e.*, sterility percentages at different time points (1–3 days, 4–5 days and >5 days), were counted separately for culturing and RT-qPCR by first adding all findings together at each time-point from all sampling sites and then calculating the number of negative cases.

Bacterial percentages in liver autopsy samples (n=42) and ascitic fluid samples (n=12) in Study III were calculated using RT-qPCR amounts. First, the number of bacterial DNA-negative (negative cases/n) was subtracted from the total (100%), and the rest were considered to be bacteria-positive. Percentages were calculated from the relative values.

2.4 Liver Histology

Histological samples from the right lobe of the liver were taken at autopsy and fixed in 10% formalin overnight in the laboratory. The liver samples from the histological paraffin-embedded blocs were cut into 5-µm sections with a microtome and attached to slides +60°C overnight. Liver sections were visualised after staining with a microscope and an Olympus DP25 camera that was connected to a computer running the Cell^D-program (CellD SARL, Sauveterre, France), at a magnification of 40X.

Staining for CD14 (Leica Biosystems, Newcastle, United Kingdom) immunohistochemistry was performed at a dilution of 1:250 with the Autostainer LV-1 (Lab Vision Corporation, California, USA). The percentage of brown peroxidase discoloration in an area of 549453.91 μm^2 in each sample was calculated with the ImmunoRatio program (Tuominen et al. 2010), and the average discoloration value was compared between the different groups. ImmunoRatio is a non-commercial program that was developed for quantitative immunohistological analyses (Tuominen et al. 2010), and it calculates the immunopositive area as a percentage of the entire nuclear area.

2.5 Statistical Analyses

The median values and statistical calculations for the different groups were calculated using PASW Statistical Software, version 18 (SPSS Ltd, Quarry Bay, Hong Kong) in Study I. For Study III, SPSS, IBM version 21 was used. Due to the skewed variation within the bacterial measurements, logarithmic values were used in Studies I and III to calculate the relative amounts of bacteria. After logarithmic transformation, the values were normally distributed (I, III).

In Study I, the Kruskal-Wallis median test was used to assess the differences among the groups, and a P-value of less than 0.05 was considered statistically significant. If P was less than 0.05, post hoc pairwise comparisons using the Mann-Whitney U-test were performed (I).

In Study III, analysis of variance (ANOVA) was used to analyse the differences in bacterial populations among the different groups, *i.e.*, healthy volunteers, alcoholic liver cirrhotics, alcoholics without cirrhosis and controls. As significant results were found in the ANOVA, pairwise comparisons were performed using the post hoc test and the least significant difference (LSD) correction. Pearson's chi-square test was used on the liver samples.

2.6 Ethics

The study design was reviewed and approved by the Ethics Committee of the Pirkanmaa Hospital District and by the National Supervisory Authority for Welfare and Health (VALVIRA). Written consent was obtained from participating healthy volunteers and patients with alcoholic liver cirrhosis.

RESULTS

1 Post-Mortem Changes in Intestinal Bacterial Populations (Study I)

In Study I, time-dependent changes in major intestinal bacterial populations (*Bacteroides* spp., *Bifidobacterium* spp., *Clostridium leptum* group, *Clostridium coccoides* group, *Lactobacillus* spp., *Enterobactericeae*) and *Streptococcus* spp. were studied with RT-qPCR in post-mortem samples of the rectum and caecum that were obtained at 61 autopsies. Additionally, the rectal autopsy samples were compared to those of healthy volunteers (n=7) to determine whether post-mortem faecal samples could be used in future basic research.

Compared to healthy controls (n=7), the median relative amounts of different bacteria in the post-mortem rectal samples (1–3 days, n=18; 4–5 days, n=21; >5 days, n=20) at different time points were not significantly different for up to seven days after death (non-parametric Kruskal-Wallis test, Table 7). However, in post-mortem rectal samples, the amount of *Enterobactericeae* started to decrease (ns) after 5 days, compared to other study groups. Relative amounts of the *C. coccoides* group, *Streptococcus* spp. and *Bifidobacterium* spp. were smaller (ns) in post-mortem samples compared to healthy volunteers. Relative amounts of *Bacteroides* spp., *C. leptum* group and *Lactobacillus* spp. were increased (ns) in autopsy samples compared to healthy volunteers at different time points.

When the amount of bacterial DNA (ng/g) in all autopsy rectal samples was compared to faecal samples from healthy volunteers, there were no significant differences (p= 0.747). However, a large amount of inter-individual variation was present at all times and for all bacterial measurements.

Table 7. Amounts of bacterial DNA measured with RT-qPCR (n-fold difference) in faecal samples of healthy volunteers and rectal samples of autopsy cases. Comparisons among the groups were calculated using the non-parametric Kruskal-Wallis test.

Bacteria measured	Healthy Volunteers (n=7)	1–3 days (n=18)	4–5 days (n=21)	>5 days (n=20)	p-value
Bacteroides spp.	1.54	2.85	4.54	3.36	0.059
C. leptum group	0.52	0.71	1.29	1.00	0.083
C. coccoides group	1.12	0.35	0.42	0.69	0.177
Bifidobacterium spp.	1.08	0.72	0.74	0.26	0.515
Enterobactericeae	2.50	7.19	5.04	0.60	0.309
Streptococcus spp.	1.29	0.20	0.15	0.16	0.185
Lactobacillus spp.	0.83	3.85	2.59	2.72	0.409

In contrast, significant post-mortem time-dependent changes were observed in the relative amounts of bacteria in caecal autopsy samples, divided according to time since death (1–3 days, n=19; 4–5 days, n=21; >5 days, n=21) and calculated using the value of the rectal sample as an internal reference (Table 8). There were significantly more *Bacteroides* spp. (p=0.014, non-parametric median, Kruskal-Wallis test) and less *Lactobacillus* spp. (p=0.024) at 4–5 days, compared to the first group (1–3 days). There were no statistically significant differences among the different study groups with respect to the *C. leptum* group, the *C. coccoides* group, *Bifidobacterium* spp., *Enterobactericeae* and *Streptococcus* spp..

Table 8. Bacteria measured with RT-qPCR (n-fold difference) in caecal samples obtained from autopsy cases. Comparisons over the groups were calculated using the non-parametric Kruskal-Wallis test.

Bacteria measured	1–3 days (n=18)	4–5 days (n=21)	>5 days (n=20)	p-value
***Bacteroides* spp.**	**0.15**	**0.53**	**0.53**	**0.014**
C. leptum group	0.59	1.09	0.60	0.472
C. coccoides group	0.64	1.27	2.81	0.421
Bifidobacterium spp.	2.03	0.61	1.03	0.054
Enterobactericeae	1.37	0.68	0.86	0.358
Streptococcus spp.	3.56	2.28	1.85	0.192
***Lactobacillus* spp.**	**1.25**	**0.30**	**1.09**	**0.024**

Table 9. The total amount of bacterial DNA in faecal samples, showing the 25th–75th interquartile range. N is the number of study subjects. Non-parametric median and Kruskal-Wallis test comparisons were performed to compare the groups.

			N	ng/g median	25th–75th	p-value
Healthy Volunteers	Stool		7	26	9.2-36.7	
Autopsies	Rectum					0.023[1)]
		1–3 days	18	8	2.0–53.6	
		4–5 days	21	8	1.7–41.4	
		>5days	20	42	12.0–124.2	
	Caecum					0.982[2)]
		1–3 days	19	51	13.3–94.1	
		4–5 days	21	68	5.1–194.7	
		>5days	21	48	6.5–113.6	

P-values (over the groups) for [1)] for rectal samples at different time points and [2)] for caecal samples at different time points.

Total bacterial DNA amounts per gram at different sampling sites (rectum, caecum and healthy volunteers, Table 9) were calculated from two standard curves. Statistically significant differences were observed in the total bacterial DNA amount in healthy volunteers versus rectal autopsy samples (p=0.044, non-parametric median, Kruskal-Wallis test). There was slightly (ns) less bacterial DNA in rectal samples that were obtained 1–3 days post-mortem (median 8 ng/g) than in the samples that were obtained from volunteers (26 ng/g, p=0.423). In the rectal samples, a statistically significant difference was observed (p=0.023) between different time points. A statistically significant increase was observed after 5 days post mortem (p=0.012), with an increase from 8 ng/g (at 4–5 days) till 42 ng/g (at >5 days). The quantity of rectal bacterial DNA at >5 days was also statistically different than at 1–3 days (p=0.031). In caecal autopsy samples, the total bacterial DNA amount tended to remain quite stable over the post-mortem period (51 ng/g, 68 ng/g and 48 ng/g, p=0.982, at 1–3 days, 4–5 days, and >5 days, respectively).

2 Post-Mortem Translocation of Bacteria into Different Organs (Study II)

In Study II, we investigated post-mortem, time-dependent intestinal bacterial translocation from the intestine into the liver, pericardial fluid, mesenteric lymph nodes (MLN), portal vein and peripheral veins, using bacterial culturing and RT-qPCR. Samples were divided into groups based on time since death: 1–3 days (n= 10), 4–5 days (n=13) and >5 days (n=10). The bacterial groups that were analysed with RT-qPCR were: *Bacteroides* spp., *Bifidobacterium* spp., *Clostridium leptum* group, *Clostridium coccoides* group, *Enterobacter* spp., *Staphylococcus aureus* and *S. epidermidis*, and *Streptococcus* spp. mainly *Str. mitis* group.

With culturing, all the bacteria that were found in different organs of 33 autopsy cases were identified at the genus level, except for one anaerobic gram-negative rod, which remained unidentified. The bacterial genera identified included *Bacillus* (n=4), *Proteus* (1), *Peptostreptococcus* (1), *Neisseria* (1), *Micrococcus* (1), *Lactobacillus* (1), *Acinetobacter* (1), *Stomatococcus* (1), *Citrobacter* (1) *Bacteroides* (4), *Candidia* (1), *Corynebacterium* (1), *Clostridium* (24), *Enterococcus* (15), *Enterobacter* (8), *Escherichia* (14), *Klebsiella* (6), *Propionibacterium* (1), *Serratia* (2), *Streptococcus* (34) and *Staphylococcus* (43). None of the staphylococcal species were *S. aureus*. Of 21 identified bacterial genera, the most common types were *Staphylococcus* spp. (26%), *Streptococcus* spp. (20%), *Clostridium* spp., *Enterococcus* spp. and *Escherichia* spp..

Evaluation of culture positivity was conducted by dividing the microbial isolates into three different groups: sterile (no detectable bacterial growth), mono-isolates (a pure growth of a single isolate); and multi-isolates (a mixed growth of 2 bacteria or more). The highest rate of bacterial culture positivity was found in blood samples at all time points after death. Ninety-four percent (31/33 cases) of pericardial fluid samples were culture-negative; the only positive mono-isolates found with cultures belonged to *Clostridium* spp. (1–3 days) and *Streptococcus* spp. (5–7 days). Up to five days after death, the liver samples were also mostly bacterial culture-negative (64%), but after 5 days, the percent of negative samples decreased to 40%, with increased amounts of mono and multi-isolates. Mono-isolates in the liver samples included *Clostridium* spp., *Streptococcus* spp., *Enterobacter* spp., *Enterococcus* spp., *Escherichia* spp., *Staphylococcus* spp., and *Streptococcus* spp.. In MLN, bacterial culture negativity rates (approximately 40%) and the predominance of mono-isolates remained mostly stable until five days post-mortem, after which the rate of culture negativity decreased, and both microbial findings increased. The cumulative bacterial negativity percentages at all sampling sites in different time points with microbial culturing were 46% (1–3 days), 66% (4–5 days) and 36% (>5 days).

With RT-qPCR, the cumulative sterility (*i.e.*, bacterial DNA negativity) percentages at all sampling sites together were 42% (1–3 days), 38% (4–5 days) and 24% (>5 days), less than the rates observe in cultures (46%, 66% and 36%, respectively). The highest increase in bacterial DNA positivity was observed in MLN. The most bacterial DNA negative sample type (according to RT-qPCR) was pericardial fluid, which also remained negative for the longest period of time. The relative amounts of intestinal bacteria (*Clostridium* spp., *Enterobacter* spp., *Bifidobacterium* spp. and *Bacteroides* spp.) detected with RT-qPCR increased with the time since death in MLN, liver, and pericardial fluid; this trend was most marked in *Bifidobacterium* spp. (Table 10). In MLN and pericardial fluid samples, intestinal bacteria genera were the most commonly (60–73%) identified bacteria at 1–3 days after death; whereas in the liver, it was the least common (<50%). In control blood and portal vein samples, the relative amounts of intestinal bacteria varied between 75–82% and 47–59%, respectively and were quite stable over time.

Table 10. The relative amounts of intestinal bacterial DNA (%) in different sampling sites, as determined by RT-qPCR specific primers and probes in samples from 33 autopsies. Bacterial percentages were calculated from the relative positive bacterial amount. MLN, mesenteric lymph node. The results from two different *Clostridium* measurements are marked as *Clostridium* spp., *Streptococcus* spp. mainly *Str. mitis* group are marked as *Streptococcus* spp., and *S. aureus* and *S. epidermidis* are marked as *Staphylococcus* spp..

	Name of the species	1–3 days (n=10)	4–5 days (n=13)	>5 days (n=10)
Control blood	Bacterial DNA negative	3/10	3/13	1/10
	Bacterial DNA positive	7/10	10/13	9/10
	Staphylococcus spp.	9%	10%	10%
	Streptococcus spp.	9%	15%	15%
	Enterobacter spp.	23%	30%	30%
	Bifidobacterium spp.	11%	6%	6%
	Bacteroides spp.	8%	15%	15%
	Clostridium spp.	40%	24%	24%
Portal venous blood	Bacterial DNA negative	5/10	5/13	4/10
	Bacterial DNA positive	5/10	8/13	6/10
	Staphylococcus spp.	8%	6%	6%
	Streptococcus spp.	33%	37%	47%
	Enterobacter spp.	19%	5%	12%
	Bifidobacterium spp.	7%	5%	7%
	Bacteroides spp.	6%	8%	11%
	Clostridium spp.	27%	39%	17%
MLN	Bacterial DNA negative	6/10	1/13	1/10
	Bacterial DNA positive	4/10	12/13	9/10
	Staphylococcus spp.	7%	7%	7%
	Streptococcus spp.	20%	25%	14%
	Enterobacter spp.	11%	16%	5%
	Bifidobacterium spp.	12%	7%	16%
	Bacteroides spp.	37%	32%	31%
	Clostridium spp.	13%	13%	27%
Liver	Bacterial DNA negative	1/10	1/13	1/10
	Bacterial DNA positive	9/10	12/13	9/10
	Staphylococcus spp.	36%	21%	5%
	Streptococcus spp.	36%	26%	32%
	Enterobacter spp.	10%	17%	17%
	Bifidobacterium spp.	6%	10%	26%
	Bacteroides spp.	4%	7%	4%
	Clostridium spp.	8%	19%	16%
Pericardial fluid	Bacterial DNA negative	6/10	9/13	5/10
	Bacterial DNA positive	4/10	4/13	5/10
	Staphylococcus spp.	7%	38%	17%
	Streptococcus spp.	33%	16%	19%
	Enterobacter spp.	17%	15%	26%
	Bifidobacterium spp.	8%	8%	13%
	Bacteroides spp.	5%	8%	4%
	Clostridium spp.	30%	15%	21%

The contamination percentages of samples obtained from autopsy tissue (liver, pericardium, MLN) and blood samples were calculated based on the bacterial culturing results of Study II. If multi-growth or non-aureus staphylococcal bacteria were present in a sample, it was marked as contaminated, *i.e.*, unreliable. The contamination percentage was calculated from the total amount of each sample type observed at each time point. Bacterial culturing

results showed that the most unreliable sampling site (*i.e.*, the site most vulnerable to contamination) at different time points (1–3 days/4–5 days/>5 days) was control blood (50/54/60), followed by portal vein (30/39/30), MLN (10/15/20) and liver (10/15/10). The pericardial fluid showed no non-aureus staphylococcal or multi-growth at all, suggesting that it is resistant to contamination and is a very reliable sampling site.

3 The Role of Intestinal Bacteria in the Development of ALC (Study III)

In Study III, the possible involvement of intestinal bacteria in the development of alcoholic liver cirrhosis was investigated using faecal and liver samples obtained from 42 autopsies, including 13 cirrhotics, 15 alcoholics, 14 controls and 7 healthy volunteers. Bacterial translocation into ascitic fluid among cirrhotics was also examined. Based on results from Studies I and II, we chose to examine changes in and translocation of the major intestinal microbiota members of *Bacteroides* spp., *Bifidobacterium* spp., *Clostridium leptum* group, *Lactobacillus* spp. and *Enterobactericeae*.

The results of RT-qPCR showed differences in *Bacteroides* spp. and *Enterobactericeae* (p=0.070 and p=0.056, respectively, ANOVA) in faecal samples across the study groups. Relative ratios of faecal bacteria in autopsy controls were comparable to those of healthy volunteers. Cirrhotics had more *Bacteroides* spp. (p=0.013) and more *Enterobactericeae* (p=0.011) compared to healthy volunteers. A similar trend was also observed when cirrhotics were compared to autopsy controls, but the differences were not statistically significant, due to high inter-individual variation. In contrast, alcoholics (p=0.033) and cirrhotics (ns) harboured less *Clostridium leptum* group members than volunteers. Autopsy cases with cirrhosis did also have statistically significantly more *Enterobacter* spp. (p=0.034), a member of *Enterobactericeae*, than alcoholics without liver cirrhosis (data not shown). The amounts of *Bifidobacterium* spp. and *Lactobacillus* spp. were similar to healthy volunteers in all the study groups. When alcoholic liver cirrhotics were compared to all other groups combined (alcoholics, autopsy controls and healthy volunteers), cirrhotics were found to have significantly more *Bacteroides* spp. (p=0.049, ANOVA), *Enterobactericeae* (p=0.037), as well as *Enterobacter* spp. (p=0.047). *Bifidobacterium* spp. and *Lactobacillus* spp. showed no differences between the different subgroups. Inter-individual variation was notable in all bacterial measurements, especially in autopsy cases.

The most common bacterial DNA findings in liver samples from alcoholic liver cirrhotics were members of *Enterobactericeae*, which was detected (Figure 3) in 49% of bacteria, followed by *Bifidobacterium* spp. (24%) and *Bacteroides* spp. (10%). Further, increased bacterial DNA-positivity in cirrhotic livers (92%) was observed compared to

autopsy controls (64%) and alcoholics (60%), although this result was not statistically significant (p=0.130, Pearson chi-square).

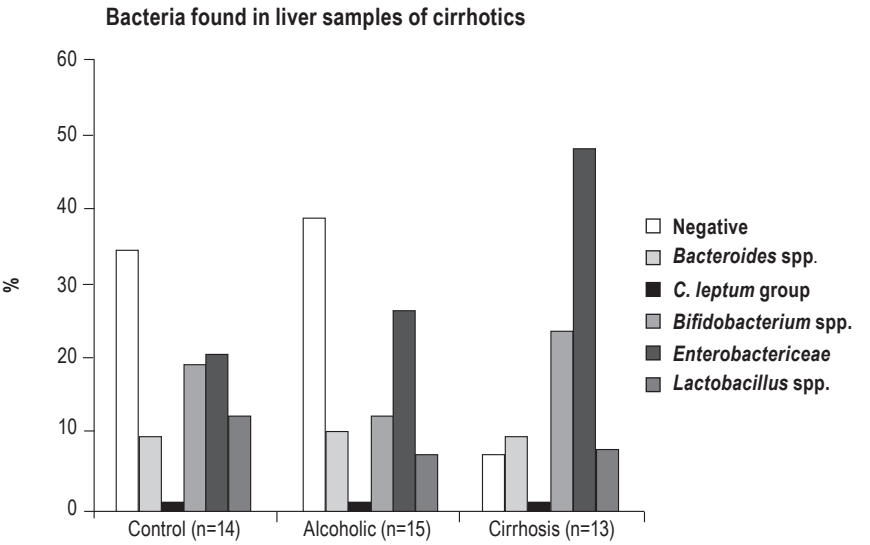

Figure 3. Bacterial DNA negativity and amounts of different bacteria found in 42 samples of the liver obtained at autopsy, detected with RT-qPCR specific primers and probes. Negative= bacterial DNA negative. Percentages have been calculated from the relative amounts. Differences are not statistically significant.

Although all ascitic fluid samples were culture-negative, 50% did contain bacterial DNA upon RT-qPCR. The species found were *Lactobacillus* spp. (18%, Figure 4), *C. leptum* group (16%) and *Enterobactericeae* (16%), whereas *Bifidobacterium* spp. and *Bacteroides* spp. were not amplified. The leucocyte count in ascitic fluid was under 250 106/l in all samples; thus, none of the patients were considered to have spontaneous bacterial peritonitis.

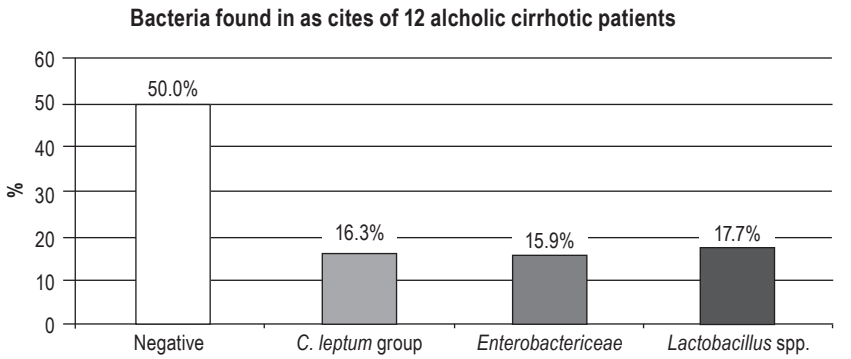

Figure 4. Bacterial DNA negativity and percentages of identified bacteria in 12 patients with alcoholic liver cirrhosis, according to RT-qPCR. Negative=bacterial DNA negative. Percentages have been calculated from the relative amounts.

CD14 expression in the liver, presented as median values of immunopositive areas compared to nuclear areas (presented as a percentage), varied significantly among alcoholic liver cirrhotics (31.9%), alcoholics (23.3%) and controls (17.6%) (p=0.012, ANOVA, Figure 5). Post hoc analyses with LSD correction revealed that the CD14 percentage was significantly higher in samples of cirrhotic livers than in control samples (p=0.004). Other significant differences were not observed among the groups. Furthermore, CD14 expression in bacterial DNA-positive liver samples was significantly higher than in negative samples (p=0.045).

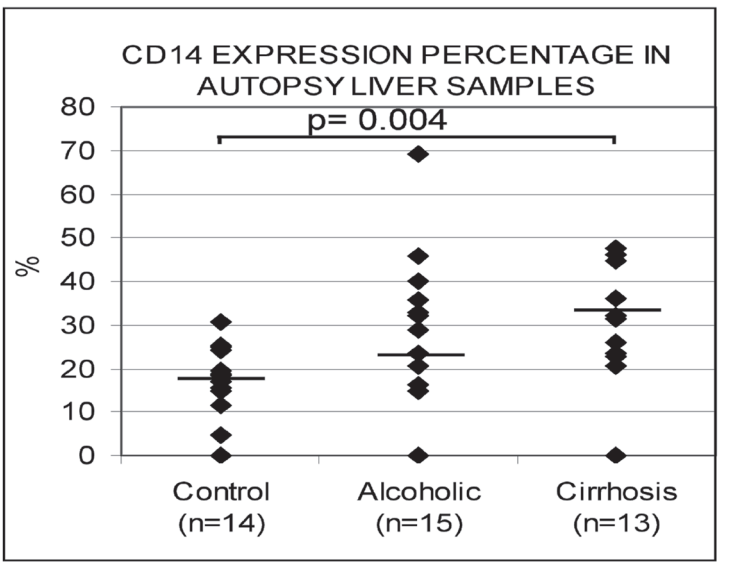

Figure 5. CD14 expression percentages in liver samples obtained at autopsy (n=42), calculated with ImmunoRatio. Individual values are shown as diamonds and median values as horizontal lines.

DISCUSSION

1 Post-Mortem Changes in Intestinal Bacterial Composition

The most abundant bacterial phyla in the human gut are *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* (NIH HMP Working Group et al. 2009). The bacterial groups investigated by RT-qPCR in this study were chosen to represent the normal intestinal microbiota in humans. *Bacteroides* spp., belonging to *Bacteroidetes*; *Clostridium* spp., *Streptococcus* spp. and *Lactobacillus* spp., belonging to *Firmicutes*; *Bifidobacterium* spp., belonging to *Actinobacteria*; and *Enterobactericeae*, belonging to *Proteobacteria*, were chosen to observe whether these major phyla in the gut change in a time-dependent fashion after death. The *Enterobactericeae* phyla consist mainly of gram-negative enteric bacteria that are involved in many disease conditions, while bifidobacteria and lactobacilli are considered important probiotic bacteria that help to prevent or ameliorate various conditions, such as malnutrition (Kerac et al. 2009, Kitajima et al. 1997), obesity (Kadooka et al. 2010), cirrhosis (Kirpich et al. 2008) and atopic dermatitis (Majamaa and Isolauri 1997). Substantial variations in species composition and distribution among living individuals have been reported (Eckburg et al. 2005, Turnbaugh et al. 2009) and were also observed in this study.

Even the use of highly sophisticated molecular biological techniques has not fully revealed the composition of the normal populations of intestinal microbiota during life (NIH HMP Working Group et al. 2009). The composition of and time-dependent post-mortem changes in intestinal bacterial populations have not been investigated. Only one study on the post-mortem intestinal microbiota has been published; this work was performed in autopsies using bacterial DNA sequencing and PCR (Hayashi et al. 2005). The present study aimed to clarify the microbiological changes after death in 61 medico-legal autopsy cases using RT-qPCR and to compare autopsy samples with samples from 7 living volunteers to determine whether post-mortem samples can be used for basic research concerning conditions during life.

After death, the body's temperature drops to the environmental level (which in a mortuary would be +4°C). Most human intestinal bacteria are temperature-dependent thermophilic bacteria and require normal body temperatures to survive and multiply. All these factors interfere with bacterial growth. This study showed that the relative amounts of bacteria in rectum and caecum remained quite stable over time up to 5 days after death and that rectal bacterial compositions were similar to those of healthy controls. Slight

differences were observed in the relative amounts of *Clostridium* spp., *Streptococcus* spp., *Bifidobacterium* spp., *Lactobacillus* spp. and *Bacteroides* spp. in rectal bacterial populations in the autopsy cases compared to the living controls. The amounts of *C. coccoides* group, *Bifidobacterium* spp. and *Streptococcus* spp. were slightly decreased after death, whereas *Bacteroides* spp., *Enterobactericeae* and *Lactobacillus* spp. were slightly increased compared to healthy volunteers. However, all these changes were minor and not statistically significant, suggesting that within this time period after death, the major bacterial populations regulate each other's growth and do not change. The amount of facultative anaerobic *Enterobactericeae* decreased after 5 days post-mortem. This finding suggests that *Enterobactericeae* are more host-dependent than the obligatory anaerobes *Bacteroides* spp., which increased slightly over the observed post-mortem interval. Furthermore, it appears that changes in oxygen levels do not completely explain the changes in the relative ratios. Our results suggest, however, that major bacterial populations (*Bacteroides* spp., *Enterobactericeae*, *Bifidobacterium* spp., *Lactobacillus* spp., *C. coccoides* group, *Streptococcus* spp. and *C. leptum* group) can be reliably measured up to 5 days after death in rectal samples. Measurements of the relative amounts of *C. coccoides* and *Enterobactericeae* groups more than 5 days after death seem to be unreliable.

Unlike the rectal samples, which showed no statistically significant differences in bacterial populations between autopsy samples and healthy living volunteers, amounts of *Bacteroides* spp. in the caecum had already increased significantly in the first group of 1–3 days after death and *Lactobacillus* spp. decreased, most likely due to changes in oxygen and nutrient levels ($p=0.014$ and 0.024, respectively). After death, interactions between the body and intestinal bacteria cease, and the concentration of oxygen begins to decrease, resulting in an imbalance in bacterial nutrient levels. *Lactobacillus* spp. might be more sensitive to oxygen levels than are the strictly anaerobic *Bacteroides* spp.. Lactobacilli are also most likely more host-dependent than *Bacteroides* spp.; death ends this symbiotic relationship, and the amount of lactobacilli consequently starts to decrease.

Earlier studies have shown fewer *Bifidobacterium* spp. and *Bacteroides* spp. in the caecum than in the rectum (Hayashi et al. 2005, Marteau et al. 2001). This was also the case with *Bacteroides* spp. in this study. The number of facultative anaerobes that tolerate low concentrations of oxygen, *e.g.*, *Escherichia* spp. and *Enterobacter* spp., are similar in caecal and faecal samples (Marteau et al. 2001). In contrast, obligatory anaerobes, including the *Clostridium leptum* and *Clostridium coccoides* groups, have been shown to be less common in the caecum than in the faeces (Marteau et al. 2001). In our study, the amounts of these obligatory anaerobes were similar in both the caecum and in the rectum, suggesting that growth conditions in the caecum change after death. Undigested nutrients and metabolites produced by bacteria accumulate in the caecum after death, which may promote bacterial growth. This is reflected in the well-known phenomenon that decomposition of the body can first be observed in the right side of the lower abdomen, just above the caecum.

In summary, we found that in post-mortem samples, the relative amounts of rectal *Bacteroides* spp., *Bifidobacterium* spp., the *C. leptum* group, the *C. coccoides* group, *Streptococcus* spp., *Lactobacillus* spp. and *Enterobactericeae* remained stable for up to 5 days after death and that rectal bacterial populations were similar to healthy controls. The total amount of bacterial DNA per gram in the rectum and the caecum remained stable for up to 5 days after death; after 5 days, bacterial populations began, to multiply in the rectum. These results suggest that major bacterial species can reliably be measured using RT-qPCR from samples obtained from the rectum after death, as long as the samples are collected no more than 5 days after death and the bodies and samples have been properly stored. The results parallel findings in living individuals. The caecal samples proved to be bacteriologically unreliable.

2 Evaluation of Post-Mortem Bacterial Migration

The purpose of Study II was to examine how soon after death bacteria migrate from the intestines into the pericardium, liver and other organs. To date, there have only been a few investigations into post-mortem microbiology (Morris et al. 2006, Weber et al. 2010). Using RT-qPCR and bacterial culturing of samples obtained at 33 autopsies, we investigated bacterial translocation in the major components of the human intestinal microbiota, including *Bacteroides* spp., *Bifidobacterium* spp., the *Clostridium leptum* group, the *Clostridium coccoides* group, *Enterobacter* spp., *Staphylococcus aureus* and *S. epidermidis*, and *Streptococcus* spp. mainly *Str. mitis* group.

Visceral organs are considered to be sterile during life, due to immune functions that rapidly clear escaped intestinal bacteria. Although these bacteria are mainly of intestinal origin, other possible sources include the skin and mucous membranes. After death, all normal immune functions, the tissue barrier and host-bacteria interactions normally vanish as a part of normal putrefaction, and bacteria begin to invade through the epithelium into the blood. In already 1964, Carpenter et al. showed that bacteria can be found in 40% of blood samples within 18 hours after death (Carpenter and Wilkins 1964). In life, gram-negative enteric bacilli such as *Escherichia coli*, *Proteus* spp., and *Enterobacter* spp. have been shown to translocate more efficiently, whereas gram-positive bacteria translocate only at intermediate levels, and obligate anaerobes translocate only at very low levels (Steffen et al. 1988). *Escherichia coli* is able to translocate particularly easily because it can adhere to the intestinal mucosa (Wells 1996). Anaerobic intestinal bacteria only rarely translocate compared to their aerobic counterparts (Steffen et al. 1988); in contrast, anaerobes tend to suppress the growth of other gut bacteria that have a greater ability to translocate, thereby limiting migration (Wells et al. 1987). The results of our cultures also suggested that post-mortem blood samples are microbiologically unreliable for the study of possible infections

during life, as 60% demonstrated multi-growth at the first analysis point (1–3 days after death), suggesting possible migration or contamination. The results of our cultures also revealed that pericardial fluid was the most culture negative sampling site at all times, with only two positive results at 1–3 days and at >5 days. Pericardial fluid remained quite stable during the entire post-mortem period, whereas culture negativity of liver samples decreased after post-mortem day 5, according to the culture results. RT-qPCR results showed that bacterial DNA negativity remained similar in the liver but decreased in the pericardial fluid after post-mortem day 5. These results clearly indicate that post-mortem blood samples are microbiologically unreliable for the evaluation of possible infection during life, as has also been suggested by other studies (Carpenter and Wilkins 1964, Wilson et al. 1993). Organs such as the pericardium and liver are the most suitable for microbiological sampling before the 5th day post mortem.

RT-qPCR showed high amounts of bacterial DNA in the liver samples. It is unknown whether the bacterial residuals measured in this study were transferred into the liver during life and engulfed by Kupffer cells or whether they arrived after death. Because the culture-based estimates of bacterial migration were stable for up to 5 days after death, it can be hypothesised that the bacteria observed with RT-qPCR were transported to the liver during life. In life, bacteria can migrate from the gut to the liver through portal venous blood. Even if gut-associated lymphoid tissues rapidly kill escaped bacteria, an unknown fraction may escape and be transported into the liver. The liver is full of innate immune cells (Suh and Jeong 2011), and bacteria and their residuals are cleared from the portal blood mainly by the sinusoidal Kupffer cells. These macrophages are capable of engulfing and killing bacteria, cancer cells and viruses (Beutler 2004, Kim et al. 2011). Furthermore, animal studies have shown that both alive and dead bacteria can escape from the intestine and be found in the liver (White et al. 2006).

This study is one of the first to describe how time since death affects bacterial findings in the pericardial fluid. According to our results pericardial fluid was the most bacterial DNA and culture-negative and remained as negative longest. Pericardial fluid differs from the other sampling sites used in this study because it can be aspirated as soon as the sternum has been removed, and no handling of organs in abdominal cavity is needed to sample this site. Furthermore, as the fluid is in a sac, it can easily be aspirated with a sterile needle into a sterile syringe, which can prevent contamination. Our results suggest that pericardial fluid could be used as a control sample for other microbial samples because it resists bacterial infiltration after death. The only bacteria detected in pericardial fluid samples were two mono-isolates belonging to *Clostridium* spp. and *Streptococcus* spp., suggesting true findings. Furthermore, bacteria detected in the pericardial fluid may provide accurate information about the true ongoing infections or even septicaemia during life. One possible reason for the rarity of finding microbes in the pericardial fluid is the fact that there is no direct blood flow to this area. Thus, bacteria cannot migrate into the pericardial

fluid through the blood; according to the literature, it is rare to have simultaneous sepsis and pericarditis (Kan et al. 2006). However, there are other possible routes for bacteria to migrate into pericardial fluid during life. They can travel from the mouth through the blood to the heart and then into the pericardial fluid (Morris et al. 2007). There are also two reports of pericarditis with local spread of infection without septicaemia. Lodha et al. showed that a bacterium that had caused a trichilemmal cyst on a patient's scalp was found only in pericardial fluid (Lodha et al. 2011). Another study by Ljenas-Garcia et al. describes a case of primary hepatic actinomycosis that caused purulent pericarditis (Llenas-Garcia et al. 2011).

3 Evaluation of Contamination of Samples and Their Clinical Use

In this thesis (Study II), contamination of post-mortem samples was evaluated with culturing results. Multi-growth and the growth of only a single *Staphylococcus* spp. (technical contamination) were considered to represent post-mortem contamination, *i.e.* unreliable sample, in this study, whereas according to literature a single bacterial finding can be considered as a true bacterial positive (Morris et al. 2006, Weber et al. 2010). The differentiation between a true finding, suggesting infection during life, and post-mortem migration is very challenging and scarcely investigated. At different time points after death (1–3 d, 4–5 d, >5 d after death, respectively), the calculated contamination percentages were as follows: 50%, 54%, and 60% in control blood samples, 30%, 39%, 30% in portal venous blood samples, 10%, 15%, 20% in MLN samples, and 10%, 15%, 10% in liver samples. There was no staphylococci growth or bacterial multi-growth in the pericardium at all. These results suggest that blood samples are often contaminated, the liver is rarely contaminated, and pericardial fluid is very rarely contaminated and may even be resistant to blood-borne post-mortem contamination over the analysed time periods. If post-mortem contamination is not present, then a detected bacterium can be considered to be a true positive result. In this study, there were two true positive findings in the pericardial fluid samples; one case had a single growth of streptococci 1–3 days after death, and another case had monoculture of clostridia (data not shown) >5 days after death. Seven samples from the liver were likely to represent true positive findings, including *Clostridium* spp., *Streptococcus* spp., *Enterobacter* spp., *Enterococcus* spp., *Escherichia* spp., *Staphylococcus* spp. and *Streptococcus* spp..

Based on the results of this work, it can be concluded that autopsy samples may be considered reliable for the study of disease associations of intestinal bacteria if they are taken within 5 days after death. For several reasons, the most reliable sampling places are the liver and the pericardial fluid. First, these 2 sampling places were the most frequently culture-negative and remained culture-negative the longest. Secondly, the liver is rarely

contaminated, and bacterial findings in the liver were mostly mono-isolates for up to 5 days after death. Pericardial fluid, according to our results, can be resistant to post-mortem bacterial contamination. Our results further suggest that the findings of RT-qPCR of liver and pericardial fluid samples represented true positives, as most of the isolates in these sampling sites were single bacterial isolates, and bacterial migration did not increase in culture. However, positive post-mortem microbiological findings must be interpreted with caution.

4 Evaluation of Techniques Used

In this study, there were differences in the results of RT-qPCR and culturing methods, specifically with respect to bacterial DNA negativity. Traditionally, bacterial cultures have been used to identify bacteria, but this approach is unreliable because most faecal bacteria are not culturable. It is estimated that 60–70% of bacteria living in the human intestinal tract cannot be easily cultured with currently available methods (Hayashi et al. 2002). The majority of intestinal bacteria are obligate anaerobes and thus cannot be grown on normal plates (Eckburg et al. 2005, Rinttilä et al. 2004). False negatives are thus a known problem of culturing (Alain and Querellou 2009). Moreover, culturing is a time-consuming method and may not provide any information regarding the bacterial composition of the sample if bacteria present have been exposed to unfavourable conditions such as the presence of oxygen or temperature changes. RT-qPCR allows the detection of parts of the sample that cannot be cultured, as well as dead bacteria and released bacterial nucleic acids, *e.g.*, DNA in macrophages. With conventional culturing, only living bacteria can be diagnosed. Therefore, the bacterial results of RT-qPCR and culturing cannot be directly compared. Moreover, culture techniques for clinical (or medical) purposes have been designed only to detect clinically important bacteria. Conventional culturing is the golden standard for diagnosing clinically relevant bacteria and identifying antibiotic sensitivity or resistance. However, RT-qPCR also detects major intestinal bacteria that are unculturable, not just pathogenic bacteria. The RT-qPCR technique used in this study was found to be a fast and accurate tool for the analysis of the faecal microbiota.

However, even if we found RT-qPCR to be a usable microbiological method, it still has some limitations. The microbial 16S rRNA genes targeted with RT-qPCR are so well conserved that RT-qPCR has a limited ability to differentiate between closely related species. Although the 16S rRNA gene can be used as a universal marker when multiplying genetic material from all species present in a sample, different bacterial species have different copy numbers of the gene. This may lead to an over- or under-representation of some bacterial species with RT-qPCR as the 16S rRNA gene is used as a target for the primers and probes (Mohania et al. 2008). Additionally, evaluation of possible contamination of a sample with

RT-qPCR is difficult because of its high sensitivity for the detection of bacteria and their residuals, not only for living bacteria. Furthermore, RT-qPCR provides no information about other bacterial species that may be present, beyond those that have been targeted with specific primers and probes.

5 The Role of Intestinal Bacteria in ALC

The involvement of major intestinal bacteria in the development of alcoholic liver cirrhosis was studied using RT-qPCR in 42 autopsies and 7 healthy volunteers; also, bacterial translocation into ascitic fluid was studied in 12 patients with clinical evidence of cirrhosis.

Today, it is thought that long-term chronic alcohol abuse can shift intestinal bacteria populations towards a more harmful gram-negative majority by increasing the amount of *Proteobacteria* and *Fusobacteria* (Chen et al. 2011). Alcohol also diminishes the diversity of bacterial species, and intestinal bacterial overgrowth has been observed in alcoholics (Bode et al. 1993). Our study showed that cirrhotics had considerably more gram-negative *Bacteroides* spp., *Enterobactericeae* and *Enterobacter* spp. in their faecal samples than healthy volunteers. Cirrhotics had a median of 27 times more *Enterobactericeae* than controls and 24 times more *Enterobacter* spp.. This finding regarding the *Enterobactericeae* phylum is in agreement with other studies (Chen et al. 2011, Zhao et al. 2004), whereas the increased amount of *Bacteroides* spp. observed in our study is not (Chen et al. 2011). Patients with cirrhosis of the liver have also been reported to have significantly decreased amounts of beneficial bacterial species, such as *Lactobacillus* (Yan et al. 2011) and *Bifidobacterium* spp. (Zhao et al. 2004), as well as simultaneously increased amounts of *Clostridium* spp. (Zhao et al. 2004). We did not observe significant decreases in the amounts of *Bifidobacterium* spp. or *Lactobacillus* spp. amounts in the different study groups, in contrast to other studies. This may be due to limited number of study subjects and high inter- and intra-individual variation. The finding that patients with alcoholic liver cirrhosis have less *Bifidobacterium* spp. and *Lactobacillus* spp. in the faeces suggests that reduced amounts of these bacteria may be involved in the pathogenesis of alcoholic liver cirrhosis. In the future, one possible treatment option for cirrhosis could be administration of the probiotics *Bifidobacterium* spp. and *Lactobacillus* spp., for which there is already evidence from human trials (Kirpich et al. 2008). Daily consumption of probiotic preparations increased the total number of these bacteria in the gut and decreased of the population of other, more harmful species, such as enterobacteria (Arora et al. 2012, Fujiwara et al. 2001). Probiotics are also known to be capable of reinforcing the tight junctions between intestinal epithelial cells that may be injured by alcohol, thereby decreasing the likelihood of bacterial translocation from the gut (Ulluwishewa et al. 2011). Probiotics can further reduce the production and translocation of endotoxins from the gut by enhancing the production of anti-inflammatory cytokines

and the secretion of antibacterial proteins (Bongaerts et al. 2005, Forsyth et al. 2009, Gratz et al. 2010, Wang et al. 2005). Furthermore, probiotics can modulate local and systemic immune responses (Forchielli and Walker 2005). *Lactobacillus* spp. have been found to increase the number of other beneficial bacteria (Rochet et al. 2006, Guerin-Danan et al. 1998) and to improve the growth and division of intestinal epithelial cells (Freitas et al. 2003) by reducing the permeability of the mucous membrane to different infectious agents (Parassol et al. 2005). In addition, probiotic bacteria reinforce the defense mechanisms of the mucous membrane by increasing the amount of lymphocytes present (Marcos et al. 2004). Thus, daily consumption of lactobacilli and bifidobacteria increases the total number of these bacteria in the gut, thereby decreasing the amount of other, more harmful, bacteria (Arora et al. 2012, Fujiwara et al. 2001).

One of the treatment goals in liver cirrhosis is to inhibit the growth of gram-negative bacteria in the gut by using broad-spectrum antibiotics, *e.g.*, neomycin, norfloxacin and ciprofloxacin (Terg et al. 2008, Madrid et al. 2001). Antibiotics have also been used to prevent infections in patients with upper gastrointestinal haemorrhage and recurrent spontaneous bacterial peritonitis (Leber et al. 2012). Antibiotics can prevent the colonisation of harmful gram-negative bacteria, *e.g.*, *Enterobacter* spp. (Hartmann et al. 2012). However, long-term use of antibiotics may lead to an increase in the amount of pathogenic bacteria in the gut (Brandl et al. 2008) and to increased antibiotic resistance (Campillo et al. 1998, Novella et al. 1997). In experimental studies, probiotics (*Bifidobacterium* spp., *Lactobacillus* spp.) have restored normal gut homeostasis and inhibited the excessive growth of gram-negative bacteria (Hartmann et al. 2012, Kirpich et al. 2008, Leber et al. 2012). However, in addition to the use of probiotics, targeted treatments against high-risk pathogens (such as *Enterobacter* spp.) need to be considered.

5.1 Bacterial Translocation and CD14 in ALC

This study also investigated bacterial translocation from the intestine into ascitic fluid and the liver. It is known that the alcohol metabolite acetaldehyde affects the function of the gut epithelial barrier by disrupting tight junctions between adjacent cells, thereby enhancing bacterial translocation (Basuroy et al. 2005). The species that escape the most easily into internal organs are the commensal intestinal bacteria, such as enterobacteria, enterococci and streptococci (Steffen et al. 1988) and are the most common organisms found in community-acquired infections in patients with alcoholic cirrhosis (Garcia-Tsao, 1992). We found that cirrhotic livers were less likely to be sterile than the livers of both control and alcoholic subjects. Alcohol ingestion has been reported to correlate with increased plasma levels of bacterial components, endotoxin (Adachi et al. 1995, Nanji et al. 1993, Tamai et al. 2000) and peptidoglycan (Tabata et al. 2002). Our results also showed that

more *Enterobactericeae* was present in liver samples obtained from cirrhotics than in those taken from controls and alcoholics. Furthermore, 50% of ascitic fluid samples were found to have bacterial DNA from *Lactobacillus* spp., *C. leptum* group and *Enterobactericeae*, suggesting bacterial translocation. One limitation of this study was that we were unable to obtain control samples from non-cirrhotic peritoneal fluid. The differences in the amounts of different bacteria in liver samples and ascitic fluid were, however, not statistically significant, possibly due to the small sample size and large inter-individual differences. Our results suggest that there may be continuous bacterial translocation from intestine into the liver that, under normal circumstances, is handled by Kupffer cells. When translocation greatly increases for one reason or another, bacteria are no longer cleared efficiently.

It has previously been shown that genetic variations in the promoter area of the CD14 bacterial recognition gene are associated with the risk of alcoholic liver cirrhosis (SNP rs2569190, -159 T>C) (Jarvelainen et al. 2001). This finding has been confirmed by other research groups (Campos et al. 2005, Desmet and Roskams 2004) and is supported by the experimental finding that alcoholic liver disease is reduced in CD14-deficient mice (Yin et al. 2001). The total bacterial DNA amount in liver samples in the present study was significantly associated with the percentage of CD14 expression (on Kuppfer cells); furthermore, the CD14 expression percentage was significantly higher in the livers of cirrhotics than in the autopsy controls. This suggests that Kupffer cells play a role in bacterial recognition. It is possible that bacterial translocation from the gut may be involved in the development of alcoholic liver cirrhosis in genetically susceptible individuals, due to cytokine production and liver inflammation that leads to fibrosis and cirrhosis.

Taken together, these findings show that there are differences in bacterial translocation and in intestinal bacterial populations between alcoholic liver cirrhotics and controls. Alcoholic liver cirrhotics have a greatly increased amount of enterobacteria in the faeces, liver and ascitic fluid. Cirrhotics also have increased expression of the bacterial recognition receptor CD14 in the liver. The level of CD14 expression was associated with total bacterial DNA amount. These findings further suggest that changes in intestinal bacterial populations and their translocation into the liver may contribute to the pathogenesis and development of alcoholic liver cirrhosis. However, we do not exactly know how detected bacteria have ended up into the liver. Have they migrated from intestine into liver because of the increased epithelial permeability due to alcohol or because of impaired hepatic immune functions due to some other reason? This still remains unanswered. However, more research on this topic is clearly needed before the interactions among ALC, the intestinal microbiota and genetic factors can be fully understood.

SUMMARY AND CONCLUSIONS

The results from this thesis reveal that certain post-mortem samples can be used for basic bacteriological research into situations in living patients, if the samples are handled with care and the results are interpreted cautiously by a person with microbiological experience and knowledge. Faecal samples from autopsies are comparable to stool samples from living volunteers for the evaluation of certain bacterial genera and species.

We found that the most reliable tissue sampling sites were liver and pericardial fluid. These sites can be used up to 5 days after death to study mechanisms of different diseases during life; pericardial fluid can even be used as a microbiological control for post-mortem contamination or bacterial migration. If mono-isolates are found at these sampling sites, they can be considered as true positive indicators of infection during life.

Our findings suggest a role for intestinal bacteria in the pathogenesis of alcoholic liver cirrhosis. We found increased amounts of *Enterobacteriaea* in cirrhotics faeces, liver and ascitic fluid samples, compared to controls. Cirrhotics also had increased expression of the CD14 bacterial recognition receptor; an association between CD14 and total bacterial amount was also noted in cirrhotic liver samples. The connection between cirrhosis and CD14 genetics has been previously demonstrated (Järvelainen et al. 2001). Our results suggest that harmful growth of intestinal *Enterobactericeae* may lead to bacterial translocation into the liver and to their increased recognition by hepatic Kupffer cells. Overproduction of cytokines may lead to liver fibrosis and possibly cirrhosis.

RT-qPCR is a useful tool for determining the bacterial ratios of human samples. This thesis suggests that RT-qPCR can be used to detect small amounts of bacteria in supposedly sterile organs. Positive bacterial RT-qPCR results may indicate the presence of non-culturable or dead bacterial cells or bacterial components that may induce long-term inflammation. Our results are preliminary due to the limited number of cases and, like all post-mortem microbiological findings, must be interpreted with caution.

ACKNOWLEDGEMENTS

This study was performed at the Department of Forensic Medicine, School of Medicine, University of Tampere, during the years 2010–2014.

This study was financially supported by the Tampere Graduate Program in Biomedicine and Biotechnology (TGPBB), the Finnish Foundation for Alcohol Studies, the Competitive Research Funding of the Pirkanmaa Hospital District, the European Union 7th Framework Program, grant number 201668 of the AtheroRemo Project and the Pirkanmaa Regional Fund of the Finnish Cultural Foundation. I would like to thank the Science Fund of the City of Tampere for covering the expenses for the printing of this book.

First, I want to sincerely express my gratitude to my mentors, Professor Pekka J. Karhunen and Docent Tanja Pessi. Pekka gave me this opportunity and taught me how to work independently, giving me the opportunity to make my own decisions while being there for my questions. Tanja has been like a lightning conductor while sharing the room with me. This work would not have been succeeded without Tanja's help and expertise.

The reviewers of my thesis work, Professor Airi Palva and Professor Helena Isoniemi, are acknowledged.

My co-authors Risto Vuento, Janne Aittoniemi and Pekka Collin are warmly thanked for their co-operation, help and excellent comments on the original articles. Jaakko Pohjoismäki and Risto Vuento are also thanked for participating in my thesis committee during the TGPBB years and providing valuable ideas and opinions on my work.

I want to thank all the personnel of the Fimlab who have been part of my thesis work. All past and present members of the Department of Forensic Medicine, especially Kari Mänttäri, Olli Penttilä and Mervi Seppänen, are thanked for their help and assistance. Mervi's mental support has been valuable over these years. She has offered me a shoulder to cry on.

I also want to acknowledge Juulia Jylhävä for helping me with this project, reading the book, providing instruction, and coping with my ever-increasing frustration with this dissertation.

I sincerely thank my dearest friends Maikki Järvinen, Ansku Autero and Jonna Kuusisto for taking my thoughts away from this work. I would also like to thank all my other friends not especially mentioned here for their general friendship and all the fun moments together over the years.

The biggest thanks go to my family, especially to my parents Ritva and Ilpo, for supporting and believing in me in all I have done for all these years: Kiitos äiti ja isä! I am also grateful to my brother Jari for all of his help during the course of my life.

Finally, I want to show my deepest gratitude from the bottom of my heart for my fiancée who has been there for me all these years, Kiitos Jyri!

Tampere, April 19, 2014

Sari Tuomisto

REFERENCES

Adachi Y, Moore LE, Bradford BU, Gao W and Thurman RG (1995): Antibiotics prevent liver injury in rats following long-term exposure to ethanol. Gastroenterology 108, 218–224.

Akira S, Takeda K and Kaisho T (2001): Toll-like receptors: Critical proteins linking innate and acquired immunity. Nature Immunology 2, 675–680.

Alain K and Querellou J (2009): Cultivating the uncultured: Limits, advances and future challenges. Extremophiles: Life Under Extreme Conditions 13, 583–594.

Alatalo PI, Koivisto HM, Hietala JP, Puukka KS, Bloigu R and Niemela OJ. (2008): Effect of moderate alcohol consumption on liver enzymes increases with increasing body mass index. The American Journal of Clinical Nutrition 88, 1097–1103.

Angelakis E, Merhej V and Raoult D (2013): Related actions of probiotics and antibiotics on gut microbiota and weight modification. The Lancet Infectious Diseases 13, 889–899.

Anthony PP, Ishak K.G, Nayak NC, Poulsen HE, Scheuer PJ and Sobin LH (1977): The morphology of cirrhosis: Definition, nomenclature, and classification. Bulletin of the World Health Organization 55, 521–540.

Arora T, Anastasovska J, Gibson G, Tuohy K, Sharma RK, Bell J and Frost G (2012): Effect of lactobacillus acidophilus NCDC 13 supplementation on the progression of obesity in diet-induced obese mice. The British Journal of Nutrition 108, 1382–9.

Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR and Bork P (2011): Enterotypes of the human gut microbiome. Nature 473, 174–180.

Asquith M, Pasala S, Engelmann F, Haberthur K, Meyer C, Park B and Messaoudi I (2013): Chronic ethanol consumption modulates growth factor release, mucosal cytokine production, and MicroRNA expression in nonhuman primates. Alcoholism, Clinical and Experimental Research 38, 980–93.

Azcarate-Peril MA, Sikes M and Bruno-Barcena JM (2011): The intestinal microbiota, gastrointestinal environment and colorectal cancer: A putative role for probiotics in prevention of colorectal cancer? American Journal of Physiology. Gastrointestinal and Liver Physiology 301, G401–24.

Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A and Gordon JI (2004): The gut microbiota as an environmental factor that regulates fat storage. Proceedings of the National Academy of Sciences of the United States of America 101, 15718–15723.

Backhed F, Ley RE, Sonnenburg JL, Peterson DA and Gordon JI (2005): Host-bacterial mutualism in the human intestine. Science 307, 1915–1920.

Bajaj JS, Heuman DM, Hylemon PB, Sanyal AJ, White MB, Monteith P, Noble NA, Unser AB, Daita K, Fisher AR, Sikaroodi M and Gillevet PM (2014): Altered profile of human gut microbiome is associated with cirrhosis and its complications. Journal of Hepatology 60, 940–7.

Bajaj JS, Ridlon JM, Hylemon PB, Thacker LR, Heuman DM, Smith S and Gillevet PM (2012): Linkage of gut microbiome with cognition in hepatic encephalopathy. American Journal of Physiology. Gastrointestinal and Liver Physiology 302, G168–75.

Bajanowski T, Vege A, Byard RW, Krous HF, Arnestad M, Bachs L and Rognum TO (2007): Sudden infant death syndrome (SIDS)--standardised investigations and classification: Recommendations. Forensic Science International 165, 129–143.

Barton PS, Cunningham SA, Lindenmayer DB and Manning AD (2013): The role of carrion in maintaining biodiversity and ecological processes in terrestrial ecosystems. Oecologia 171, 761–772.

Bas S, Gauthier BR, Spenato U, Stingelin S and Gabay C (2004): CD14 is an acute-phase protein. Journal of Immunology 172, 4470–4479.

Basuroy S, Sheth P, Mansbach CM and Rao RK (2005): Acetaldehyde disrupts tight junctions and adherens junctions in human colonic mucosa: Protection by EGF and L-glutamine. American Journal of Physiology.Gastrointestinal and Liver Physiology 289, G367–75.

Benten D and Wiest R (2012): Gut microbiome and intestinal barrier failure – the "achilles heel" in hepatology? Journal of Hepatology 56, 1221–1223.

Berg RD and Garlington AW (1979): Translocation of certain indigenous bacteria from the gastrointestinal tract to the mesenteric lymph nodes and other organs in a gnotobiotic mouse model. Infection and Immunity 23, 403–411.

Beutler B (2004): Innate immunity: An overview. Molecular Immunology 40, 845–859.

Bjarnason I, Peters TJ and Wise RJ (1984): The leaky gut of alcoholism: Possible route of entry for toxic compounds. Lancet 1, 179–182.

Blaser MJ (2010): Harnessing the power of the human microbiome. Proceedings of the National Academy of Sciences of the United States of America 107, 6125–6126.

Bode C and Bode JC (2003): Effect of alcohol consumption on the gut. Best Practice & Research. Clinical Gastroenterology 17, 575–592.

Bode C, Kolepke R, Schafer K, and Bode JC (1993): Breath hydrogen excretion in patients with alcoholic liver disease – evidence of small intestinal bacterial overgrowth. Zeitschrift Fur Gastroenterologie 31, 3–7.

Bode C, Kugler V and Bode JC (1987): Endotoxemia in patients with alcoholic and non-alcoholic cirrhosis and in subjects with no evidence of chronic liver disease following acute alcohol excess. Journal of Hepatology 4, 8–14.

Bode JC (1980): Alcohol and the gastrointestinal tract. Ergebnisse Der Inneren Medizin Und Kinderheilkunde 45, 1–75.

Bongaerts G, Severijnen R and Timmerman H (2005): Effect of antibiotics, prebiotics and probiotics in treatment for hepatic encephalopathy. Medical Hypotheses 64, 64–68.

Bouskra D, Brezillon C, Berard M, Werts C, Varona R, Boneca IG and Eberl G (2008): Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature 456, 507–510.

Brandl K, Plitas G, Mihu CN, Ubeda C, Jia T, Fleisher M and Pamer EG (2008): Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. Nature 455, 804–807.

Brunk CF, Li J and Avaniss-Aghajani E (2002): Analysis of specific bacteria from environmental samples using a quantitative polymerase chain reaction. Current Issues in Molecular Biology 4, 13–18.

Bungert HJ (1973): Absorption of hemoglobin and hemoglobin iron in alcohol-induced liver injury. Digestion 9, 293–308.
Butzbach DM (2010): The influence of putrefaction and sample storage on post-mortem toxicology results. Forensic Science, Medicine, and Pathology 6, 35–45.
Campillo B, Dupeyron C, Richardet JP, Mangeney N and Leluan G (1998): Epidemiology of severe hospital-acquired infections in patients with liver cirrhosis: Effect of long-term administration of norfloxacin. Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America 26, 1066–1070.
Campos J, Gonzalez-Quintela A, Quinteiro C, Gude F, Perez LF, Torre JA and Vidal C (2005): The -159C/T polymorphism in the promoter region of the CD14 gene is associated with advanced liver disease and higher serum levels of acute-phase proteins in heavy drinkers. Alcoholism, Clinical and Experimental Research 29, 1206–1213.
Canny GO and McCormick BA (2008): Bacteria in the intestine, helpful residents or enemies from within? Infection and Immunity 76, 3360–3373.
Carpenter HM and Wilkins RM (1964): Autopsy bacteriology: Review of 2,033 cases. Archives of Pathology 77, 73–81.
Carter DO, Yellowlees D and Tibbett M (2010): Moisture can be the dominant environmental parameter governing cadaver decomposition in soil. Forensic Science International 200, 60–66.
Chen CC, Teng LJ and Chang TC (2004): Identification of clinically relevant viridans group streptococci by sequence analysis of the 16S–23S ribosomal DNA spacer region. Journal of Clinical Microbiology 42, 2651–2657.
Chen CC, Teng LJ, Kaiung S and Chang TC (2005): Identification of clinically relevant viridans streptococci by an oligonucleotide array. Journal of Clinical Microbiology 43, 1515–1521.
Chen Y, Yang F, Lu H, Wang B, Chen Y, Lei D and Li L (2011): Characterization of fecal microbial communities in patients with liver cirrhosis. Hepatology 54, 562–572.
Chesta J, Defilippi C and Defilippi C (1993): Abnormalities in proximal small bowel motility in patients with cirrhosis. Hepatology 17, 828–832.
Chiva M, Soriano G, Rochat I, Peralta C, Rochat, F, Llovet T and Balanzo J (2002): Effect of lactobacillus johnsonii La1 and antioxidants on intestinal flora and bacterial translocation in rats with experimental cirrhosis. Journal of Hepatology 37, 456–462.
Chung H, Pamp SJ, Hill JA, Surana NK, Edelman SM, Troy EB and Kasper DL (2012): Gut immune maturation depends on colonization with a host-specific microbiota. Cell 149, 1578–1593.
Claesson MJ, O'Sullivan O, Wang Q, Nikkila J, Marchesi JR, Smidt H, and O'Toole PW (2009): Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PloS One 4, e6669.
Cleveland MG, Gorham JD, Murphy TL, Tuomanen E and Murphy KM (1996): Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. Infection and Immunity 64, 1906–1912.
Cummings JH and Macfarlane GT (1997): Colonic microflora: Nutrition and health. Nutrition 13, 476–478.

Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW and Greenberg EP (1998): The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280, 295–298.

Debard N, Sierro F, Browning J and Kraehenbuhl JP (2001): Effect of mature lymphocytes and lymphotoxin on the development of the follicle-associated epithelium and M cells in mouse peyer's patches. Gastroenterology 120, 1173–1182.

Delgado S, Suarez A, Otero L and Mayo B (2004): Variation of microbiological and biochemical parameters in the faeces of two healthy people over a 15 day period. European Journal of Nutrition 43, 375–380.

Desmet VJ and Roskams T (2004): Cirrhosis reversal: A duel between dogma and myth. Journal of Hepatology 40, 860–867.

Dickson GC, Poulter RT, Maas EW, Probert PK and Kieser JA (2011): Marine bacterial succession as a potential indicator of postmortem submersion interval. Forensic Science International 209, 1–10.

Durban A, Abellan JJ, Jimenez-Hernandez N, Ponce M, Ponce J, Sala T and Moya A (2011): Assessing gut microbial diversity from feces and rectal mucosa. Microbial Ecology 61, 123–133.

Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M and Relman DA (2005): Diversity of the human intestinal microbial flora. Science 308, 1635–1638.

Eisen JA (2007): Environmental shotgun sequencing: Its potential and challenges for studying the hidden world of microbes. PLoS Biology 5, e82.

Elamin EE, Masclee AA, Dekker J and Jonkers DM (2013): Ethanol metabolism and its effects on the intestinal epithelial barrier. Nutrition Reviews 71, 483–499.

El-Nezami H, Mykkanen H, Kankaanpaa P, Salminen S and Ahokas J (2000): Ability of lactobacillus and propionibacterium strains to remove aflatoxin B, from the chicken duodenum. Journal of Food Protection 63, 549–552.

Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA and Smith TF (2006): Real-time PCR in clinical microbiology: Applications for routine laboratory testing. Clinical Microbiology Reviews 19, 165–256.

Fagarasan S and Honjo T (2004): Regulation of IgA synthesis at mucosal surfaces. Current Opinion in Immunology 16, 277–283.

Finegold S M, Molitoris D, Song Y, Liu C, Vaisanen ML, Bolte E and Kaul A (2002): Gastrointestinal microflora studies in late-onset autism. Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America 35, S6–S16.

Forchielli ML and Walker WA (2005): The role of gut-associated lymphoid tissues and mucosal defence. The British Journal of Nutrition 93 Suppl 1, S41–8.

Forsyth CB, Farhadi A, Jakate SM, Tang Y, Shaikh M and Keshavarzian A (2009): Lactobacillus GG treatment ameliorates alcohol-induced intestinal oxidative stress, gut leakiness, and liver injury in a rat model of alcoholic steatohepatitis. Alcohol 43, 163–172.

Forsyth CB, Tang Y, Shaikh, M, Zhang L and Keshavarzian A (2011): Role of snail activation in alcohol-induced iNOS-mediated disruption of intestinal epithelial cell permeability. Alcoholism, Clinical and Experimental Research 35, 1635–1643.

Franks AH, Harmsen HJ, Raangs GC, Jansen GJ, Schut F and Welling GW (1998): Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. Applied and Environmental Microbiology 64, 3336–3345.

Freitas M, Tavan E, Cayuela C, Diop L, Sapin C and Trugnan G (2003): Host-pathogens cross-talk. indigenous bacteria and probiotics also play the game. Biology of the Cell / Under the Auspices of the European Cell Biology Organization 95, 503–506.

Fujiwara S, Seto Y, Kimura A and Hashiba H (2001): Intestinal transit of an orally administered streptomycin-rifampicin-resistant variant of bifidobacterium longum SBT2928: Its long-term survival and effect on the intestinal microflora and metabolism. Journal of Applied Microbiology 90, 43–52.

Fujiya M, Musch MW, Nakagawa Y, Hu S, Alverdy J, Kohgo Y and Chang, EB (2007): The bacillus subtilis quorum-sensing molecule CSF contributes to intestinal homeostasis via OCTN2, a host cell membrane transporter. Cell Host & Microbe 1, 299–308.

Fukuda S, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K and Ohno H (2011): Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature 469, 543–547.

Furet JP, Firmesse O, Gourmelon M, Bridonneau C, Tap J, Mondot S and Corthier G (2009): Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR. FEMS Microbiology Ecology 68, 351–362.

Furrie E (2006): A molecular revolution in the study of intestinal microflora. Gut 55, 141–143.

Färkkilä M (2013): Alkoholihepatiitti on vaikea ja yleinen maksasairaus. Suomen lääkärilehti, 68, 25–32.

Garcia-Tsao G (1992): Spontaneous bacterial peritonitis. Gastroenterology Clinics of North America 21, 257–275.

Garrett WS, Gordon JI and Glimcher LH (2010): Homeostasis and inflammation in the intestine. Cell 140, 859–870.

Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS and Nelson KE (2006): Metagenomic analysis of the human distal gut microbiome. Science 312, 1355–1359.

Gomez F, Ruiz P, and Schreiber AD (1994): Impaired function of macrophage fc gamma receptors and bacterial infection in alcoholic cirrhosis. The New England Journal of Medicine 331, 1122–1128.

Gomez-Hurtado I, Santacruz A, Peiro G, Zapater P, Gutierrez A, Perez-Mateo M and Frances R (2011): Gut microbiota dysbiosis is associated with inflammation and bacterial translocation in mice with CCl4-induced fibrosis. PloS One 6, e23037.

Gordon FD (2012): Ascites. Clinics in Liver Disease 16, 285–299.

Gratz SW, Mykkanen H and El-Nezami HS (2010): Probiotics and gut health: A special focus on liver diseases. World Journal of Gastroenterology: WJG 16, 403–410.

Grenham S, Clarke G, Cryan JF and Dinan TG (2011): Brain-gut-microbe communication in health and disease. Frontiers in Physiology 2, 94.

Guarner C, Runyon BA, Young S. Heck M and Sheikh MY (1997): Intestinal bacterial overgrowth and bacterial translocation in cirrhotic rats with ascites. Journal of Hepatology 26, 1372–1378.

Guarner F and Malagelada JR (2003): Gut flora in health and disease. Lancet 361, 512–519.

Guerin-Danan C, Chabanet C, Pedone C, Popot F, Vaissade P, Bouley C and Andrieux C (1998): Milk fermented with yogurt cultures and lactobacillus casei compared with yogurt and gelled milk: Influence on intestinal microflora in healthy infants. The American Journal of Clinical Nutrition 67, 111–117.

Hartmann P, Chen P, Wang HJ, Wang L, McCole DF, Brandl K and Schnabl B (2013): Deficiency of intestinal mucin-2 ameliorates experimental alcoholic liver disease in mice. Hepatology 58, 108–119.

Hartmann P, Chen WC and Schnabl B (2012): The intestinal microbiome and the leaky gut as therapeutic targets in alcoholic liver disease. Frontiers in Physiology 3, 402.

Haslam TC and Tibbett M (2009): Soils of contrasting pH affect the decomposition of buried mammalian (ovis aries) skeletal muscle tissue. Journal of Forensic Sciences 54, 900–904.

Hassner A, Kletter Y, Shlag D, Yedvab M, Aronson M and Shibolet S (1981): Impaired monocyte function in liver cirrhosis. British Medical Journal (Clinical Research Ed.) 282, 1262–1263.

Hauge T, Persson J and Danielsson D (1997): Mucosal bacterial growth in the upper gastrointestinal tract in alcoholics (heavy drinkers). Digestion 58, 591–595.

Hayashi H, Sakamoto M and Benno Y (2002): Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. Microbiology and Immunology 46, 535–548.

Hayashi H, Takahashi R, Nishi T, Sakamoto M and Benno Y (2005): Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism. Journal of Medical Microbiology 54, 1093–1101.

Hayashi T, Kishiwada M, Fujii K, Yuasa H, Nishioka J, Ido M and Suzuki K (2006): Lipopolysaccharide-induced decreased protein S expression in liver cells is mediated by MEK/ERK signaling and NFkappaB activation: Involvement of membrane-bound CD14 and toll-like receptor-4. Journal of Thrombosis and Haemostasis: JTH 4, 1763–1773.

Haziot A, Rong GW, Silver J and Goyert SM (1993): Recombinant soluble CD14 mediates the activation of endothelial cells by lipopolysaccharide. Journal of Immunology 151, 1500–1507.

Heine H and Lien E (2003): Toll-like receptors and their function in innate and adaptive immunity. International Archives of Allergy and Immunology 130, 180–192.

Heumann D and Roger T (2002): Initial responses to endotoxins and gram-negative bacteria. Clinica Chimica Acta; International Journal of Clinical Chemistry 323, 59–72.

Heyman M, Crain-Denoyelle AM, Corthier G, Morgat JL and Desjeux JF (1986): Postnatal development of protein absorption in conventional and germ-free mice. The American Journal of Physiology 251, G326–31.

Hoefs JC, Canawati HN, Sapico FL, Hopkins RR Weiner J and Montgomerie JZ (1982): Spontaneous bacterial peritonitis. Hepatology 2, 399–407.

Hoffmann JA, Kafatos FC, Janeway CA and Ezekowitz RA (1999): Phylogenetic perspectives in innate immunity. Science 284, 1313–1318.

Holmes E, Li JV, Athanasiou T, Ashrafian H and Nicholson JK (2011): Understanding the role of gut microbiome-host metabolic signal disruption in health and disease. Trends in Microbiology 19, 349–359.

Hooper LV and Gordon JI (2001): Commensal host-bacterial relationships in the gut. Science 292, 1115–1118.

Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG and Gordon JI (2001): Molecular analysis of commensal host-microbial relationships in the intestine. Science 291, 881–884.

Humblot C, Murkovic M, Rigottier-Gois L, Bensaada M, Bouclet A, Andrieux C and Rabot S (2007): Beta-glucuronidase in human intestinal microbiota is necessary for the colonic genotoxicity of the food-borne carcinogen 2-amino-3-methylimidazo[4,5-f]quinoline in rats. Carcinogenesis 28, 2419–2425.

Hyde ER, Haarmann DP, Lynne AM, Bucheli SR and Petrosino JF (2013): The living dead: Bacterial community structure of a cadaver at the onset and end of the bloat stage of decomposition. PloS One 8, e77733.

Hyndman DL and Mitsuhashi M (2003): PCR primer design. Methods in Molecular Biology 226, 81–88.

Ikenouchi J, Matsuda M, Furuse M and Tsukita S (2003): Regulation of tight junctions during the epithelium-mesenchyme transition: Direct repression of the gene expression of claudins/occludin by snail. Journal of Cell Science 116, 1959–1967.

Ilan Y (2012): Leaky gut and the liver: A role for bacterial translocation in nonalcoholic steatohepatitis. World Journal of Gastroenterology: WJG 18, 2609–2618.

Jahr TG, Ryan L, Sundan A, Lichenstein HS, Skjak-Braek G and Espevik T (1997): Induction of tumor necrosis factor production from monocytes stimulated with mannuronic acid polymers and involvement of lipopolysaccharide-binding protein, CD14, and bactericidal/permeability-increasing factor. Infection and Immunity 65, 89–94.

Jakobsson HE, Abrahamsson TR, Jenmalm MC, Harris K, Quince C, Jernberg C and Andersson AF (2013): Decreased gut microbiota diversity, delayed bacteroidetes colonisation and reduced Th1 responses in infants delivered by caesarean section. Gut 63, 559–66.

Järvelainen HA, Orpana A, Perola M, Savolainen VT, Karhunen PJ and Lindros KO (2001): Promoter polymorphism of the CD14 endotoxin receptor gene as a risk factor for alcoholic liver disease. Hepatology (Baltimore, Md.) 33, 1148–1153.

Jokelainen K, Matysiak-Budnik T, Makisalo H, Hockerstedt K and Salaspuro M (1996): High intracolonic acetaldehyde values produced by a bacteriocolonic pathway for ethanol oxidation in piglets. Gut 39, 100–104.

Kadooka Y, Sato M, Imaizumi K, Ogawa A, Ikuyama K, Akai Y and Tsuchida T (2010): Regulation of abdominal adiposity by probiotics (lactobacillus gasseri SBT2055) in adults with obese tendencies in a randomized controlled trial. European Journal of Clinical Nutrition 64, 636–643.

Kakiyama G, Pandak WM, Gillevet PM, Hylemon PB, Heuman DM, Daita K and Bajaj JS (2013): Modulation of the fecal bile acid profile by gut microbiota in cirrhosis. Journal of Hepatology 58, 949–955.

Kalis C, Kanzler B, Lembo A, Poltorak A, Galanos C and Freudenberg MA (2003): Toll-like receptor 4 expression levels determine the degree of LPS-susceptibility in mice. European Journal of Immunology 33, 798–805.

Kan B, Ries J, Normark BH, Chang FY, Feldman C, Ko WC, and Ortqvist A (2006): Endocarditis and pericarditis complicating pneumococcal bacteraemia, with special reference to the adhesive abilities of pneumococci: Results from a prospective study. Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases 12, 338–344.

Kau AL, Ahern PP, Griffin NW, Goodman AL and Gordon JI (2011): Human nutrition, the gut microbiome and the immune system. Nature 474, 327–336.

Keelan M, Walker K and Thomson AB (1985): Effect of chronic ethanol and food deprivation on intestinal villus morphology and brush border membrane content of lipid and marker enzymes. Canadian Journal of Physiology and Pharmacology 63, 1312–1320.

Kelly P (2010): Nutrition, intestinal defence and the microbiome. The Proceedings of the Nutrition Society 69, 261–268.

Kerac M, Bunn J, Seal, A, Thindwa M, Tomkins A, Sadler K and Collins S (2009): Probiotics and prebiotics for severe acute malnutrition (PRONUT study): A double-blind efficacy randomised controlled trial in malawi. Lancet 374, 136–144.

Khan J, Pikkarainen P, Karvonen AL, Makela T, Peraaho M, Pehkonen E and Collin P (2009): Ascites: Aetiology, mortality and the prevalence of spontaneous bacterial peritonitis. Scandinavian Journal of Gastroenterology 44, 970–974.

Kim TH, Lee SH, and Lee SM (2011): Role of kupffer cells in pathogenesis of sepsis-induced drug metabolizing dysfunction. The FEBS Journal 278, 2307–17.

Kirpich IA, Solovieva NV, Leikhter SN, Shidakova NA, Lebedeva OV, Sidorov PI and Cave M (2008): Probiotics restore bowel flora and improve liver enzymes in human alcohol-induced liver injury: A pilot study. Alcohol 42, 675–682.

Kitajima H, Sumida Y, Tanaka R, Yuki N, Takayama H and Fujimura M (1997): Early administration of bifidobacterium breve to preterm infants: Randomised controlled trial. Archives of Disease in Childhood.Fetal and Neonatal Edition 76, F101–7.

Knights D, Lassen KG and Xavier RJ (2013): Advances in inflammatory bowel disease pathogenesis: Linking host genetics and the microbiome. Gut 62, 1505–1510.

Knolle PA and Limmer A (2003): Control of immune responses by savenger liver endothelial cells. Swiss Medical Weekly 133, 501–506.

Krous HF, Beckwith JB, Byard RW, Rognum TO. Bajanowski T, Corey T and Mitchell EA (2004): Sudden infant death syndrome and unclassified sudden infant deaths: A definitional and diagnostic approach. Pediatrics 114, 234–238.

Kusunoki T and Wright SD (1996): Chemical characteristics of staphylococcus aureus molecules that have CD14-dependent cell-stimulating activity. Journal of Immunology 157, 5112–5117.

Landmann R, Knopf HP, Link S, Sansano S, Schumann R and Zimmerli W (1996): Human monocyte CD14 is upregulated by lipopolysaccharide. Infection and Immunity 64, 1762–1769.

Lata J, Jurankova J, Kopacova M and Vitek P (2011): Probiotics in hepatology. World Journal of Gastroenterology: WJG 17, 2890–2896.

Lay C, Sutren M, Rochet V, Saunier K, Dore J and Rigottier-Gois L (2005): Design and validation of 16S rRNA probes to enumerate members of the clostridium leptum subgroup in human faecal microbiota. Environmental Microbiology 7, 933–946.

Leber B, Spindelboeck W and Stadlbauer V (2012): Infectious complications of acute and chronic liver disease. Seminars in Respiratory and Critical Care Medicine 33, 80–95.

Leist TP, Frei K, Kam-Hansen S, Zinkernagel RM and Fontana A (1988): Tumor necrosis factor alpha in cerebrospinal fluid during bacterial, but not viral, meningitis. Evaluation in murine model infections and in patients. The Journal of Experimental Medicine 167, 1743–1748.

Leser TD and Molbak L (2009): Better living through microbial action: The benefits of the mammalian gastrointestinal microbiota on the host. Environmental Microbiology 11, 2194–2206.

LeVan TD, Bloom JW, Bailey TJ, Karp CL. Halonen M, Martinez FD and Vercelli D (2001): A common single nucleotide polymorphism in the CD14 promoter decreases the affinity of sp protein binding and enhances transcriptional activity. Journal of Immunology 167, 5838–5844.

Lewis CM Jr, Obregon-Tito A, Tito RY, Foster MW and Spicer PG. (2012): The human microbiome project: Lessons from human genomics. Trends in Microbiology 20, 1–4.

Ley RE, Backhed F, Turnbaugh P, Lozupone CA, Knight RD and Gordon JI (2005): Obesity alters gut microbial ecology. Proceedings of the National Academy of Sciences of the United States of America 102, 11070–11075.

Ley RE, Lozupone CA, Hamady M, Knight R and Gordon JI (2008): Worlds within worlds: Evolution of the vertebrate gut microbiota. Nature Reviews. Microbiology 6, 776–788.

Ley RE, Peterson DA and Gordon JI (2006): Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124, 837–848.

Li X, Wang C, Nie J, Lv D, Wang T and Xu Y (2013): Toll-like receptor 4 increases intestinal permeability through up-regulation of membrane PKC activity in alcoholic steatohepatitis. Alcohol 47, 459–465.

Lien E and Ingalls RR (2002): Toll-like receptors. Critical Care Medicine 30, S1–S11.

Liu J, Wu D, Ahmed A, Li X, Ma Y, Tang L and Xin Y (2012): Comparison of the gut microbe profiles and numbers between patients with liver cirrhosis and healthy individuals. Current Microbiology 65, 7–13.

Liu Q, Duan ZP, Ha DK, Bengmark S, Kurtovic J and Riordan SM (2004): Synbiotic modulation of gut flora: Effect on minimal hepatic encephalopathy in patients with cirrhosis. Hepatology 39, 1441–1449.

Lindsay DT (1995): Functional Human Anatomy. Mosby

Llenas-Garcia J, Lalueza-Blanco A, Fernandez-Ruiz M, Villar-Silva J, Ochoa M, Lozano F and Aguado JM (2011): Primary hepatic actinomycosis presenting as purulent pericarditis with cardiac tamponade. Infection 40, 339–41.

Llovet JM, Bartoli R, Planas R, Cabre E, Jimenez M, Urban A and Gassull MA (1994): Bacterial translocation in cirrhotic rats. its role in the development of spontaneous bacterial peritonitis. Gut 35, 1648–1652.

Lloyd KL and Kubes P (2006): GPI-linked endothelial CD14 contributes to the detection of LPS. American Journal of Physiology.Heart and Circulatory Physiology 291, H473–81.

Lobmaier IV K, Vege Å, Gaustad P and Rognum TO (2009): Bacteriological investigation-significance of time lapse after death. European Journal of Clinical Microbiology and Infectious Diseases 28, 1191–1198.

Lodha A, Enakpene E, Haran M, Sadiq A and Shani J (2011): Bacterial pericarditis caused by infected trichilemmal cyst. Journal of Infection and Chemotherapy 18, 251–4.

Loppnow H, Stelter F, Schonbeck U, Schluter C, Ernst M, Schutt C and Flad HD (1995): Endotoxin activates human vascular smooth muscle cells despite lack of expression of CD14 mRNA or endogenous membrane CD14. Infection and Immunity 63, 1020–1026.

Lu H, Wu, Z, Xu W, Yang J, Chen Y and Li L (2011): Intestinal microbiota was assessed in cirrhotic patients with hepatitis B virus infection. intestinal microbiota of HBV cirrhotic patients. Microbial Ecology 61, 693–703.

Lukkari TA, Jarvelainen HA, Oinonen T, Kettunen E and Lindros KO (1999): Short-term ethanol exposure increases the expression of kupffer cell CD14 receptor and lipopolysaccharide binding protein in rat liver. Alcohol and Alcoholism 34, 311–319.

Macfarlane GT and Macfarlane S (1997): Human colonic microbiota: Ecology, physiology and metabolic potential of intestinal bacteria. Scandinavian Journal of Gastroenterology. Supplement 222, 3–9.

Madrid AM, Hurtado C, Venegas M, Cumsille F and Defilippi C (2001): Long-term treatment with cisapride and antibiotics in liver cirrhosis: Effect on small intestinal motility, bacterial overgrowth, and liver function. The American Journal of Gastroenterology 96, 1251–1255.

Majamaa H and Isolauri E (1997): Probiotics: A novel approach in the management of food allergy. The Journal of Allergy and Clinical Immunology 99, 179–185.

Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L and Dore J (2006): Reduced diversity of faecal microbiota in crohn's disease revealed by a metagenomic approach. Gut 55, 205–211.

Marcos A, Warnberg J, Nova E, Gomez S, Alvarez A, Alvarez R and Cobo JM (2004): The effect of milk fermented by yogurt cultures plus lactobacillus casei DN-114001 on the immune response of subjects under academic examination stress. European Journal of Nutrition 43, 381–389.

Markle JG, Frank DN, Mortin-Toth S. Robertson CE, Feazel LM, Rolle-Kampczyk U and Danska JS (2013): Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. Science.) 339, 1084–1088.

Marteau P, Pochart P, Dore J, Bera-Maillet C, Bernalier A and Corthier G (2001): Comparative study of bacterial groups within the human cecal and fecal microbiota. Applied and Environmental Microbiology 67, 4939–4942.

Mazmanian SK, Liu CH, Tzianabos AO and Kasper DL (2005): An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell 122, 107–118.

Mestecky J, Russell, MW and Elson CO (1999): Intestinal IgA: Novel views on its function in the defence of the largest mucosal surface. Gut 44, 2–5.

Milani S, Herbst H, Schuppan D, Surrenti C, Riecken EO and Stein H (1990): Cellular localization of type I III and IV procollagen gene transcripts in normal and fibrotic human liver. The American Journal of Pathology 137, 59–70.

Mitsuoka T (1992): Intestinal flora and aging. Nutrition Reviews 50, 438–446.
Mohania D, Nagpal R, Kumar M, Bhardwaj A, Yadav M, Jain S and Yadav H (2008): Molecular approaches for identification and characterization of lactic acid bacteria. Journal of Digestive Diseases 9, 190–198.
Moore WE and Holdeman LV (1974a): Human fecal flora: The normal flora of 20 japanese-hawaiians. Applied Microbiology 27, 961–979.
Moreau MC, Ducluzeau R, Guy-Grand D and Muller MC (1978) Increase in the population of duodenal immunoglobulin A plasmocytes in axenic mice associated with different living or dead bacterial strains of intestinal origin. Infection and Immunity 21, 532–539.
Morris JA, Harrison LM, Biswas J and Telford DR (2007): Transient bacteraemia: A possible cause of sudden life threatening events. Medical Hypotheses 69, 1032–1039.
Morris JA, Harrison LM and Partridge SM (2006) Postmortem bacteriology: A re-evaluation. Journal of Clinical Pathology 59, 1–9.
Mutlu EA, Gillevet PM, Rangwala H, Sikaroodi M, Naqvi A, Engen PA and Keshavarzian A (2012): Colonic microbiome is altered in alcoholism. American Journal of Physiology. Gastrointestinal and Liver Physiology 302, G966–78.
Nakanishi N, Tashiro K, Kuhara S, Hayashi T, Sugimoto N and Tobe T (2009): Regulation of virulence by butyrate sensing in enterohaemorrhagic escherichia coli. Microbiology 155, 521–530.
Nanji AA, Khettry U, Sadrzadeh SM and Yamanaka T (1993): Severity of liver injury in experimental alcoholic liver disease. correlation with plasma endotoxin, prostaglandin E2, leukotriene B4, and thromboxane B2. The American Journal of Pathology 142, 367–373.
National Research Council (US) Committee on Metagenomics: Challenges and Functional Applications. (2007).
NIH HMP Working Group, Peterson J, Garges S, Giovanni M, McInnes P, Wang L and Guyer M (2009): The NIH human microbiome project. Genome Research 19, 2317–2323.
Nosova T, Jokelainen K, Kaihovaara P, Jousimies-Somer H, Siitonen A, Heine R and Salaspuro M (1996): Aldehyde dehydrogenase activity and acetate production by aerobic bacteria representing the normal flora of human large intestine. Alcohol and Alcoholism 31, 555–564.
Novella M, Sola R, Soriano G, Andreu M, Gana J, Ortiz J and Vilardell F (1997): Continuous versus inpatient prophylaxis of the first episode of spontaneous bacterial peritonitis with norfloxacin. Hepatology 25, 532–536.
O'Hara AM and Shanahan F (2006): The gut flora as a forgotten organ. EMBO Reports 7, 688–693.
Orrhage K and Nord CE (2000): Bifidobacteria and lactobacilli in human health. Drugs Under Experimental and Clinical Research 26, 95–111.
Page RB and Stromberg AJ (2011): Linear methods for analysis and quality control of relative expression ratios from quantitative real-time polymerase chain reaction experiments. The Scientific World Journal 11, 1383–1393.
Pai R and Kang G (2008): Microbes in the gut: A digestable account of host-symbiont interactions. The Indian Journal of Medical Research 128, 587–594.

Pande C, Kumar A and Sarin SK (2009): Small-intestinal bacterial overgrowth in cirrhosis is related to the severity of liver disease. Alimentary Pharmacology & Therapeutics 29, 1273–1281.

Parassol N, Freitas M, Thoreux K, Dalmasso G, Bourdet-Sicard R and Rampal P (2005): Lactobacillus casei DN-114 001 inhibits the increase in paracellular permeability of enteropathogenic escherichia coli-infected T84 cells. Research in Microbiology 156, 256–262.

Pechal JL, Crippen TL, Benbow ME, Tarone AM, Dowd S and Tomberlin JK (2014): The potential use of bacterial community succession in forensics as described by high throughput metagenomic sequencing. International Journal of Legal Medicine 128, 193–205.

Pereira F, Carneiro J, Matthiesen R, van Asch B, Pinto N, Gusmao L and Amorim A (2010): Identification of species by multiplex analysis of variable-length sequences. Nucleic Acids Research 38, e203.

Pessi T, Isolauri E, Sutas Y, Kankaanranta H, Moilanen E and Hurme M (2001): Suppression of T-cell activation by lactobacillus rhamnosus GG-degraded bovine casein. International Immunopharmacology 1, 211–218.

Pessi T, Sutas Y, Hurme M and Isolauri E (2000): Interleukin-10 generation in atopic children following oral lactobacillus rhamnosus GG. Clinical and Experimental Allergy: Journal of the British Society for Allergy and Clinical Immunology 30, 1804–1808.

Pessi T, Sutas Y, Marttinen A and Isolauri E (1998): Probiotics reinforce mucosal degradation of antigens in rats: Implications for therapeutic use of probiotics. The Journal of Nutrition 128, 2313–2318.

Pijls KE, Koek GH, Elamin EE, de Vries H, Masclee AA and Jonkers DM (2014): Large intestine permeability is increased in patients with compensated liver cirrhosis. American Journal of Physiology. Gastrointestinal and Liver Physiology 306, G147–53.

Proctor LM (2011): The human microbiome project in 2011 and beyond. Cell Host & Microbe 10, 287–291.

Pugin J, Heumann ID, Tomasz A, Kravchenko VV, Akamatsu Y, Nishijima M and Ulevitch RJ (1994): CD14 is a pattern recognition receptor. Immunity 1, 509–516.

Pugin J, Schurer-Maly CC, Leturcq D, Moriarty A, Ulevitch RJ and Tobias PS (1993): Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. Proceedings of the National Academy of Sciences of the United States of America 90, 2744–2748.

Purohit V, Gao B and Song BJ (2009): Molecular mechanisms of alcoholic fatty liver. Alcoholism, Clinical and Experimental Research 33, 191–205.

Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C and Wang J (2010): A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464, 59–65.

Rajkovic IA and Williams R (1986): Abnormalities of neutrophil phagocytosis, intracellular killing and metabolic activity in alcoholic cirrhosis and hepatitis. Hepatology 6, 252–262.

Rea MC, Sit CS, Clayton E, O'Connor PM, Whittal RM, Zheng J and Hill C (2010): Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against clostridium difficile. Proceedings of the National Academy of Sciences of the United States of America 107, 9352–9357.

Rinttilä T, Kassinen A, Malinen E, Krogius L and Palva A (2004): Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. Journal of Applied Microbiology 97, 1166–1177.

Rioux KP, Madsen KL and Fedorak RN (2005): The role of enteric microflora in inflammatory bowel disease: Human and animal studies with probiotics and prebiotics. Gastroenterology Clinics of North America 34, 465–82, ix.

Roberts FJ (1998): Procurement, interpretation, and value of postmortem cultures. European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology 17, 821–827.

Rochet V, Rigottier-Gois L, Sutren M, Krementscki MN, Andrieux C, Furet J. P and Dore J (2006): Effects of orally administered lactobacillus casei DN-114 001 on the composition or activities of the dominant faecal microbiota in healthy humans. The British Journal of Nutrition 95, 421–429.

Rolandelli RH, Koruda MJ, Settle RG, Leskiw MJ, Stein TP and Rombeau JL (1989): The effect of pectin on hepatic lipogenesis in the enterally-fed rat. The Journal of Nutrition 119, 89–93.

Rosenfeld ME and Campbell LA (2011): Pathogens and atherosclerosis: Update on the potential contribution of multiple infectious organisms to the pathogenesis of atherosclerosis. Thrombosis and Haemostasis 106, 858–867.

Round JL and Mazmanian SK (2009): The gut microbiota shapes intestinal immune responses during health and disease. Nature Reviews.Immunology 9, 313–323.

Saito Y, Sakamoto M, Takizawa S and Benno Y (2004): Monitoring the cell number and viability of lactobacillus helveticus GCL1001 in human feces by PCR methods. FEMS Microbiology Letters 231, 125–130.

Salaspuro V, Nyfors S, Heine R, Siitonen A, Salaspuro M and Jousimies-Somer H (1999): Ethanol oxidation and acetaldehyde production in vitro by human intestinal strains of escherichia coli under aerobic, microaerobic, and anaerobic conditions. Scandinavian Journal of Gastroenterology 34, 967–973.

Salyers AA (1984): Bacteroides of the human lower intestinal tract. Annual Review of Microbiology 38, 293–313.

Sansonetti PJ (2008): War and peace at the intestinal epithelial surface: An integrated view of bacterial commensalism versus bacterial pathogenicity. Journal of Pediatric Gastroenterology and Nutrition 46, E6–7.

Savage DC (2001). Microbial biota of the human intestine: A tribute to some pioneering scientists. Current Issues in Intestinal Microbiology 2, 1–15.

Schaffert CS, Duryee MJ, Hunter CD, Hamilton BC 3rd, DeVeney AL, Huerter MM and Thiele GM (2009): Alcohol metabolites and lipopolysaccharide: Roles in the development and/or progression of alcoholic liver disease. World Journal of Gastroenterology: WJG 15, 1209–1218.

Schenker S and Bay MK (1995): Alcohol and endotoxin: Another path to alcoholic liver injury? Alcoholism, Clinical and Experimental Research 19, 1364–1366.

Schild S, Tamayo R, Nelson EJ, Qadri F, Calderwood SB and Camilli A (2007): Genes induced late in infection increase fitness of vibrio cholerae after release into the environment. Cell Host & Microbe 2, 264–277.

Schloss PD and Handelsman J (2005): Metagenomics for studying unculturable microorganisms: Cutting the gordian knot. Genome Biology 6, 229.
Sears CL (2005): A dynamic partnership: Celebrating our gut flora. Anaerobe 11, 247–251.
Seitz HK and Becker P (2007): Alcohol metabolism and cancer risk. Alcohol Research & Health: The Journal of the National Institute on Alcohol Abuse and Alcoholism 30, 38–41, 44–7.
Seitz HK, Egerer G, Oneta C, Kramer S, Sieg A, Klee F and Simanowski UA (1996): Alcohol dehydrogenase in the human colon and rectum. Digestion 57, 105–108.
Sekirov I, Russell SL, Antunes LC and Finlay BB (2010): Gut microbiota in health and disease. Physiological Reviews 90, 859–904.
Sen K and Asher DM (2001): Multiplex PCR for detection of enterobacteriaceae in blood. Transfusion 41, 1356–1364.
Sghir A, Gramet G, Suau A, Rochet V, Pochart P and Dore J (2000): Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. Applied and Environmental Microbiology 66, 2263–2266.
Sharma R and Schumacher U (1995): Morphometric analysis of intestinal mucins under different dietary conditions and gut flora in rats. Digestive Diseases and Sciences 40, 2532–2539.
Sharma R, Schumacher U, Ronaasen V and Coates M (1995): Rat intestinal mucosal responses to a microbial flora and different diets. Gut 36, 209–214.
Shimizu K, Ogura H, Goto M, Asahara T, Nomoto K, Morotomi M and Sugimoto H (2006): Altered gut flora and environment in patients with severe SIRS. The Journal of Trauma 60, 126–133.
Soell M, Lett E, Holveck F, Scholler M, Wachsmann D and Klein JP (1995): Activation of human monocytes by streptococcal rhamnose glucose polymers is mediated by CD14 antigen, and mannan binding protein inhibits TNF-alpha release. Journal of Immunology 154, 851–860.
Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ and Langella P (2008): Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of crohn disease patients. Proceedings of the National Academy of Sciences of the United States of America 105, 16731–16736.
Son G, Kremer M and Hines IN (2010): Contribution of gut bacteria to liver pathobiology. Gastroenterology Research and Practice 2010, Article ID 453563.
Stanier RY, Ingraham JL, Wheelis ML. and Painter PR (1990): General Microbiology, Fifth Edition, p. 69. Macmillan Education LTD.
Steffen EK, Berg RD and Deitch EA (1988): Comparison of translocation rates of various indigenous bacteria from the gastrointestinal tract to the mesenteric lymph node. The Journal of Infectious Diseases 157, 1032–1038.
Steinhoff U (2005): Who controls the crowd? New findings and old questions about the intestinal microflora. Immunology Letters 99, 12–16.
Strauch UG, Obermeier F, Grunwald N, Gurster S, Dunger N, Schultz M and Rath HC (2005): Influence of intestinal bacteria on induction of regulatory T cells: Lessons from a transfer model of colitis. Gut 54, 1546–1552.
Suh YG and Jeong WI (2011): Hepatic stellate cells and innate immunity in alcoholic liver disease. World Journal of Gastroenterology: WJG 17, 2543–2551.

Suzuki N, Yoshida A and Nakano Y (2005): Quantitative analysis of multi-species oral biofilms by TaqMan real-time PCR. Clinical Medicine & Research 3, 176–185.
Swidsinsky A, Loening-Baucke V, Lochs H and Hale LP (2005): Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence in situ hybridization study in mice. World Journal of Gastroenterology 11, 1131–1140.
Tabata T, Tani T, Endo Y and Hanasawa K (2002): Bacterial translocation and peptidoglycan translocation by acute ethanol administration. Journal of Gastroenterology 37, 726–731.
Tamai H, Kato S, Horie Y, Ohki E, Yokoyama H and Ishii H (2000): Effect of acute ethanol administration on the intestinal absorption of endotoxin in rats. Alcoholism, Clinical and Experimental Research 24, 390–394.
Taschuk R and Griebel PJ (2012): Commensal microbiome effects on mucosal immune system development in the ruminant gastrointestinal tract. Animal Health Research Reviews/ Conference of Research Workers in Animal Diseases 13, 129–141.
Terg R, Fassio E, Guevara M, Cartier M, Longo C, Lucero R and Abecasis R (2008): Ciprofloxacin in primary prophylaxis of spontaneous bacterial peritonitis: A randomized, placebo-controlled study. Journal of Hepatology 48, 774–779.
Thurman RG (1998): II. alcoholic liver injury involves activation of kupffer cells by endotoxin. The American Journal of Physiology 275, G605–11.
Tichopad A, Bar T, Pecen L, Kitchen RR, Kubista M and Pfaffl MW (2010): Quality control for quantitative PCR based on amplification compatibility test. Methods 50, 308–312.
Tillonen J, Homann N, Rautio M, Jousimies-Somer H and Salaspuro M (1999): Ciprofloxacin decreases the rate of ethanol elimination in humans. Gut 44, 347–352.
Tsokos M and Puschel K (2001): Postmortem bacteriology in forensic pathology: Diagnostic value and interpretation. Legal Medicine 3, 15–22.
Tuominen VJ, Ruotoistenmaki S, Viitanen A, Jumppanen M and Isola J (2010): ImmunoRatio: A publicly available web application for quantitative image analysis of estrogen receptor (ER), progesterone receptor (PR), and ki-67. Breast Cancer Research: BCR 12, R56.
Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE and Gordon JI (2009): A core gut microbiome in obese and lean twins. Nature 457, 480–484.
Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER and Gordon JI (2006): An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444, 1027–1031.
Uematsu S and Akira S (2006): Toll-like receptors and innate immunity. Journal of Molecular Medicine 84, 712–725.
Ulluwishewa D, Anderson RC, McNabb WC, Moughan PJ, Wells JM and Roy NC (2011): Regulation of tight junction permeability by intestinal bacteria and dietary components. The Journal of Nutrition 141, 769–776.
Utne HE and Winkler K (1980): Hepatic and extrahepatic elimination of ethanol in cirrhosis. With estimates of intrahepatic shunts and km for ethanol elimination. Scandinavian Journal of Gastroenterology 15, 297–304.
Vass AA, Barshick SA, Sega G, Caton J, Skeen JT, Love JC and Synstelien JA (2002): Decomposition chemistry of human remains: A new methodology for determining the postmortem interval. Journal of Forensic Sciences 47, 542–553.

Velayudhan J, Jones MA, Barrow PA and Kelly DJ (2004): L-serine catabolism via an oxygen-labile L-serine dehydratase is essential for colonization of the avian gut by campylobacter jejuni. Infection and Immunity 72, 260–268.

Vilstrup H (2003): Cirrhosis and bacterial infections. Romanian Journal of Gastroenterology 12, 297–302.

Vinolo MA, Rodrigues HG, Nachbar RT and Curi R (2011) Regulation of inflammation by short chain fatty acids. Nutrients 3, 858–876.

Wang F, Graham WV, Wang Y, Witkowski ED, Schwarz BT and Turner JR (2005): Interferon-gamma and tumor necrosis factor-alpha synergize to induce intestinal epithelial barrier dysfunction by up-regulating myosin light chain kinase expression. The American Journal of Pathology 166, 409–419.

Wang JH, Batey RG and George J (2006) Role of ethanol in the regulation of hepatic stellate cell function. World Journal of Gastroenterology: WJG 12, 6926–6932.

Weber MA, Hartley JC, Brooke I, Lock PE. Klein NJ, Malone M and Sebire NJ (2010): Post-mortem interval and bacteriological culture yield in sudden unexpected death in infancy (SUDI). Forensic Science International 198, 121–125.

Wells CL (1996): Colonization and translocation of intestinal bacterial flora. Transplantation Proceedings 28, 2653–2656.

Wells CL and Erlandsen SL (1991): Localization of translocating escherichia coli, proteus mirabilis, and enterococcus faecalis within cecal and colonic tissues of monoassociated mice. Infection and Immunity 59, 4693–4697.

Wells CL, Maddaus MA, Reynolds CM, Jechorek RP and Simmons RL (1987): Role of anaerobic flora in the translocation of aerobic and facultatively anaerobic intestinal bacteria. Infection and Immunity 55, 2689–2694.

Wershil BK and Furuta GT (2008): 4. gastrointestinal mucosal immunity. The Journal of Allergy and Clinical Immunology 121, S380–3; quiz S415.

White JS, Hoper M, Parks RW, Clements WD and Diamond T (2006): Patterns of bacterial translocation in experimental biliary obstruction. The Journal of Surgical Research 132, 80–84.

Whitman WB, Coleman DC and Wiebe WJ (1998): Prokaryotes: The unseen majority. Proceedings of the National Academy of Sciences of the United States of America 95, 6578–6583.

Wiest R and Garcia-Tsao G (2005): Bacterial translocation (BT) in cirrhosis. Hepatology 41, 422–433.

Willing BP, Antunes LC, Keeney KM, Ferreira RB and Finlay BB (2011): Harvesting the biological potential of the human gut microbiome. BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology 33, 414–418.

Wilson SJ, Wilson ML and Reller LB (1993): Diagnostic utility of postmortem blood cultures. Archives of Pathology & Laboratory Medicine 117, 986–988.

Winter SE and Baumler AJ (2011): A breathtaking feat: To compete with the gut microbiota, salmonella drives its host to provide a respiratory electron acceptor. Gut Microbes 2, 58–60.

Winter SE, Thiennimitr P, Winter MG, Butler BP, Huseby DL, Crawford RW and Baumler AJ (2010): Gut inflammation provides a respiratory electron acceptor for salmonella. Nature 467, 426–429.

Woese CR and Fox GE (1977): Phylogenetic structure of the prokaryotic domain: The primary kingdoms. Proceedings of the National Academy of Sciences of the United States of America 74, 5088–5090.

Worthington BS, Meserole L and Syrotuck JA (1978): Effect of daily ethanol ingestion on intestinal permeability to macromolecules. The American Journal of Digestive Diseases 23, 23–32.

Wostmann BS, Larkin C, Moriarty A and Bruckner-Kardoss E (1983): Dietary intake, energy metabolism, and excretory losses of adult male germfree wistar rats. Laboratory Animal Science 33, 46–50.

Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y., Keilbaugh SA and Lewis JD (2011): Linking long-term dietary patterns with gut microbial enterotypes. Science 334, 105–108.

Yan AW, Fouts DE, Brandl J, Starkel P, Torralba M, Schott E and Schnabl B (2011): Enteric dysbiosis associated with a mouse model of alcoholic liver disease. Hepatology 53, 96–105.

Yan AW and Schnabl B (2012): Bacterial translocation and changes in the intestinal microbiome associated with alcoholic liver disease. World Journal of Hepatology 4, 110–118.

Yang S, Lin S, Kelen GD, Quinn TC, Dick JD, Gaydos CA and Rothman RE (2002): Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. Journal of Clinical Microbiology 40, 3449–3454.

Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M and Gordon JI (2012): Human gut microbiome viewed across age and geography. Nature 486, 222–227.

Yin M, Bradford BU, Wheeler MD, Uesugi T, Froh M, Goyert SM and Thurman RG (2001): Reduced early alcohol-induced liver injury in CD14-deficient mice. Journal of Immunology166, 4737–4742.

Yoshida A, Suzuki N, Nakano Y, Kawada M, Oho T and Koga T (2003): Development of a 5' nuclease-based real-time PCR assay for quantitative detection of cariogenic dental pathogens streptococcus mutans and streptococcus sobrinus. Journal of Clinical Microbiology 41, 4438–4441.

Zhang W, Gu Y, Chen Y, Deng H, Chen L, Chen S and Gao Z (2010): Intestinal flora imbalance results in altered bacterial translocation and liver function in rats with experimental cirrhosis. European Journal of Gastroenterology & Hepatology 22, 1481–1486.

Zhang X, Yu W P, Gao L, Wei K B, Ju JL and Xu JZ (2004): Effects of lipopolysaccharides stimulated kupffer cells on activation of rat hepatic stellate cells. World Journal of Gastroenterology: WJG 10, 610–613.

Zhang Y, Doerfler M, Lee TC, Guillemin B and Rom WN (1993): Mechanisms of stimulation of interleukin-1 beta and tumor necrosis factor-alpha by mycobacterium tuberculosis components. The Journal of Clinical Investigation 91, 2076–2083.

Zhao HY, Wang HJ, Lu Z and Xu SZ (2004): Intestinal microflora in patients with liver cirrhosis. Chinese Journal of Digestive Diseases 5, 64–67.

Web page references

Suomen virallinen tilasto (SVT) (2012a): Kuolemansyyt. Liitetaulukko 1B. Kuolleet peruskuolemansyyn mukaan 2012, miehet. Helsinki:Tilastokeskus. http://www.stat.fi/til/ksyyt/2012/index.html (accessed 12.8.2014)

Suomen virallinen tilasto (SVT) (2012b): Kuolemansyyt. Liitetaulukko 1C. Kuolleet peruskuolemansyyn mukaan 2012, naiset. Helsinki:Tilastokeskus. http://www.stat.fi/til/ksyyt/2012/index.html (accessed 12.8.2014)

Suomen virallinen tilasto (SVT) (2012c): Kuolemansyyt. 4. Alkoholikuolleisuus kääntyi nousuun. Helsinki:Tilastokeskus. http://www.stat.fi/til/ksyyt/2012/index.html (accessed 12.8.2014)

Terveyden ja hyvinvoinnin laitos (THL) (2013): Alkoholijuomien kulutus. http://www.julkari.fi/bitstream/handle/10024/116083/Tr09_14.pdf?sequence=4 (accessed 12.8.2014)

ORIGINAL PUBLICATIONS

These original articles have been reprinted according to the permissions of the following publishers and are gratefully acknowledged:

I, III BioMed Central journal copyright and license agreement of Open Access

II The kind permission of John Wiley and Sons

Gut Pathogens

RESEARCH Open Access

Time-dependent post mortem changes in the composition of intestinal bacteria using real-time quantitative PCR

Sari Tuomisto[1,2*], Pekka J Karhunen[1,2] and Tanja Pessi[1,2]

Abstract

Post mortem or even normal changes during life occurring in major gut bacterial populations are not known. We investigated *Bacteroides* sp., *Bifidobacterium* sp., *Clostridium leptum, Clostridium coccoides*, *Streptococcus* sp., *Lactobacillus* sp. and *Enterobacteriacaea* ratios in 7 fecal samples from healthy volunteers and in 61 autopsies rectum and cecum samples and studied the effect of post mortem time using quantitative real-time PCR. Bacterial ratios in stool samples from volunteers and rectum samples from autopsy cases were similar and did not change significantly up to 5 days post mortem. In cecum, significant post mortem time-dependent differences were observed in ratios of *Bacteroides* sp. ($p = 0.014$) and *Lactobacillus* sp. ($p = 0.024$). Our results showed that ratios of *Bacteroides* sp., *Bifidobacterium* sp., *Clostridium leptum*, *Clostridium coccoides*, *Streptococcus* sp., *Lactobacillus* sp. and *Enterobacteriacaea* can be investigated in autopsy rectum samples up to 5 days after death.

Keywords: Forensic science, Post mortem microbiology, Fecal sample, Real-time quantitative polymerase chain reaction, Bacterial relative amount, Time-dependent changes

Background

Basic knowledge on the composition of intestinal bacterial populations and changes occurring after death is lacking. Even the normal composition of intestinal microbiota in life is not fully known [1]. Only one study exists in which intestinal bacterial populations have been studied in three elderly women after death using PCR and sequencing [2].

Resident micro-organisms living in the intestinal tract influence host's normal well-being and physiology including gut metabolism and the regulation of epithelial cell growth [3]. Intestinal microbiota functions as a physical barrier against invading pathogens. It has been suggested that gut microbiota may have a role on the development of diseases, *e.g.* alcoholic liver cirrhosis [4] and atherosclerosis [5]. Detailed bacterial population studies on the intestinal tract have mostly concentrated on fecal samples because they are easy to collect. Intestinal microbiota consists of a large and diverse community containing hundreds of commensal bacterial species [6]. From sequencing libraries of 16S rRNA genes Durban et al. found that two dominant phyla, *Firmicutes* and *Bacteroidetes* accounted for nearly 85% of all sequences in stool samples [7]. Compared to these two major phyla, *Bifidobacterium* genus is present in eight to ten-fold lower numbers [8]. Although *Bacteroides* sp., *Bifidobacterium* sp. and bacteria belonging to the *Clostridium coccoides*–group (cluster XIVa) and *Clostridium leptum*–group (cluster IV) dominate in colon [9,10] there is substantial inter- and intra-individual variation in species composition and distribution [7,11].

This study aimed to investigate ratios of major intestinal bacterial populations in healthy volunteers and in rectum and cecum autopsy samples. Post mortem time-dependent changes were studied in order to see whether autopsy samples can be used for basic research concerning lifetime. Six species: *Bacteroides* sp. (phylum Bacteroidetes), *Clostridium* sp. (Firmicutes), *Streptococcus* sp. (Firmicutes), *Lactobacillus* sp. (Firmicutes), *Bifidobacterium* sp. (Actinobacteria) and *Enterobactericaea* (Proteobacteria) were chosen since they represent the major intestinal bacterial phyla [12].

* Correspondence: Sari.Tuomisto@uta.fi
[1]Department of Forensic Medicine, School of Medicine, University of Tampere, Medisiinarinkatu 3, Tampere 33014, Finland
[2]Fimlab Ltd, Pirkanmaa Hospital District, Biokatu 4, Tampere 33520, Finland

Table 1 Demographic characteristics of the study subjects divided by post mortem time

	N	PM mean	Age mean (range)	BMI mean (range)	Basic cause of death		
					Heart diseases %	Other diseases %	Violent deaths (suicide, accident, poisoning) %
Autopsy cases:							
1–3 days	19	2.3	55 (18–79)	29.3 (20.4–42.1)	7 (37%)	4 (21%)	8 (42%)
4–5 days	21	4.5	58 (20–86)	28.4 (18.4–43.6)	10 (48%)	9 (43%)	2 (10%)
>5 days	21	6.5	61 (28–76)	30.7 (21.1–50.3)	15 (71%)	2 (10%)	4 (19%)
p-value			0.373	0.543	0.079	0.086	0.096
Control volunteers	7		45 (26–57)	27.1 (20.8–37.2)			

PM mean = Post mortem mean time.

Findings

Study design and results

This study comprises of 61 male cases collected in the Department of Forensic Medicine of the University of Tampere and 7 male volunteers. The selection criteria for the autopsies have been described elsewhere [13]. None of the controls or cases was reported to has been used antibiotics. Deceased had been stored in +4°C within 24 hours after death. Written consent was obtained from the volunteers.

Samples of the autopsy cases were taken from rectum and cecum. All samples were frozen immediately at −80°C until further processing. On the basis of time post mortem the cases were divided into groups: 1–3 days, 4–5 days and >5 days. Demographic characteristics of these groups are shown in the Table 1.

Fecal samples were weighed to be 150 mg (wet weight). Bacterial DNA was extracted from the samples using Zymo Fecal DNA Kit (Zymo Research Corporation, Irvine, California, USA). The bacterial ratios were determined by RT-qPCR using specific primers and probes (Table 2). The primers and probes for *Enterobacteriacaea* and *Lactobacillus* sp. were designed and confirmed by using BLAST (http://www.ncbi.nlm.nih.gov/) and Ribosomal Database Project (http://rdp.cme.msu.edu/probematch/search.jsp). Specificity and cross reactivity of the designed primers and probes were tested using bacterial cultures from clinical samples [13]. PCR assays were performed with AbiPrism 7000 HT Sequence Detection System (Taqman, AppliedBiosystems, California, USA) with Taqman Environmental MasterMix. Endogen and DNA-free water was used as a negative control.

The comparative Ct method (ΔΔCt, $\Delta Ct_{\text{sample}} - \Delta Ct_{\text{reference sample}}$) [17], was used where mean values from healthy male volunteers were calculated and used as a reference to determine bacterial relative amount in rectum samples. The differences of the Ct values between the bacteria and the universal bacteria measurement (ΔCt) for each sample were calculated; the comparative Ct (ΔΔCt) for sample and reference samples was

Table 2 Used primers and probes

Primer and probe	Sequence (5′-3′)	Reference
***Bacteroides* sp.**		[14]
Forward	TGGTAGTCCACACAGTAAACGATGA	
Reverse	CGTACTCCCCAGGTGGAATACTT	
Probe	GTTTGCCATATACAGTAAGCGGCCAAGCG	
***Bifidobacterium* sp.**		[15]
Forward	CGGGTGAGTAATGCGTGACC	
Reverse	TGATAGGACGCGACCCCA	
Probe	CTCCTGGAAACGGGTG	
Clostridium leptum		[15]
Forward	CCTTCCGTGCCGSAGTTA	
Reverse	GAATTAAACCACATACTCCACTGCTT	
Probe	CACAATAAGTAATCCACC	
Clostridium coccoides		[15]
Forward	GACGCCGCGTGAAGGA	
Reverse	AGCCCCAGCCTTTCACATC	
Probe	CGGTACCTGACTAAGAAG	
Enterobactericaea		This study
Forward	GCGGTAGCACAGAGAGCTT	
Reverse	GGCAGTTTCCCAGACATTACTCA	
Probe	CCGCCGCTCGTCACC	
***Lactobacillus* sp.**		This study
Forward	GCTAGGTGTTGGAGGGTTTCC	
Reverse	CCAGGCGGAATGCTTAATGC	
Probe	TCAGTGCCGCAGCTAA	
Streptococcus sp.* mainly *Str. mitis*-group		[13]
Forward	CCAGCAGCCGCGGTAATA	
Reverse	CCTGCGCTCGCTTTACG	
Probe	ACGCTCGGGACCTACG	
Universal		[16]
Forward	TGGAGCATGTGGTTTAATTCGA	
Reverse	TGCGGGACTTAACCCAACA	
Probe	CACGAGCTGACGACA[A/G]CCATGCA	

*This was abbreviated as *Streptococcus* sp. in the text.

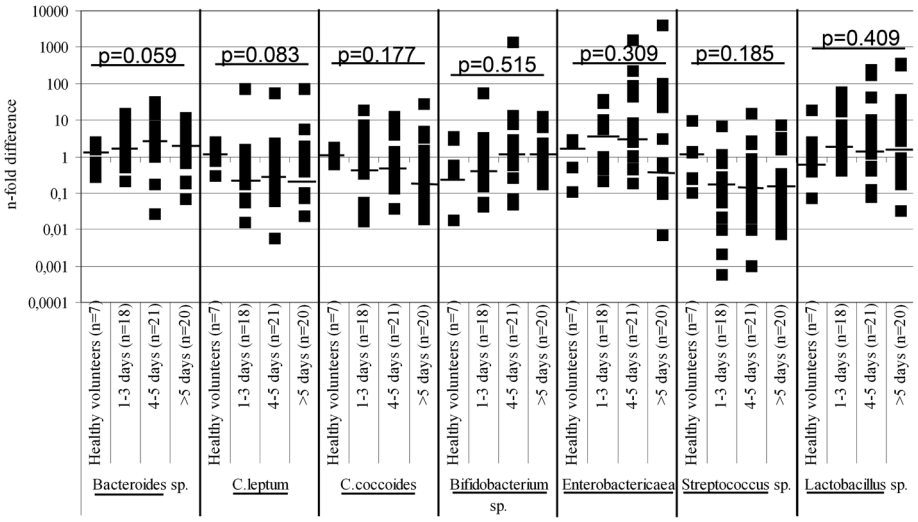

Figure 1 Relative amounts (n-fold difference) of measured bacteria (*Bacteroides* sp., *C. leptum*, *C. coccoides*, *Bifidobacterium* sp., *Enterobactericaea*, *Streptococcus* sp. and *Lactobacillus* sp.) in fecal samples of controls and rectum of autopsy cases. Individual values are presented as boxes, median values with horizontal lines. Comparisons over the groups were calculated using non-parametric Kruskal-Wallis test.

calculated. To determine relative amounts of bacteria in cecum samples the rectal sample was used as an inner reference.

Two standard curves were used to determine the total amount of bacteria. Tenfold dilution series of between 33 ng/ml and 0.00033 ng/ml from *E. coli* genomic DNA (ATCC 35401–5) as well as between 10^9 and 10^5 colony forming units (CFU) per milliliter from *E .coli* (ATCC 25922) were applied. The amount of CFU or bacterial DNA in the sample was calculated using values from universal measurement and the equation y = slope log (X) + intercept [18].

Statistical analyses were performed with Kruskal-Wallis median test with PASW Statistical Software, version 18 (SPSS Ltd, Quarry Bay, Hong Kong). If P-value was less than 0.05 (considered significant) pairwise Post Hoc comparisons using Mann–Whitney U-test were done.

Median values of different bacteria in the stool of healthy controls and in post mortem rectum samples

Table 3 The relative amounts (n-fold difference) of measured bacteria in cecum samples compared to rectum samples over post mortem time

		Bacterial Group						
		Bacteroides sp.	*C. leptum*	*C. coccoides*	*Bifidobacterium* sp.	*Enterobactericaea*	*Streptococcus* sp.	*Lactobacillus* sp.
All	**Median**	**0.32**	**0.72**	**1.29**	**1.18**	**0.86**	**2.19**	**0.82**
	25th–75th	**0.13–1.06**	**0.41–1.61**	**0.27–3.94**	**0.52–2.66**	**0.09–3.38**	**0.52–7.50**	**0.25–3.61**
1–3 days								
	Median	0.15	0.59	0.64	2.03	1.37	3.56	1.25
	25th–75th	0.01–0.43	0.31–2.94	0.20–4.55	0.84–35.63	0.26–14.77	0.85–35.32	0.46–7.11
4–5 days								
	Median	0.53	1.09	1.27	0.61	0.68	2.28	0.30
	25th–75th	0.17–1.60	0.60–1.81	0.15–2.30	0.26–2.34	0.03–1.85	0.34–8.07	0.16–1.95
>5 days								
	Median	0.53	0.60	2.81	1.03	0.86	1.85	1.09
	25th–75th	0.21–1.45	0.41–1.39	0.59–4.27	0.45–1.61	0.16–7.94	0.27–5.68	0.65–7.84
	p-value	0.014	0.472	0.421	0.054	0.358	0.192	0.024

Results are presented as median and 25^{th}-75^{th} interquartile range. Non-parametric median, Kruskal-Wallis-test comparisons between groups.

Table 4 The total amount of bacterial DNA in fecal samples

			N	ng/g median*	25^{th} -75^{th}	p-value[1)]	p-value[2)]
Healthy volunteers	Stool	Control	7	26	9.2-36.7		
Autopsy cases	Rectum	1-3 days	18	8	2.0-53.6		
		4-5 days	21	8	1.7-41.4		
		>5 days	20	42	12.0-124.2	0.044	0.023
Autopsy cases	Cecum	1-3 days	19	51	13-3-94.1		
		4-5 days	21	68	5.1-194.7		
		>5 days	21	48	6.5-113.6		0.982

*1 ng/g corresponds to $4.8x10^{10}$ colony forming units using *E. coli* as a standard. P-values (over the groups) for [1)]healthy volunteers and autopsy cases, [2)]autopsy cases only.
25^{th} -75^{th} interquartile range. Non-parametric median, Kruskal-Wallis-test comparisons over the groups.

showed no statistically significant changes over post mortem time (Figure 1). In cecum, significant post mortem time-dependent differences were observed over the groups in the relative amounts of *Bacteroides* sp. ($p = 0.014$) and *Lactobacillus* sp. ($p = 0.024$, Table 3). There were significantly more *Bacteroides* sp. ($p = 0.012$) and less *Lactobacillus* sp. ($p = 0.015$) already in 4–5 days. Statistically significant differences in the total amount of bacterial DNA were seen in healthy volunteers and autopsy rectum samples ($p = 0.044$, Table 4). In autopsy rectum, the amount of bacterial DNA remained quite stable with time elapsing post mortem except for a high increase observed after day 5 post mortem ($p = 0.023$). A slightly higher total amount of bacterial DNA (measured as a wet weight) in stool samples donated by the volunteers compared to autopsy rectum samples might be due to lower water concentration in stool compared to rectum without changes in bacterial ratios [19]. Inter-individual variation was great at all time points and in all bacterial measurements.

Conclusion

This study showed that relative amounts of major intestinal bacteria in rectum of autopsy cases were similar to stool donated by volunteers and remained quite stable over post mortem time up to 5 days, after which the total amount of bacteria started to increase. In contrast, in cecum significant post mortem time-dependent differences were observed as increase in ratio of strictly anaerobic *Bacteroides* sp. and decrease of facultative *Lactobacillus* sp. due to hypoxia after death. In cecum there is accumulation of undigested nutrients and metabolites produced by bacteria after death, which may be conducive to anaerobic bacterial growth. This study showed that autopsy rectum samples can be used to evaluate major intestinal bacterial populations concerning lifetime up to 5 days after death.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ST performed experiments and analyses, helped in collection of the autopsy samples and wrote the manuscript. PK was the iniator of the project and group leader and participated in writing the script. TP was the guarantor of the microbiological part of the study, designed the sample collection and experiments, and participated in writing the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The excellent technical assistance from the personnel of the Department of Forensic Medicine, especially Mervi Seppänen, Kari Mänttäri and Olli Penttilä, University of Tampere, Finland is gratefully acknowledged. We also appreciate the participation of the volunteers.

Funding

This study was financially supported by the Tampere Graduate Program in Biomedicine and Biotechnology (TGPBB), the Finnish Foundation for Alcohol Studies, the Competitive Research Funding of the Pirkanmaa Hospital District, the European Union 7th Framework Program grant number 201668 for the AtheroRemo Project, the Pirkanmaa Regional Fund of the Finnish Cultural Foundation.

Received: 30 August 2013 Accepted: 20 November 2013
Published: 25 November 2013

References

1. Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA, Bonazzi V, McEwen JE, Wetterstrand KA, Deal C, Baker CC, Di Francesco V, Howcroft TK, Karp RW, Lunsford RD, Wellington CR, Belachew T, Wright M, Giblin C, David H, Mills M, Salomon R, Mullins C, Akolkar B, Begg L, Davis C, Grandison L, Humble M, Khalsa J, Little AR, *et al*: **The NIH human microbiome project.** *Genome Res* 2009, **19:**2317–2323.
2. Hayashi H, Takahashi R, Nishi T, Sakamoto M, Benno Y: **Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism.** *J Med Microbiol* 2005, **54:**1093–1101.
3. Morris JA, Harrison LM, Biswas J, Telford DR: **Transient bacteraemia: a possible cause of sudden life threatening events.** *Med Hypotheses* 2007, **69:**1032–1039.
4. Chen Y, Yang F, Lu H, Wang B, Chen Y, Lei D, Wang Y, Zhu B, Li L: **Characterization of fecal microbial communities in patients with liver cirrhosis.** *Hepatology* 2011, **54:**562–572.
5. Rosenfeld ME, Campbell LA: **Pathogens and atherosclerosis: Update on the potential contribution of multiple infectious organisms to the pathogenesis of atherosclerosis.** *Thromb Haemost* 2011, **106:**858–867.
6. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman D: **Diversity of the human intestinal microbial flora.** *Science* 2005, **308:**1635–1638.
7. Durbán A, Abellán JJ, Jiménez-Hernández N, Ponce M, Ponce J, Sala T, D'Auria G, Latorre A, Moya A: **Assessing gut microbial diversity from feces and rectal mucosa.** *Microb Ecol* 2011, **61:**123–133.

8. Mariat D, Firmesse O, Levenez F, Guimarães V, Sokol H, Doré J, Corthier G, Furet JP: **The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age.** *BMC Microbiol* 2009, **9:**123.
9. Lay C, Sutren M, Rochet V, Saunier K, Dore J, Rigottier-Gois L: **Design and validation of 16S rRNA probes to enumerate members of the clostridium leptum subgroup in human faecal microbiota.** *Environ Microbiol* 2005, **7:**933–946.
10. Orrhage K, Nord CE: **Bifidobacteria and lactobacilli in human health.** *Drugs Exp Clin Res* 2000, **26:**95–111.
11. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI: **A core gut microbiome in obese and lean twins.** *Nature* 2009, **457:**480–484.
12. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM, Bertalan M, Borruel N, Casellas F, Fernandez L, Gautier L, Hansen T, Hattori M, Hayashi T, Kleerebezem M, Kurokawa K, Leclerc M, Levenez F, Manichanh C, Nielsen HB, Nielsen T, Pons N, Poulain J, Qin J, Sicheritz-Ponten T, Tims S, *et al*: **Enterotypes of the human gut microbiome.** *Nature* 2011, **12:**174–180.
13. Tuomisto S, Karhunen P, Vuento R, Aittoniemi J, Pessi T: **Evaluation of post mortem bacterial migration using culturing and real-time quantitative PCR.** *J Forensic Sci* 2013, **58:**910–916.
14. Brunk CF, Li J, Avaniss-Aghajani E: **Analysis of specific bacteria from environmental samples using a quantitative polymerase chain reaction.** *Curr Issues Mol Biol* 2002, **4:**13–18.
15. Furet JP, Firmesse O, Gourmelon M, Bridonneau C, Tap J, Mondot S, Doré J, Corthier G: **Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR.** *FEMS Microbiol Ecol* 2009, **68:**351–362.
16. Yang S, Lin S, Kelen GD, Quinn TC, Dick JD, Gaydos CA, Rothman RE: **Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens.** *J Clin Microbiol* 2002, **40:**3449–3454.
17. Suzuki N, Yoshida A, Nakano Y: **Quantitative analysis of multi-species oral biofilms by TaqMan real-time PCR.** *Clin Med Res* 2005, **3:**176–185.
18. Yoshida A, Suzuki N, Nakano Y, Kawada M, Oho T, Koga T: **Development of a 5′ nuclease-based real-time PCR assay for quantitative detection of cariogenic dental pathogens streptococcus mutans and streptococcus sobrinus.** *J Clin Microbiol* 2003, **41:**4438–4441.
19. Geibel JP: **Secretion and absorption by colonic crypts.** *Annu Rev Physiol* 2005, **67:**471–490.

doi:10.1186/1757-4749-5-35
Cite this article as: Tuomisto *et al.*: Time-dependent post mortem changes in the composition of intestinal bacteria using real-time quantitative PCR. *Gut Pathogens* 2013 **5**:35.

JOURNAL OF FORENSIC SCIENCES

J Forensic Sci, July 2013, Vol. 58, No. 4
doi: 10.1111/1556-4029.12124
Available online at: onlinelibrary.wiley.com

PAPER

PATHOLOGY/BIOLOGY

Sari Tuomisto,[1,2] *M.Sc.; Pekka J. Karhunen,*[1,2] *M.D., Ph.D.; Risto Vuento,*[2] *M.D., Ph.D.; Janne Aittoniemi,*[2] *M.D., Ph.D.; and Tanja Pessi,*[1,2] *Ph.D.*

Evaluation of Postmortem Bacterial Migration Using Culturing and Real-Time Quantitative PCR*

ABSTRACT: Postmortem bacteriology can be a valuable tool for evaluating deaths due to bacterial infection or for researching the involvement of bacteria in various diseases. In this study, time-dependent postmortem bacterial migration into liver, mesenteric lymph node, pericardial fluid, portal, and peripheral vein was analyzed in 33 autopsy cases by bacterial culturing and real-time quantitative polymerase chain reaction (RT-qPCR). None suffered or died from bacterial infection. According to culturing, pericardial fluid and liver were the most sterile samples up to 5 days postmortem. In these samples, multigrowth and staphylococci were not or rarely detected. RT-qPCR was more sensitive and showed higher bacterial positivity in all samples. Relative amounts of intestinal bacterial DNA (bifidobacteria, bacteroides, enterobacter, clostridia) increased with time. Sterility of blood samples was low during the studied time periods (1–7 days). The best postmortem microbiological sampling sites were pericardial fluid and liver up to 5 days after death.

KEYWORDS: forensic science, postmortem microbiology, bacterial culturing, real-time quantitative polymerase chain reaction, sampling place, sample reliability

Bacterial migration from the intestine or locus of infection into the bloodstream and internal organs is not uncommon, but in most cases, bacteria are rapidly eliminated by immune defenses. Sometimes bacteria can escape the immune system causing life-threatening events such as circulatory collapse, disseminated intravascular coagulation, and septicemia (1). Postmortem bacteriology can be used for evaluating deaths due to bacterial infection or for researching the involvement of bacteria in various diseases. There is already postmortem bacteriological investigation protocol for sudden unexpected death in infancy (2). Postmortem bacterial migration has, however, been poorly studied and false, misleading positive results may occur (3).

There are four ways in which positive bacterial results may occur in autopsy samples: true positive, agonal spread, postmortem migration, and contamination (4). In a genuine positive result, the bacteria have already been present in life and can also be found after death. Normally, genuine positive isolate will be pure growth of a recognized pathogen. Agonal spread indicates bacterial invasion at the time of the death struggle or during resuscitation, when circulation is artificially maintained. The mucosal integrity decreases and bacterial invasion may occur. This may generate mixed bacterial growth (4). In postmortem migration, bacteria migrate mainly from the gut into the blood, and body tissues after the circulation have stopped as a part of the normal putrefaction (4). The likely result is polymicrobial growth in cultures and decreased sample sterility (5). Contamination of the samples from external sources is a major problem and cannot be totally avoided either in life or after death. Bacteria may be introduced into the samples through inadequate sampling techniques or unskilled staff. Usually, a single bacterial finding is considered as a genuine result, whereas mixed bacterial finding indicates postmortem contamination or postmortem migration.

It has been proposed that bacterial migration through the gut mucosal surfaces into the blood, and internal organs may be detected more frequently as time postmortem increases (5). The studies, however, are controversial. Morris et al. suggested in literature review that postmortem migration is not a problem if the body is appropriately stored soon after death and that the postmortem period has only a minor effect on the bacterial isolation rate (4). Similarly, Weber et al. (6) in their retrospective review reported that a longer postmortem interval is neither associated with increased frequency of positive cultures nor with increased polymicrobial culture findings. Clearly, more investigation of bacterial behavior after death is needed.

The optimal postmortem microbial sampling sites are also under debate. Different places for sampling have been proposed. For example, cerebrospinal fluid, blood, spleen, lung, and liver tissue have been considered as valuable sampling sites (6–9).

[1]School of Medicine, University of Tampere, Medisiinarinkatu 3, 33014, Tampere, Finland.
[2]Centre for Laboratory Medicine, Pirkanmaa Hospital District, Biokatu 4, 33520, Tampere, Finland.

*Financial support provided by the Tampere Graduate Program in Biomedicine and Biotechnology (TGPBB), the Finnish Foundation for Alcohol Studies, the Competitive Research Funding of Pirkanmaa Hospital District, the European Union 7th Framework Program grant number 201668 for the AtheroRemo Project, the Pirkanmaa Regional Fund of the Finnish Cultural Foundation, and the Finnish Foundation for Cardiovascular Research.

Received 21 Sept. 2011; and in revised form 24 April 2012; accepted 5 May 2012.

Tsokos and Puschel recommended that the collection of samples should be performed from at least two different sampling sites. This is a standard procedure in cases where there is a suspicion of bacteria causing the death (9).

The standard method for diagnosing the presence of bacteria in clinical samples currently relies on bacterial culturing technique, but its reliability has been severely criticized due to false negative findings. Active research is ongoing to apply new methods, such as real-time quantitative polymerase chain reaction (RT-qPCR) to increase assay specificity, sensitivity, and reliability (10).

In this study, we examined how soon bacteria migrate to different organs after death by studying the emergence of major commensal bacteria from the gut (*Bacteroides* sp., *Bifidobacterium* sp., *Clostridium* sp., and *Enterobacter* sp.) as well as the clinically important pathogens staphylococci and streptococci using RT-qPCR and bacterial culturing. These results can be used to evaluate the presence of true positive bacterial findings in autopsies.

Materials and Methods

The present prospective autopsy series comprises 33 consecutive cases at the Department of Forensic Medicine of the University of Tampere during the period from 2009 to 2010 meeting the study criteria: out-of-hospital death, male sex, age over 18 years, time elapsed postmortem under 7 days, time interval between death and storage of the body in the mortuary less than 24 h, intact middle torso and bowel, no use of antibiotics for 2 weeks before death, no signs of bacterial infections or drug addiction, and no visible wounds or necrosis. The mean age was 56.7 years (range 34–82 years). Hospital protocols were used in the assessment of cause of death. Of the cases, 16 (49%) died of heart disease, 12 (36%) of other noncardiac disease, and five (15%) suffered violent death (suicide, accident, poisoning). Liver, mesenteric lymph node (MLN), portal vein blood, pericardial fluid, and control blood (right cardiac ventricular blood $n = 20$, jugular vein $n = 11$, or femoral vein $n = 2$) samples were collected using sterile techniques.

All bodies in the mortuary were kept refrigerated at around +4°C. The bodies were not generally cooled during transportation to the mortuary. The average time elapsed postmortem was 2.4 days (range 1–7 days). On the basis of the time elapsed postmortem, the samples were divided into three groups: 1–3 days, 4–5 days, and 5–7 days.

After sampling, samples for culturing were immediately transported at room temperature to the laboratory for bacteriological assessment. Samples for RT-qPCR were placed on ice and transferred to −80°C until analyzed. Bacteriological culturing was performed in an accredited laboratory using their standard procedures. Bacterial culturing from autopsy samples was performed using standard protocols in the Department of Microbiology, Centre for Laboratory Medicine, Pirkanmaa Hospital District, Tampere, Finland (JA, RV). For evaluation of culture positivity, microbial isolates were divided into three groups: sterile, mono-isolates (a pure culture growth); multi-isolates (a mixed growth). All bacteria (except one unidentified anaerobic gram negative rod) were identified at genus level (*Bacillus*, *Proteus*, *Peptostreptococcus*, *Neisseria*, *Micrococcus*, *Lactobacillus*, *Acinetobacter*, *Stomatococcus* and *Citrobacter*), and in cases positive for *Bacteroides*, *Candidia*, *Corynebacterium*, *Clostridium*, *Enterococcus*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Propionibacterium*, *Serratia*, *Staphylococcus,* and *Streptococcus*, identification was performed to the species level using standard biochemical tests. None of the detected staphylococcal species were *S. aureus.*

For RT-qPCR, bacterial DNA was extracted from the autopsy samples using a commercially available Zymo Bacterial/Fungal DNA Kit (Zymo Research Corporation, Irvine, CA) according to the instructions provided. Oligonucleotide primers and probes for RT-qPCR are listed in Table 1. The staphylococci and streptococci primers and probes were designed and confirmed using BLAST with the National Centre for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/) and/or Ribosomal Database Project (http://rdp.cme.msu.edu/probematch/search.jsp). Specificity and cross-reactivity of the designed primers and probes were tested using bacterial cultures from clinical samples (*Staphylococcus epidermidis*, *S. aureus, Streptococcus mitis*, *Streptococcus sanguinis*, *Streptococcus anginosus*) and reference bacteria from ATCC collection (*Streptococcus mitis* ATCC 49456, *Streptococcus sanguinis* ATCC 10556, *Streptococcus anginosus* ATCC 33397). Amplification primers/probes designed by other laboratories were synthesized according to the sequences published by the authors and examined in the same manner.

Assays were performed under standard conditions using specific Taqman allele hybridization according to the instructions provided with the assay with ABI PRISM 7900 HT Sequence Detection System (Taqman; Applied Biosystems, Carlsbad, CA). MasterMix was prepared using Taqman Environmental MasterMix adding at final concentrations of 1000 nM of each primer and 250 nM of each fluorescence-labeled probe. All amplifications and detections were carried out as duplicates or quadruples (in uncertain cases) depending on test runs in a MicroAmp

TABLE 1—*Primers and probes used.*

Primer and Probe	Sequence (5′-3′)	Reference
Bacteroides sp.		
Forward	TGGTAGTCCACACAGTAAACGATGA	11
Reverse	CGTACTCCCCAGGTGGAATACTT	
Probe	GTTTGCCATATACAGTAAGCGGCCAAGCG	
Bifidobacterium sp.		
Forward	CGGGTGAGTAATGCGTGACC	12
Reverse	TGATAGGACGCGACCCCA	
Probe	CTCCTGGAAACGGGTG	
Clostridium leptum		
Forward	CCTTCCGTGCCGSAGTTA	12
Reverse	GAATTAAACCACATACTCCACTGCTT	
Probe	CACAATAAGTAATCCACC	
Clostridium coccoides		
Forward	GACGCCGCGTGAAGGA	12
Reverse	AGCCCCAGCCTTTCACATC	
Probe	CGGTACCTGACTAAGAAG	
Enterobacter sp.		
Forward	GCGGTAGCACAGAGAGCTT	13
Reverse	GGCAGTTTCCCAGACATTACTCA	
Probe	CCGCCGCTCGTCACC	
Staphylococcus aureus and *S. epidermidis*		
Forward	GCGTTTTTCACGTGGAATATC	This study
Reverse	AATCCAAAACACAAACAAAGACAAGGT	
Probe	ACGTGCCATATTAATTTAC	
Streptococcus sp. mainly *Str. mitis*-group		
Forward	CCAGCAGCCGCGGTAATA	This study
Reverse	CCTGCGCTCGCTTTACG	
Probe	ACGCTCGGGACCTACG	
Universal		
Forward	TGGAGCATGTGGTTTAATTCGA	14
Reverse	TGCGGGACTTAACCCAACA	
Probe	CACGAGCTGACGACA[A/G]CCATGCA	

optical 384-well reaction plate (Applied Biosystems) with optical caps (Sarsted, Nümbrecht, Germany) in a reaction volume of 5 μL.

Amplification data were analyzed with SDS 2.2 software (Applied Biosystems), which calculates ΔRn using the equation Rn(+)−Rn(−). Rn(+) is the emission intensity of the reporter divided by the emission intensity of the quencher at any given time, whereas Rn(−) is the value of Rn(+) prior to PCR amplification. Thus, ΔRn indicates the magnitude of the signal generated. The critical threshold cycle (Ct) is the cycle at which a statistically significant increase in ΔRn is first detected and at which the fluorescence becomes detectable above background. Ct is inversely proportional to the logarithm of the initial number of template molecules, that is, the initial amount of sample DNA. The detection limits for Ct values were between 15 and 35 for the detection of *Bacteroides* sp., *Bifidobacterium* sp., *Enterobacter* sp., *Clostridium leptum*, *S. aureus,* and *S. epidermidis* as well as 15–40 for *Clostridium coccoides* and *Streptococcus* sp. mainly *Str. mitis*-group.

The comparative Ct method (ΔΔCt, $\Delta Ct_{sample} - \Delta Ct_{reference\ sample}$), (15) was used with a simplification where a blood sample from healthy person was used as a reference to determine bacterial DNA positivity and their relative amount in samples. The differences of the Ct values between the candidate bacteria and the universal bacteria measurement (ΔCt) for each sample were calculated; the comparative Ct (ΔΔCt) for sample and reference samples was calculated (16). The results from two different *Clostridium* measurements were marked as *Clostridium* sp., findings from *S. aureus* and *S. epidermidis* were marked as *Staphylococcus* sp. In a similar manner, the results from *Streptococcus* detections were marked as *Streptococcus* sp.

The study was approved by the Ethics Committee of Pirkanmaa Hospital District and the National Supervisory Authority for Welfare and Health (VALVIRA).

Results

By culturing, 21 different bacteria genera were found (Fig. 1). The most common findings were *Staphylococcus* sp. (26%), *Streptococcus* sp. (20%), followed by *Clostridium* sp., *Enterococcus* sp., and *Escherichia* sp.

Findings from bacterial culturing were divided into three groups: sterile, mono-isolates (single bacterial growth), and multi-isolates (two bacterial findings or more). It turned out that 31 of 33 (94%) pericardial fluid samples were sterile. *Clostridium* sp. (1–3 days) and *Streptococcus* sp. (5–7 days) were the only single isolates found. Liver showed high sterility (64%) up to 5 days, after which the percentages of mono- and multi-isolates increased and sterility decreased to 40%. The single isolates found in liver were *Clostridium* sp., *Streptococcus* sp., *Enterobacter* sp., *Enterococcus* sp., *Escherichia* sp., *Staphylococcus* sp., and *Streptococcus* sp. MLN was less sterile (40%) than liver. In MLN, the percentages of mono-isolates remained mainly the same for up to 5 days. The sterility was lowest in both the portal vein and in the control blood samples.

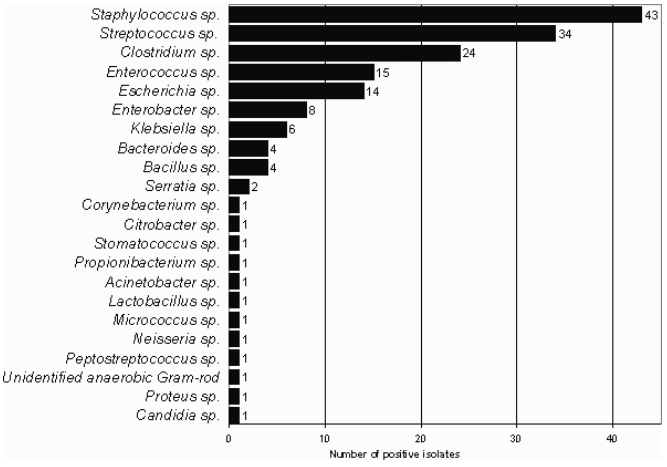

FIG. 1—*Bacterial isolates found in all samples with culturing.*

The sterility obtained using RT-qPCR was considerably less compared with culturing (Fig. 2). The cumulative sterility percentages at all sampling sites according to RT-qPCR results were 42% (1–3 days), 38% (4–5 days), and 24% (>5 days), whereas the corresponding values with microbial culturing were 46, 66, and 36%. In RT-qPCR, the decrease in sterility was most clearly seen in MLN. Pericardial fluid was the most sterile sampling site and remained sterile longest.

The relative amounts of intestinal bacteria (*Clostridium* sp., *Enterobacter* sp., *Bifidobacterium* sp., and *Bacteroides* sp.) increased with time postmortem in MLN, liver, and pericardial fluid (Fig. 3). The increase was seen especially in the relative amount of *Bifidobacterium* sp. In the liver, the relative amount of intestinal bacteria was smallest (<50%) at 1–3 days. In MLN and pericardial fluid, intestinal bacterial genera was the most common (60–73%) bacterial type in the first time period. In blood samples, the relative amount of intestinal bacteria remained quite stable, varying 75–82% in control blood and 47–59% in portal vein blood.

Discussion

In this study, we examined how bacteria migrate after death mainly from the gut into the blood, liver, portal vein, MLN, and pericardium. Other possible sources of postmortem bacterial migration include skin, respiratory tract, oral cavity, and other mucous membranes. There have been only a few postmortem microbiological investigations on how time elapsing postmortem affects the behavior of bacteria (4,6). In life, the internal organs are considered to be sterile. After death, tissue barriers and host immunity vanish and bacteria start to migrate through the epithelium. The blood is the first place where bacteria can be found soon after death. Carpenter and Wilkins (17) showed that after 18 h, 40% of the blood samples were already contaminated with bacteria. According to our culture results, 60% of blood samples showed multigrowth of bacteria, suggesting postmortem contamination. According to culturing, the sterility of pericardial fluid remained stable, whereas that of the liver samples decreased markedly after 5 days. According to RT-qPCR, the sterility of liver samples remained quite stable, but a decrease in the sterility of the pericardium samples was seen after 5 days. Our results confirm earlier findings on the unreliability of blood samples and suggest that pericardium and liver are the most suitable for microbiological sampling, at least before 5 days postmortem. Moreover, the bacterial result from one organ cannot be extrapolated to other organs.

RT-qPCR has the potential to reveal the uncultivable portion of the microbial community as well as species which are more difficult to culture. With culturing, only a minor percentage of all bacteria can be grown on plates because most obligate anaerobes dominating the intestinal ecosystem fail to grow

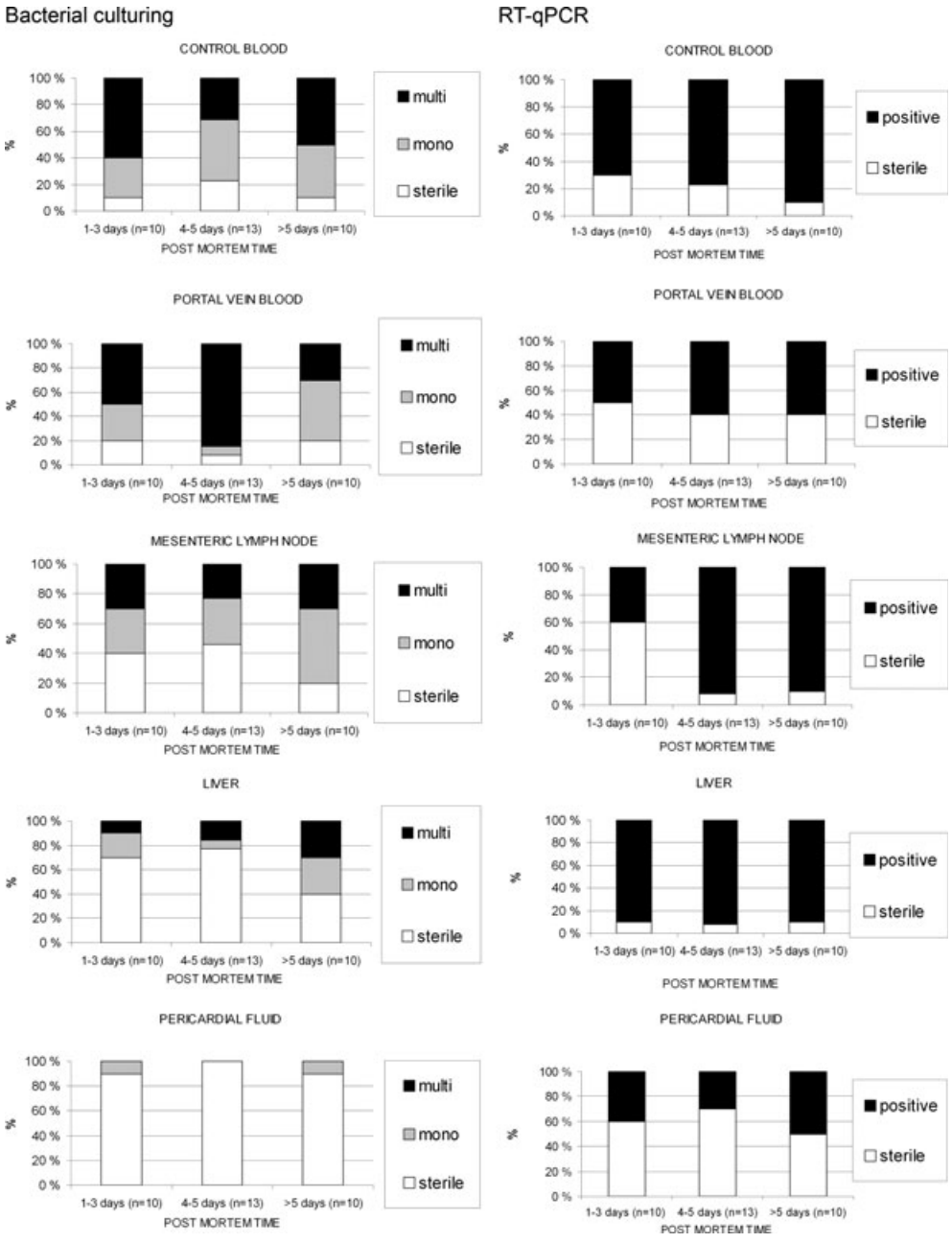

FIG. 2—*Percentage of bacterial positive findings in different organs using bacterial culturing as well as RT-qPCR with specific primers and probes. In culturing, the number of bacterial isolates per sample is presented as sterile = no bacterial growth, mono = single isolates; multi = culture with more than one isolates. In RT-qPCR, sterile means no bacterial DNA present, whereas positive means positive finding for bacterial DNA.*

(18,19). Therefore, false negatives are a known problem in bacterial culturing (20). Moreover, only living bacteria can be diagnosed by conventional culturing, whereas RT-qPCR also detects dead bacteria and fragmented bacterial DNA, for example, in macrophages. Therefore, culturing and RT-qPCR results cannot be directly compared. At present, there are no other studies available on this topic. Moreover, bacterial culturing has been designed to detect bacteria that are clinically important, whereas RT-qPCR here was designed not only to detect pathogenic bacteria like streptococci and staphylococci but also to detect major intestinal bacteria that are uncultivable or difficult to culture. This explains the discrepancy in the results between the methods.

Intestinal microbiota is taxonomically complex and constitutes an ecologically dynamic community that has been shown to have a major impact on the human immune system (21). A current estimate of their numbers lies between 1200 and 2000 species. The predominant genera are *Bacteroides*, *Clostridium*, *Bifidobacterium*, *Enterobacter*, *Eubacterium*, *Ruminococcus*, *Peptostreptococcus,* and *Fusobacterium* (10). Several studies on living individuals point toward a strong interaction between the host and its indigenous microbiota. Anaerobic rods such as *Bacteroides* and *Bifidobacteria* are "beneficial" in the maintenance of colonization resistance and gut epithelium integrity (22,23), whereas minor gut bacteria such as *Staphylococcus* sp. are thought to be "pathogenic" for gut barrier maintenance (10,24).

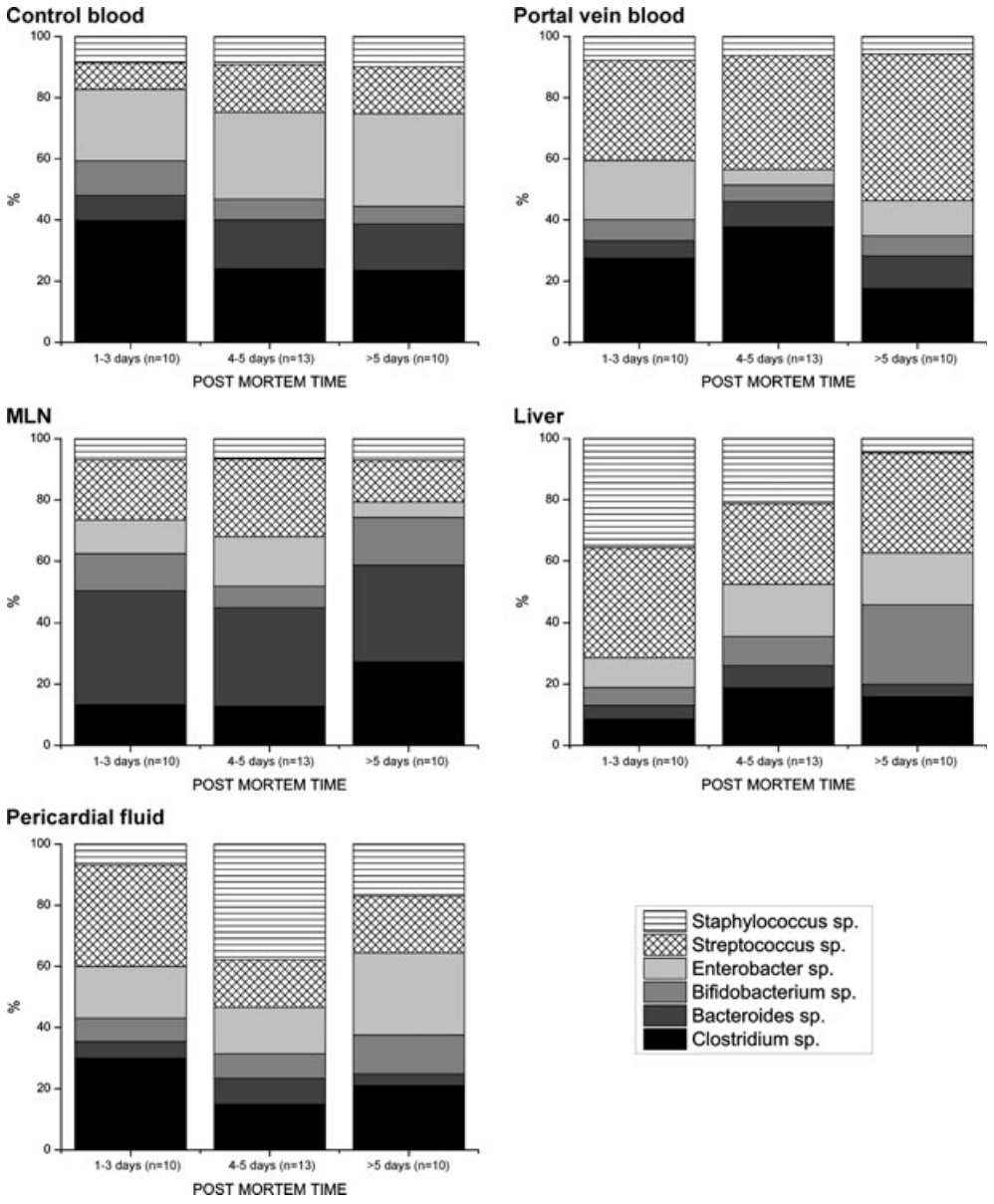

FIG. 3—*Relative amount of different genera (%) found in bacterial DNA positive samples in RT-qPCR. MLN, mesenteric lymph node. The results from two different Clostridium measurements were marked as* Clostridium *sp., findings from* S. aureus *and* S. epidermidis *were marked as* Staphylococcus *sp. as well as results from* Streptococcus *sp. mainly* Str. mitis*-group were marked as* Streptococcus *sp.*

In MLN, liver, and pericardial fluid, the relative amount of intestinal bacteria also increased with time postmortem. In the blood samples, the relative amount of intestinal bacteria decreased, whereas *Staphylococcus* sp. and *Streptococcus* sp. increased.

Differentiation between postmortem microbiological migration and true positive findings is challenging and is still mainly unexplored. According to the literature (4,6), a single bacterial finding in the sample can be considered as a true result, whereas multigrowth is believed to be postmortem migration. The contamination of samples was here evaluated using culturing results. If more than one different isolates or staphylococcal species other than *S. aureus* were detected in the sample, it was marked as unreliable, that is, contaminated. In control blood contamination, percentages for different time points (1–3 days/4–5 days/ >5 days) were (50/54/60), for MLN (10/15/20), for portal vein blood (30/39/30) and for liver (10/15/10). In pericardial fluid, there was no multigrowth or nonaureus staphylococci growth, suggesting that it is resistant to postmortem contamination. If postmortem contamination is not detected, then the bacterium found can be considered as a true finding. In our study, two pericardial samples were most probably true positive because there was only a single bacterial growth either *Streptococcus* sp. or *Clostridium* sp. In the liver, there were seven samples likely to be true positive (*Clostridium* sp., *Streptococcus* sp., *Enterobacter* sp., *Enterococcus* sp., *Escherichia* sp., *Staphylococcus* sp., or *Streptococcus* sp.).

There were signatures from several bacteria detected by qPCR in liver. Bacteria may pass through the gut epithelium and migrate to the liver in portal vein blood during life. Animal studies have shown that not only living but also dead bacteria can escape from the intestine and be found in the liver (25). The liver is enriched with innate immune cells (e.g., Kuppfer

cells, natural killer cells) and hepatic stellate cells (26). Bacteria and their residuals are cleared from portal blood mainly by Kupffer cells in sinusoids. These macrophages are able to engulf and kill bacteria, cancer cells, and viruses, but their prominent task is to recruit other immune cells for the cascade (27,28). We do not know when the bacterial residuals measured by RT-qPCR have been transferred into the liver. However, because the bacterial migration measured by culturing remained stable until 5 days, it can be assumed that bacterial residuals measured by RT-qPCR have been transported during the lifetime into the organ.

To the best of our knowledge, our study is the first to evaluate postmortem bacterial findings in pericardial fluid. Pericardial fluid differs from other sampling sites as samples can be taken as soon as the sternum has been removed, and no handling of organs in the abdominal cavity is needed, and it can easily be aspirated with a sterile needle. Pericardial fluid is produced by the thin fibrous mesothelium of the external surface of the heart. Therefore, postmortem microbial contamination or migration from the blood is prevented. Mono-isolates found in pericardial fluid in this study could indicate a transient bacteremia or even true ongoing infection during lifetime. During life, bacteria may enter the pericardial fluid causing purulent pericarditis spreading from an intrathoracic, myocardial or subdiaphragmatic focus and by hematogenous dissemination. The most frequent causes are *Staphylococcus* sp., *Streptococcus* sp, *Haemophilus* sp., and *Mycobacterium tuberculosis* (29). There are two reports of pericarditis with local infections without septicemia. Lodha et al. showed that the bacterium *S. aureus* from a purulent cyst of the scalp was found only in pericardial fluid (30). In another study, primary hepatic actinomycosis caused purulent pericarditis (31). According to the literature, if the patient has sepsis, it is rare that he also has pericarditis (32).

As a conclusion, postmortem samples ought to be taken within 5 days postmortem and the best sampling places are liver and pericardial fluid. These two sampling sites were the most sterile and also remained sterile longest. Liver is rarely contaminated, and the bacterial findings were mostly mono-isolates up to 5 days postmortem. Because bacterial migration remained stable in pericardial fluid according to culturing, all the bacterial results detected by RT-qPCR could be true findings. This can also be applied to the RT-qPCR results of liver samples before 5 days postmortem. Our results suggested that true bacterial findings could be found in the pericardial fluid and in the liver. The microbiological results from autopsy cases must, however, be interpreted by an experienced microbiologist and forensic specialist.

Acknowledgments

The excellent technical assistance performed by the personnel of Centre for Laboratory Medicine, Pirkanmaa Hospital District and the Department of Forensic Medicine, especially Kari Mänttäri and Olli Penttilä, Tampere University is gratefully acknowledged. We also thank ADP Designer Jukka Lehtiniemi from Institute of Biomedical Technology, University of Tampere for his technical assistance with the figures.

References

1. Morris JA, Harrison LM, Biswas J, Telford DR. Transient bacteraemia: a possible cause of sudden life threatening events. Med Hypotheses 2007;69:1032–9.
2. Bajanowski T, Vege A, Byard RW, Krous HF, Arnestad M, Bachs L, et al. Sudden infant death syndrome (SIDS)—standardised investigations and classification: recommendations. Forensic Sci Int 2007;165:129–43.
3. Krous HF, Beckwith JB, Byard RW, Rognum TO, Bajanowski T, Corey T, et al. Sudden infant death syndrome and unclassified sudden infant deaths: a definitional and diagnostic approach. Pediatrics 2004;114:234–8.
4. Morris JA, Harrison LM, Partridge SM. Postmortem bacteriology: a re-evaluation. J Clin Pathol 2006;59:1–9.
5. Wilson SJ, Wilson ML, Reller LB. Diagnostic utility of postmortem blood cultures. Arch Pathol Lab Med 1993;117:986–8.
6. Weber MA, Hartley JC, Brooke I, Lock PE, Klein NJ, Malone M, et al. Post mortem interval and bacteriological culture yield in sudden unexpected death in infancy (SUDI). Forensic Sci Int 2010;198:121–5.
7. Roberts FJ. Procurement, interpretation, and value of postmortem cultures. Eur J Clin Microbiol Infect Dis 1998;17:821–7.
8. Lobmaier IV, Vege A, Gaustad P, Rognum TO. Bacteriological investigation—significance of time lapse after death. Eur J Clin Microbiol Infect Dis 2009;28:1191–8.
9. Tsokos M, Puschel K. Postmortem bacteriology in forensic pathology: diagnostic value and interpretation. Leg Med (Tokyo) 2001;3:15–22.
10. Sekirov I, Russell SL, Antunes LC, Finlay BB. Gut microbiota in health and disease. Physiol Rev 2010;90:859–904.
11. Brunk CF, Li J, Avaniss-Aghajani E. Analysis of specific bacteria from environmental samples using a quantitative polymerase chain reaction. Curr Issues Mol Biol 2002;4:13–8.
12. Furet JP, Firmesse O, Gourmelon M, Bridonneau C, Tap J, Mondot S, et al. Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR. FEMS Microbiol Ecol 2009;68:351–62.
13. Sen K, Asher DM. Multiplex PCR for detection of enterobacteriaceae in blood. Transfusion 2001;41:1356–64.
14. Yang S, Lin S, Kelen GD, Quinn TC, Dick JD, Gaydos CA, et al. Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens. J Clin Microbiol 2002;40:3449–54.
15. Suzuki N, Yoshida A, Nakano Y. Quantitative analysis of multi-species oral biofilms by TaqMan real-time PCR. Clin Med Res 2005;3: 176–85.
16. Tichopad A, Bar T, Pecen L, Kitchen RR, Kubista M, Pfaffl MW. Quality control for quantitative PCR based on amplification compatibility test. Methods 2010;50:308–12.
17. Carpenter HM, Wilkins RM. Autopsy bacteriology: review of 2,033 cases. Arch Pathol 1964;77:73–81.
18. Rinttila T, Kassinen A, Malinen E, Krogius L, Palva A. Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. J Appl Microbiol 2004;97:1166–77.
19. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the human intestinal microbial flora. Science 2005;308:1635–8.
20. Alain K, Querellou J. Cultivating the uncultured: limits, advances and future challenges. Extremophiles 2009;13:583–94.
21. Kinross JM, Darzi AW, Nicholson JK. Gut microbiome-host interactions in health and disease. Genome Med 2011;3:14.
22. Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI. Molecular analysis of commensal host-microbial relationships in the intestine. Science 2001;291:881–4.
23. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. Science 2005;307:1915–20.
24. Shimizu K, Ogura H, Goto M, Asahara T, Nomoto K, Morotomi M, et al. Altered gut flora and environment in patients with severe SIRS. J Trauma 2006;60:126–33.
25. White JS, Hoper M, Parks RW, Clements WD, Diamond T. Patterns of bacterial translocation in experimental biliary obstruction. J Surg Res 2006;132:80–4.
26. Suh YG, Jeong WI. Hepatic stellate cells and innate immunity in alcoholic liver disease. World J Gastroenterol 2011;17:2543–51.
27. Beutler B. Innate immunity: an overview. Mol Immunol 2004;40:845–59.
28. Kim TH, Lee SH, Lee SM. Role of kupffer cells in pathogenesis of sepsis-induced drug metabolizing dysfunction. FEBS J 2011;278: 2307–17.
29. Pankuweit S, Ristić AD, Seferović PM, Maisch B. Bacterial pericarditis: diagnosis and management. Am J Cardiovasc Drugs 2005;5:103–12.
30. Lodha A, Enakpene E, Haran M, Sadiq A, Shani J. Bacterial pericarditis caused by infected trichilemmal cyst. J Infect Chemother 2012;18(2): 251–4.

31. Llenas-Garcia J, Lalueza-Blanco A, Fernandez-Ruiz M, Villar-Silva J, Ochoa M, Lozano F, et al. Primary hepatic actinomycosis presenting as purulent pericarditis with cardiac tamponade. Infection 2012;40(3): 339–41.
32. Kan B, Ries J, Normark BH, Chang FY, Feldman C, Ko WC, et al. Endocarditis and pericarditis complicating pneumococcal bacteraemia, with special reference to the adhesive abilities of pneumococci: results from a prospective study. Clin Microbiol Infect 2006;12:338–44.

Additional information and reprint requests:
Sari Tuomisto, M.Sc.
School of Medicine
University of Tampere
Medisiinarinkatu 3
Tampere 33014
Finland
E-mail: Sari.Tuomisto@uta.fi

BMC Gastroenterology

RESEARCH ARTICLE Open Access

Changes in gut bacterial populations and their translocation into liver and ascites in alcoholic liver cirrhotics

Sari Tuomisto[1,3*], Tanja Pessi[1,3], Pekka Collin[2], Risto Vuento[3], Janne Aittoniemi[3] and Pekka J Karhunen[1,3]

Abstract

Background: The liver is the first line of defence against continuously occurring influx of microbial-derived products and bacteria from the gut. Intestinal bacteria have been implicated in the pathogenesis of alcoholic liver cirrhosis. Escape of intestinal bacteria into the ascites is involved in the pathogenesis of spontaneous bacterial peritonitis, which is a common complication of liver cirrhosis. The association between faecal bacterial populations and alcoholic liver cirrhosis has not been resolved.

Methods: Relative ratios of major commensal bacterial communities (*Bacteroides* spp., *Bifidobacterium* spp., *Clostridium leptum* group, *Enterobactericaea* and *Lactobacillus* spp.) were determined in faecal samples from post mortem examinations performed on 42 males, including cirrhotic alcoholics (n = 13), non-cirrhotic alcoholics (n = 15), non-alcoholic controls (n = 14) and in 7 healthy male volunteers using real-time quantitative PCR (RT-qPCR). Translocation of bacteria into liver in the autopsy cases and into the ascites of 12 volunteers with liver cirrhosis was also studied with RT-qPCR. CD14 immunostaining was performed for the autopsy liver samples.

Results: Relative ratios of faecal bacteria in autopsy controls were comparable to those of healthy volunteers. Cirrhotics had in median 27 times more bacterial DNA of *Enterobactericaea* in faeces compared to the healthy volunteers (p = 0.011). *Enterobactericaea* were also the most common bacteria translocated into cirrhotic liver, although there were no statistically significant differences between the study groups. Of the ascites samples from the volunteers with liver cirrhosis, 50% contained bacterial DNA from *Enterobactericaea*, *Clostridium leptum* group or *Lactobacillus* spp.. The total bacterial DNA in autopsy liver was associated with the percentage of CD14 expression (p = 0.045). CD14 expression percentage in cirrhotics was significantly higher than in the autopsy controls (p = 0.004).

Conclusions: Our results suggest that translocation of intestinal bacteria into liver may be involved as a one factor in the pathogenesis of alcoholic liver cirrhosis.

Keywords: Alcoholic liver cirrhosis, Gut microbiota, RT-qPCR, Bacterial translocation, Microbiology, CD14

Background

Liver cirrhosis is the irreversible end stage of chronic liver disease. In Western countries more than 90% of cirrhosis is caused by excessive ethanol consumption. However, only a minority of alcohol abusers ever develop liver cirrhosis during their lives. Our group has previously reported a link between alcoholic liver cirrhosis and bacterial recognition receptor *CD14* genetics [1] supporting the hypothesis of the involvement of intestinal bacteria and their endotoxins in the pathogenesis of cirrhosis [2]. This observation has been confirmed by other groups [3,4].

In healthy individuals, normal gut microbiota consists mainly of bacteria belonging to the *Clostridium coccoides* (cluster XIVa) and *Clostridium leptum* group (cluster IV), *Bacteroides* spp., *Bifidobacterium* spp. and *Enterobacter* spp. [5,6]. *Enterobacter* spp. belongs to a

* Correspondence: Sari.Tuomisto@uta.fi
[1]Department of Forensic Medicine, University of Tampere, School of Medicine, Medisiinarinkatu 3, 33014 Tampere, Finland
[3]Fimlab Laboratories, Pirkanmaa Hospital District, Biokatu 4, 33520 Tampere, Finland
Full list of author information is available at the end of the article

large *Enterobactericaea* family consisting of several gram-negative bacteria [7]. The liver is continually exposed to gut derived bacteria and bacterial components because ca. 70% of its blood supply is from the portal vein, which is the direct venous outflow of the intestine [8]. Chronic alcohol abuse has been shown to change intestinal bacterial population by decreasing the numbers of *Clostridium* (gram-positive, anaerobe) and *Bacteroidetes* (gram-negative, anaerobe) and increasing the numbers of aerobic *Proteobacteria* (gram-negative, facultatively or obligately anaerobic) [9].

Alcohol can cause leakage of intestinal cell junctions [10]. Gram-negative bacteria [11] and their endotoxins [12] may then translocate through portal blood into the liver and induce inflammation leading to fibrosis and cirrhosis in genetically susceptible individuals [1]. The quantities of *Bacteroides* spp. and *Bifidobacterium* spp. have been reported to be decreased and those of *Enterobacter* spp. and *Clostridium* spp. to be increased in the faeces of cirrhotics [13,14], but it is not known whether these bacteria may translocate into the liver.

Spontaneous bacterial peritonitis is a complication of cirrhosis. It is thought to be due to bacterial overgrowth in the gut and their translocation into the ascites [15]. Its frequency has been 7% in cirrhotic patients with ascites [15]. Bacterial culture is unreliable, and the diagnosis is based on a polymorphonuclear cell count of 250/mm^3 or more in the ascites fluid, regardless of the result of received by bacterial culturing. Bacterial contamination may be far more common than manifest in spontaneous bacterial peritonitis or positive bacterial culture. It is possible that bacteria which may be associated with the pathogenesis of cirrhosis may also be present in ascites when measured by molecular biological methods instead of conventional culturing.

We have previously reported that liver samples from autopsies can be reliably used in bacteriological analyses up to 5 days post mortem [16], and that the faecal bacterial composition of certain bacteria remains stable during that period [17]. In an earlier paper we also have shown in the present autopsy series that bacteria can be detected by culturing and qPCR in mesenteric lymph nodes but also in the portal vein and liver [16]. The aim of this study was to characterize changes in intestinal microbiota (*Bacteroides* spp., *Bifidobacterium* spp., *Clostridium leptum* group, *Enterobacteriacaea,* and *Lactobacillus* spp.) in alcoholic liver cirrhotics compared to alcoholics without cirrhosis, non-alcoholic autopsy controls and in healthy volunteers, and to study the translocation of bacteria into liver and ascites samples. In order to confirm the significance of our bacterial findings, the activity of bacteria recognizing receptor CD14 was studied by immunostaining of autopsy liver samples and correlated with the presence and amount of bacterial DNA in liver.

Methods

The present study comprised a prospective autopsy series of 42 males at the Department of Forensic Medicine of the University of Tampere, and 7 male healthy volunteers and 12 male clinical volunteers with alcoholic liver cirrhosis with ascites at the Department of Gastroenterology and Alimentary Tract Surgery (Table 1). From the routine autopsies, we selected cases with alcoholic cirrhosis (n = 13), alcoholics (n = 15) without liver cirrhosis and a control series (n = 14) of non-alcoholic men. Criteria for including autopsies were: out-of-hospital death, male sex, age over 18 years, time elapsed post mortem 5 days or less, time interval between death and storage of the body in the mortuary less than 24 hours, intact middle torso and bowel, no signs of bacterial infections or visible wounds or necrosis and no signs or reports of drug addiction. None of the study subjects were reported to have taken antibiotics for 2 weeks prior to sampling. Two of the 42 men died of alcohol intoxication and in 17 cases alcohol was a contributory factor for death. One subject died of alcoholic liver cirrhosis. The hospital records of the autopsy cases were available, and were scrutinized for mentions of alcohol use. The criterion for the definition of

Table 1 Demographic characteristics of the study subjects

	Autopsy cases			Volunteers		
	Cirrhotics	Alcoholics	Non-alcoholic controls	Healthy controls	Liver cirrhosis	P-value
N	13	15	14	7	12	
PM time mean	3.6	3.3	3.8			0.537
Age mean (range)	56 (39–77)	54 (34–77)	58 (18–86)	45 (26–57)	58 (39–73)	0.383
BMI mean (range	29.1 (19.6-41.9)	30.1 (20.4-42.1)	27.4 (18.4-43.6)	27.1 (20.8-37.2)	28.1 (19.7-39.2)	0.573
Cause of death						
Heart disease%	5 (38.5%)	3 (20.0%)	10 (71.4%)			0.007
Other disease%	5 (38.5%)	8 (53.3%)	1 (7.1%)			0.017
Non-natural death[1]%	3 (23.1%)	4 (26.7%)	3 (21.4%)			0.885

[1]suicide, accident, poisoning.

alcoholism or heavy alcohol consumption was a comment in the hospital records/police reports or alcoholism-related microscopic findings such as presence of alcoholic liver disease or cerebellar atrophy, along with positive post-mortem alcohol test. Of the 15 alcoholics, 11 had mentions of alcoholism in their documentation, 12 had positive post mortem alcohol test and 6 also had microscopic findings (fatty liver, cerebellar atrophy or chronic pancreatitis) of alcoholism.

Written consent was obtained from the volunteers. None of the healthy volunteers drank more than 3 drinks (12 g of alcohol) per week. Volunteers with liver cirrhosis reported excessive alcohol consumption. None of the study subjects were reported to have taken antibiotics for 2 weeks prior to sampling.

Samples from rectum and liver were taken aseptically from the autopsy cases. The healthy volunteers provided samples after defecation. All faecal samples were frozen (−20°C) immediately after sampling and were transferred to −80°C until further processing. Ascites fluid samples were collected from patients during their hospital stay with sterile instruments.

Ascites fluid samples were drawn aseptically at the bedside from the volunteers with a diagnosis of alcohol-related cirrhosis, which was based on drinking history and clinical findings. Bacterial conventional culturing as well as albumin and polymorphonuclear measurements from ascites samples were performed.

Faecal samples were weighed to be 150 mg (wet weight). Bacterial DNA was extracted from the faecal samples using a commercial DNA extraction kit (Zymo Fecal DNA Kit (Zymo Research Corporation, Irvine, California, USA)) according to the instructions provided. DNA from liver, blood and ascites fluid samples was extracted using Zymo Bacterial/Fungal DNA Kit (Zymo Bacterial/Fungal DNA Kit (Zymo Research Corporation, Irvine, California, USA)).

The quantity of bacteria was determined by RT-qPCR using published oligonucleotide primers and probes for *Bacteroides* spp. [18], *Bifidobacterium* spp. [19], *Clostridium leptum* group (cluster IV) [19], and *Enterobacter* spp. [20]. The primers and probe for *Enterobacteriacaea* (Forward: GCGGTAGCACAGAGAGCTT, Reverse: GG CAGTTTCCCAGACATTACTCA, PROBE:6FAM-CCG CCGCTCGTCACC-BHQ), and *Lactobacillus* spp. (Forward: GCTAGGTGTTGGAGGGTTTCC, Reverse: CCA GGCGGAATGCTTAATGC, PROBE:6FAM- TCAGTG CCGCAGCTAA-BHQ) were designed and confirmed using BLAST with the National Centre for Biotechnology Information server (http://www.ncbi.nlm.nih.gov) and Ribosomal Database Project (http://rdp.cme.msu.edu/probematch/search.jsp). Specificity and cross reactivity of all the primers and probes were tested using bacteria from clinical samples and reference bacteria [16,21]. Total amount of bacteria was measured using universal bacterial primers and a probe [22]. Assays from faecal samples and from ascites were performed with AbiPrism 7000 HT Sequence Detection System (Taqman™, AppliedBiosystems, California, USA) in reaction volume of 20 μl in 96-well reaction plate under standard conditions with Taqman. Faecal samples were diluted 1:100. In the liver samples, assays were performed with AbiPrism 7900 HT Sequence Detection System (Taqman®, AppliedBiosystems) in a reaction volume of 5 μl. One micro litre of DNA was added into the reactions for the detection system. MasterMix was prepared using Taqman™ Environmental MasterMix adding at final concentrations of 1000 nM of each primer, and 250 nM of each fluorescence labelled probe. All amplifications and detections were carried out in duplicate or quadruplicate (in uncertain cases).

The relative amount of bacterial DNA in a sample was determined with comparative Ct method (ΔΔCt, $\Delta Ct_{\text{sample}} - \Delta Ct_{\text{reference sample}}$) [23]. In autopsy faecal samples, mean ΔC values of the faecal samples of healthy volunteers were calculated and used as a reference. In ascites fluid and liver samples the blood sample from a healthy individual was used as a reference as previously described [16]. Bacterial positivity of liver and ascites samples was determined as previously described [16].

Histological samples from the right lobe of the liver were taken at autopsy, fixed in 10% formalin overnight and processed for CD14 (Leica Biosystems, Newcastle, United Kingdom) immunohistochemistry, using dilution of 1:250 with Autostainer LV-1 (Lab Vision Corporation, California, USA). The percentage of brown peroxidase positivity in each sample was calculated with the ImmunoRatio program [24] and average discolouration value was compared between different groups.

The study was approved by the Ethics Committee of Pirkanmaa Hospital District and the National Supervisory Authority for Welfare and Health (VALVIRA).

Statistics

Due to the skewed variation within the bacterial measurements, logarithmic values were used for the calculations [IBM SPSS Statistics version 21 (IBM, New York, United States)]. ANOVA was used to measure significant differences between groups (healthy volunteers, alcoholic cirrhotics, alcoholics and non-alcoholic controls). When ANOVA showed a significant difference, pairwise comparisons with Post Hoc test with Least Significant Difference (LSD) corrections were made. With liver samples, Pearson's Chi-square test was used.

Results

The median values of relative amounts of measured bacterial groups in the faeces of the autopsy controls were comparable to those in the healthy volunteers (Figure 1). Although the numbers in the groups were small, there

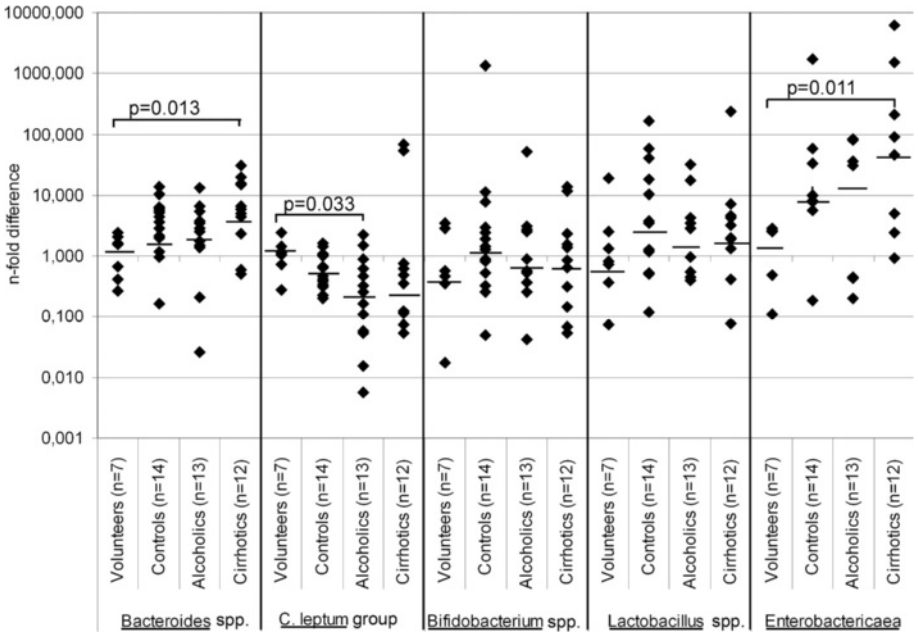

Figure 1 Relative amounts (n-fold difference) of bacteria measured (*Bacteroides* spp., *C. leptum* group, *Bifidobacterium* spp., *Lactobacillus* spp., *Enterobactericaea*) in faecal samples of cirrhotic, alcoholic and autopsy control cases and in healthy volunteers. Individual values are presented as diamond, median values with horizontal lines.

were differences across study groups (healthy volunteers, controls, alcoholics and cirrhotics) in the relative amounts of *Bacteroides* spp. (p = 0.070) and *Enterobactericaea* (p = 0.056). Post Hoc analysis showed that males with cirrhosis had significantly more gram-negative *Bacteroides* spp. (p = 0.013) and gram-negative *Enterobactericaea* (p = 0.011) in autopsy faecal samples than did the healthy volunteers. Of *Enterobactericaea*, autopsy faecal samples from males with cirrhosis contained considerably more gram-negative *Enterobacter* spp. (p = 0.034) than those from alcoholics without liver cirrhosis (data not shown). When comparison was done against all other groups combined (alcoholics, control autopsies and healthy volunteers), the faeces of alcoholic cirrhotics contained statistically significantly more gram-negative *Enterobactericaea* (p = 0.037), *Enterobacter* spp. (p = 0.047) and *Bacteroides* spp. (p = 0.049). No differences were seen in the proportion of *Bifidobacterium* spp. and *Lactobacillus* spp. between different groups. Inter-individual variation was great in all bacterial measurements.

Bacterial DNA positivity was in liver samples of cirrhotics 92%, alcoholics 60% and in the controls 64% (Figure 2). These differences did not reach statistical significance (p = 0.130, Pearson Chi Square). The most common bacterial DNA detected in the liver samples of cirrhotics was *Enterobactericaea* (49%) and *Bifidobacterium* spp. (24%). Differences between detected bacteria in the groups did not reach statistical significance; wide inter-individual variation was detected.

Of the ascites fluid samples, 50% contained bacterial DNA. Of the bacteria measured *Lactobacillus* spp., *C. leptum* group and *Enterobactericaea* were found, whereas *Bifidobacterium* spp. and *Bacteroides* spp. were not amplified (Figure 3). In all 12 samples, the ascites leukocyte count was below 250 10E6/l, thus none of the patients fulfilled the criteria of spontaneous bacterial peritonitis. Ascites bacterial culturing was negative in each case.

CD14 percentage differed significantly (p = 0.012) among the controls (median 17.6%), alcoholics (23.3%) and cirrhotics (31.9%). In Post Hoc analyses using LSD correction the CD14 expression percentage was significantly higher in the cirrhotics than in the autopsy controls (p = 0.004) but there were no statistically significant differences between the controls and alcoholics without cirrhosis or between the alcoholics and cirrhotics. Figure 4 presents examples of CD14 staining of control, alcoholic and alcoholic liver cirrhosis cases. Expression of CD14 was higher (p = 0.045) in bacterial DNA-positive liver samples than in DNA- negative samples (Figure 5).

Discussion

In this study we measured with RT-qPCR the composition of gut microbiota and bacterial translocation into the liver in alcoholic liver cirrhotics and non-cirrhotic alcoholics compared to controls. We found that liver cirrhotics harboured considerably more gram-negative *Bacteroides* and *Enterobactericaea* including *Enterobacter*

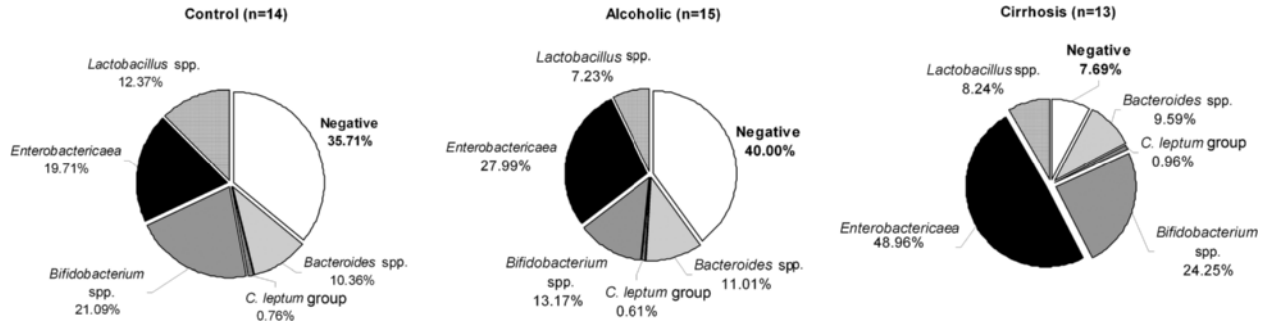

Figure 2 Percentages of bacteria found in the liver samples of autopsy cases (n = 42) with RT-qPCR.

spp. in their faeces than did the controls. This result is in line with those of other studies [13,14,25] showing increased prevalence of *Enterobactericaea* in the phylum level and also an increase in the counts of *Enterobacter* spp. in the faeces of cirrhotics. Cirrhosis is associated with a decreased conversion of primary to secondary faecal bile acids, which is associated with abundance of major gut microbiome taxa. Bile acids in general, and cholestasis, which is common in cirrhotic patients, may be one mechanistic explanation for the altered microbiota in liver diseases [25]. However, our results do not confirm earlier reports of decreased amounts of gram-negative *Bacteroides* spp. and gram-positive *Bifidobacterium* spp. and increased amounts of gram-positive *Clostridium* spp. in the faeces of cirrhotics [13, 14,].

We also found that the total amount of bacterial DNA in liver was associated with CD14 expression percentage in immunohistochemical stainings. The CD14 expression percentage in cirrhotics was significantly higher due to possible bacterial load compared to that of the autopsy controls. While CD14 macrophages in liver, Kupffer cells, are also capable of recognizing e.g. human cell debris, apoptotic cells and other activating agents [26], they have developed an efficient phagocytic capacity to remove endotoxin from portal circulation. Moreover, since Kupffer cells are continuously exposed to endotoxin and other activators, they show constitutive low-level CD14 activation [27], as is seen in our control liver samples. As far as we know, there are no previous studies reporting the simultaneous measurement of amounts of bacterial DNA and CD14 activity in the liver. Our results suggest that bacteria may have a role as inducers of the CD14 mediated inflammation process that may lead to fibrosis and cirrhosis [28].

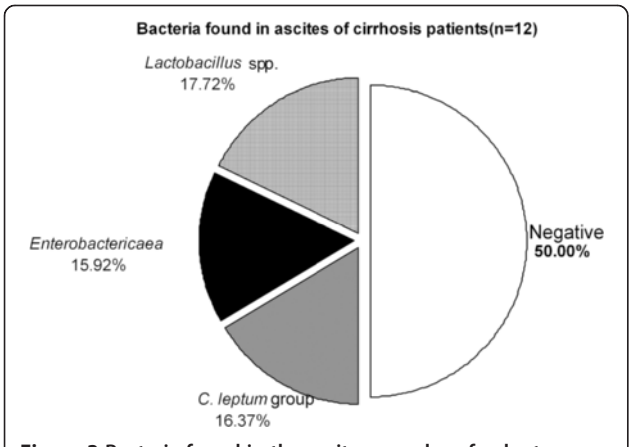

Figure 3 Bacteria found in the ascites samples of volunteers with liver cirrhosis (n = 12) with RT-qPCR specific primers and probes. A blood sample from a healthy volunteer was used as a reference.

It is known that intestinal bacteria contribute to intestinal homeostasis and that alcohol affects microbial populations by disturbing this balance [2]. Alcohol metabolite, acetaldehyde, has a direct effect on gut epithelial cell function by disrupting tight junctions, and may thus enhance bacterial translocation [10] from the intestines. Alcohol ingestion has been reported to correlate with increased levels of bacterial endotoxins [29-31] and peptidoglycan [32] in plasma. Of commensal intestinal bacteria, gram-negative enterobacteria and gram-positive enterococci are the most effective to escape into the organs [11] and are the most commonly found in community-acquired infections in patients with alcoholic liver cirrhosis [33]. Moreover, sepsis is a 20 times more common cause of death in cirrhotics than in general population [34]. Our results showed that *Enterobactericaea* was the most commonly detected in livers of cirrhotics. These differences were not statistically significant, most probably due to small sample size and wide inter-individual variations in bacterial populations. Furthermore, bacterial DNA from *Enterobactericaea* was also detected in ascites.

In cirrhotic patients, attempts have been made to inhibit the growth of gram-negative bacteria in the gut by the use of broad-spectrum antibiotics such as fluorokinones e.g. neomysin, norfloxacin and ciprofloxacin [35,36]. However, long-term use of antibiotics may lead to an increase of pathogenic bacteria in the gut [37] and to increased antibiotic resistance [38,39]. In experimental studies,

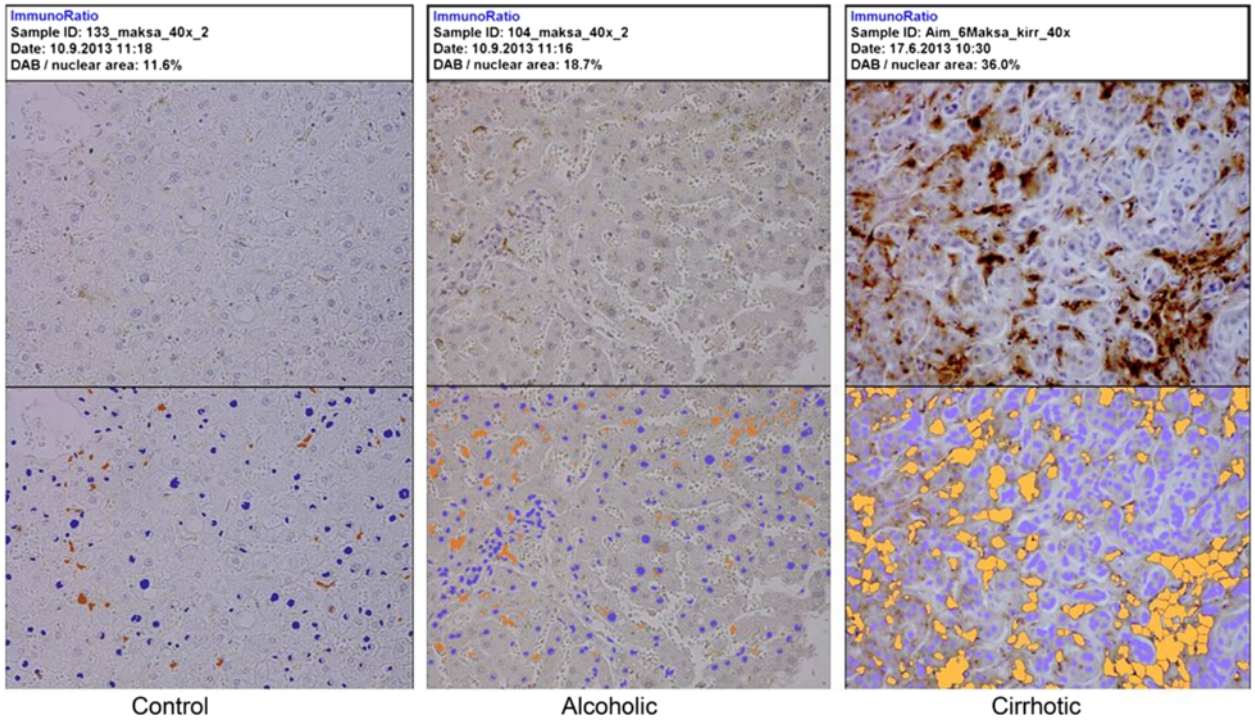

Figure 4 Immunohistological staining of CD14 as DAB/nuclear area percentage calculated with ImmunoRatio program.

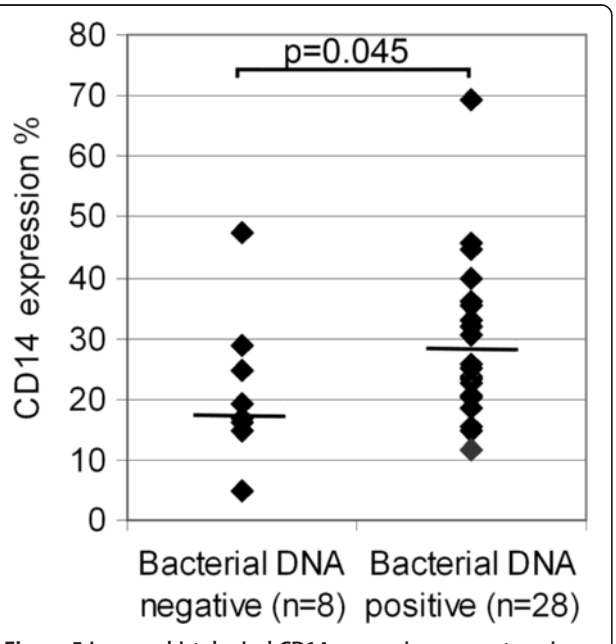

Figure 5 Immunohistological CD14 expression percentage in bacterial DNA positive and bacterial DNA negative autopsy liver samples calculated with ImmunoRatio software. Medians are presented as lines.

probiotics *(Bifidobacterium* spp., *Lactobacillus* spp.), have restored normal gut homeostasis and have inhibited excessive growth of gram-negative bacteria [40-42]. In the future, targeted treatments using probiotics or better focused antibiotics against risk pathogens, like *Enterobactericaea*, may be available.

In the present study, relative amounts of commensal gut bacteria and their translocation into liver and ascites was investigated with RT-qPCR. The frequency of bacterial DNA in ascites was 50%, even though none of the volunteers with liver cirrhosis had positive culture or spontaneous bacterial peritonitis. We used universal bacterial primers and probes [22] to amplify all bacterial DNA in the samples. Therefore we believe that our negative cases were genuinely bacterial negative and ascites were sterile in these cases. We did not have control samples from non-cirrhotic ascites, which is a limitation of the study, but except in malignancy, such samples are difficult to obtain. Nevertheless, the same bacteria were found to leak in ascites which were found translocated in liver samples. RT-qPCR provides a fast and accurate tool for the determination of the faecal bacterial composition of clinical patients. Conventional culturing, which is used in many hospital labs, provides only limited opportunities to study certain bacterial strains [16]. Culturing is a relatively slow method and may not provide any information if the bacteria in the samples have been exposed to unfavourable conditions like oxygen or

temperature changes leading to their death. Because only 30-40% of the bacteria in the human intestinal tract are culturable, bacterial detection diagnostics can be improved with the DNA-detection based approach [43]. We also found that there were remarkable inter-individual variations in bacterial population ratios, as also reported by earlier studies [44,45].

We have previously shown that e.g. genetic polymorphism in the bacterial CD14 receptor may play a role in susceptibility to cirrhosis [1]. This suggests that susceptibility to alcoholic liver cirrhosis due to bacterial influx into the liver may also be genetically determined. A continuous bacterial translocation from the gut may not be enough per se to cause the development of cirrhosis.

Conclusions

In conclusion, alcoholic liver cirrhotics had increased amounts of gram-negative enterobacteria in faeces and DNA from enterobacteria was detected in liver and ascites. Total bacterial DNA amount in the liver was associated with immunohistochemical CD14 expression, which was significantly higher in liver cirrhotics than in autopsy controls.

These results suggest that intestinal microbiota and bacterial translocation into the liver may be involved in the pathogenesis of alcoholic liver cirrhosis.

Abbreviations

RT-qPCR: Real-time quantitative polymerase chain reaction.

Competing interests

The authors declared that they have no competing interests.

Authors' contributions

ST performed the experiments and analyses, wrote the manuscript and helped in the collection of the autopsy samples. TP designed the sample collection and experiments and participated in writing the manuscript. PC participated in the planning of the study and in samples collection from patients. RV and JA provided comments. PK was the initiator of the project and participated in writing the manuscript and collected the autopsy series. All authors have read and approved the final version.

Acknowledgements

The excellent technical assistance from the personnel of Department of Forensic Medicine, especially Kari Mänttäri, Olli Penttilä and Mervi Seppänen, Tampere University is gratefully acknowledged. We also appreciate the participation of the volunteers and the personnel of the Tampere University Hospital.

Grant support

The study was financially supported by the Tampere Graduate Program in Biomedicine and Biotechnology (TGPBB), the Finnish Foundation for Alcohol Studies, Foundation of Mary and Georg C. Ehrnrooth, the Competitive State Research Financing of the Expert Responsibility Area of Tampere University Hospital, the European Union 7th Framework Program grant number 201668 for the AtheroRemo Project, the Pirkanmaa Regional Fund of the Finnish Cultural Foundation.

Author details

[1]Department of Forensic Medicine, University of Tampere, School of Medicine, Medisiinarinkatu 3, 33014 Tampere, Finland. [2]Department of Gastroenterology and Alimentary Tract Surgery, Tampere University Hospital, Tampere, Finland. [3]Fimlab Laboratories, Pirkanmaa Hospital District, Biokatu 4, 33520 Tampere, Finland.

Received: 9 October 2013 Accepted: 19 February 2014
Published: 24 February 2014

References

1. Jarvelainen HA, Orpana A, Perola M, Savolainen VT, Karhunen PJ, Lindros KO: **Promoter polymorphism of the CD14 endotoxin receptor gene as a risk factor for alcoholic liver disease.** *Hepatology* 2001, **33:**1148–1153.
2. Yan AW, Schnabl B: **Bacterial translocation and changes in the intestinal microbiome associated with alcoholic liver disease.** *World J Hepatol* 2012, **4:**110–118.
3. Campos J, Gonzalez-Quintela A, Quinteiro C, Gude F, Perez LF, Torre JA, Vidal C: **The -159C/T polymorphism in the promoter region of the CD14 gene is associated with advanced liver disease and higher serum levels of acute-phase proteins in heavy drinkers.** *Alcohol Clin Exp Res* 2005, **29:**1206–1213.
4. LeVan TD, Bloom JW, Bailey TJ, Karp CL, Halonen M, Martinez FD, Vercelli D: **A common single nucleotide polymorphism in the CD14 promoter decreases the affinity of Sp protein binding and enhances transcriptional activity.** *J Immunol* 2001, **167:**5838–5844.
5. Lay C, Sutren M, Rochet V, Saunier K, Dore J, Rigottier-Gois L: **Design and validation of 16S rRNA probes to enumerate members of the Clostridium leptum subgroup in human faecal microbiota.** *Environ Microbiol* 2005, **7:**933–946.
6. Orrhage K, Nord CE: **Bifidobacteria and lactobacilli in human health.** *Drugs Exp Clin Res* 2000, **26:**95–111.
7. Williams KP, Gillespie JJ, Sobral BW, Nordberg EK, Snyder EE, Shallom JM, Dickerman AW: **Phylogeny of gammaproteobacteria.** *J Bacteriol* 2010, **192:**2305–2314.
8. Son G, Kremer M, Hines IN: **Contribution of gut bacteria to liver pathobiology.** *Gastroenterol Res Pract* 2010, 2010 doi:10.1155/2010/453563. Epub 2010 Jul 28.
9. Mutlu EA, Gillevet PM, Rangwala H, Sikaroodi M, Naqvi A, Engen PA, Kwasny M, Lau CK, Keshavarzian A: **Colonic microbiome is altered in alcoholism.** *Am J Physiol Gastrointest Liver Physiol* 2012, **302:**G966–G978.
10. Basuroy S, Sheth P, Mansbach CM, Rao RK: **Acetaldehyde disrupts tight junctions and adherens junctions in human colonic mucosa: protection by EGF and L-glutamine.** *Am J Physiol Gastrointest Liver Physiol* 2005, **289:**G367–G375.
11. Steffen EK, Berg RD, Deitch EA: **Comparison of translocation rates of various indigenous bacteria from the gastrointestinal tract to the mesenteric lymph node.** *J Infect Dis* 1988, **157:**1032–1038.
12. Schaffert CS, Duryee MJ, Hunter CD, Hamilton BC 3rd, DeVeney AL, Huerter MM, Klassen LW, Thiele GM: **Alcohol metabolites and lipopolysaccharide: roles in the development and/or progression of alcoholic liver disease.** *World J Gastroenterol* 2009, **15:**1209–1218.
13. Zhao HY, Wang HJ, Lu Z, Xu SZ: **Intestinal microflora in patients with liver cirrhosis.** *Chin J Dig Dis* 2004, **5:**64–67.
14. Chen Y, Yang F, Lu H, Wang B, Chen Y, Lei D, Wang Y, Zhu B, Li L: **Characterization of fecal microbial communities in patients with liver cirrhosis.** *Hepatology* 2011, **54:**562–572.
15. Khan J, Pikkarainen P, Karvonen AL, Mäkelä T, Peräaho M, Pehkonen E, Collin P: **Ascites: aetiology, mortality and the prevalence of spontaneous bacterial peritonitis.** *Scand J Gastroenterol* 2009, **44:**970–974.
16. Tuomisto S, Karhunen PJ, Vuento R, Aittoniemi J, Pessi T: **Evaluation of postmortem bacterial migration using culturing and real-time quantitative PCR.** *J Forensic Sci* 2013, **58:**910–916.
17. Tuomisto S, Karhunen PJ, Pessi T: **Time-dependent post mortem changes in the composition of intestinal bacteria using real-time quantitative PCR.** *Gut Pathogens* 2013, **5:**35.
18. Brunk CF, Li J, Avaniss-Aghajani E: **Analysis of specific bacteria from environmental samples using a quantitative polymerase chain reaction.** *Curr Issues Mol Biol* 2002, **4:**13–18.
19. Furet JP, Firmesse O, Gourmelon M, Bridonneau C, Tap J, Mondot S, Doré J, Corthier G: **Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR.** *FEMS Microbiol Ecol* 2009, **68:**351–362.
20. Sen K, Asher DM: **Multiplex PCR for detection of Enterobacteriaceae in blood.** *Transfusion* 2001, **41:**1356–1364.
21. Pessi T, Karhunen V, Karjalainen PP, Ylitalo A, Airaksinen JK, Niemi M, Pietila M, Lounatmaa K, Haapaniemi T, Lehtimäki T, Laaksonen R, Karhunen PJ,

Mikkelsson J: **Bacterial signatures in thrombus aspirates of patients with myocardial infarction.** *Circulation* 2013, **127:**1219–1228.
22. Yang S, Lin S, Kelen GD, Quinn TC, Dick JD, Gaydos CA, Rothman RE: **Quantitative multiprobe PCR assay for simultaneous detection and identification to species level of bacterial pathogens.** *J Clin Microbiol* 2002, **40:**3449–3454.
23. Suzuki N, Yoshida A, Nakano Y: **Quantitative analysis of multi-species oral biofilms by TaqMan real-time PCR.** *Clin Med Res* 2005, **3:**176–185.
24. Tuominen VJ, Ruotoistenmaki S, Viitanen A, Jumppanen M, Isola J: **ImmunoRatio: a publicly available web application for quantitative image analysis of estrogen receptor (ER), progesterone receptor (PR), and Ki-67.** *Breast Cancer Res* 2010, **12:**R56.
25. Kakiyama G, Pandak WM, Gillevet PM, Hylemon PB, Heuman DM, Daita K, Takei H, Muto A, Nittono H, Ridlon JM, White MB, Noble NA, Monteith P, Fuchs M, Thacker LR, Sikaroodi M, Bajaj JS: **Modulation of the fecal bile acid profile by gut microbiota in cirrhosis.** *J Hepatol* 2013, **58:**949–955.
26. Hume DA, Ross IL, Himes SR, Sasmono RT, Wells CA, Ravasi T: **The mononuclear phagocyte system revisited.** *J Leukoc Biol* 2002, **72:**621–627.
27. Laskin DL, Weinberger B, Laskin JD: **Functional heterogeneity in liver and lung macrophages.** *J Leukoc Biol* 2001, **70:**163–170.
28. Levy E, Xanthou G, Petrakou E, Zacharioudaki V, Tsatsanis C, Fotopoulos S, Xanthou M: **Distinct roles of TLR4 and CD14 in LPS-induced inflammatory responses of neonates.** *Pediatr Res* 2009, **66:**179–184.
29. Adachi Y, Moore LE, Bradford BU, Gao W, Thurman RG: **Antibiotics prevent liver injury in rats following long-term exposure to ethanol.** *Gastroenterology* 1995, **108:**218–224.
30. Nanji AA, Khettry U, Sadrzadeh SM, Yamanaka T: **Severity of liver injury in experimental alcoholic liver disease. Correlation with plasma endotoxin, prostaglandin E2, leukotriene B4, and thromboxane B2.** *Am J Pathol* 1993, **142:**367–373.
31. Tamai H, Kato S, Horie Y, Ohki E, Yokoyama H, Ishii H: **Effect of acute ethanol administration on the intestinal absorption of endotoxin in rats.** *Alcohol Clin Exp Res* 2000, **24:**390–394.
32. Tabata T, Tani T, Endo Y, Hanasawa K: **Bacterial translocation and peptidoglycan translocation by acute ethanol administration.** *J Gastroenterol* 2002, **37:**726–731.
33. Garcia-Tsao G: **Spontaneous bacterial peritonitis.** *Gastroenterol Clin North Am* 1992, **21:**257–275.
34. Vilstrup H: **Cirrhosis and bacterial infections.** *Rom J Gastroenterol* 2003, **12:**297–302.
35. Terg R, Fassio E, Guevara M, Cartier M, Longo C, Lucero R, Landeira C, Romero G, Dominguez N, Muñoz A, Levi D, Miguez C, Abecasis R: **Ciprofloxacin in primary prophylaxis of spontaneous bacterial peritonitis: a randomized, placebo-controlled study.** *J Hepatol* 2008, **48:**774–779.
36. Madrid AM, Hurtado C, Venegas M, Cumsille F, Defilippi C: **Long-Term treatment with cisapride and antibiotics in liver cirrhosis: effect on small intestinal motility, bacterial overgrowth, and liver function.** *Am J Gastroenterol* 2001, **96:**1251–1255.
37. Brandl K, Plitas G, Mihu CN, Ubeda C, Jia T, Fleisher M, Schnabl B, DeMatteo RP, Pamer EG: **Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits.** *Nature* 2008, **455:**804–807.
38. Novella M, Solà R, Soriano G, Andreu M, Gana J, Ortiz J, Coll S, Sàbat M, Vila MC, Guarner C, Vilardell F: **Continuous versus inpatient prophylaxis of the first episode of spontaneous bacterial peritonitis with norfloxacin.** *Hepatology* 1997, **25:**532–536.
39. Campillo B, Dupeyron C, Richardet JP, Mangeney N, Leluan G: **Epidemiology of severe hospital-acquired infections in patients with liver cirrhosis: effect of long-term administration of norfloxacin.** *Clin Infect Dis* 1998, **26:**1066–1070.
40. Kirpich IA, Solovieva NV, Leikhter SN, Shidakova NA, Lebedeva OV, Sidorov PI, Bazhukova TA, Soloviev AG, Barve SS, McClain CJ, Cave M: **Probiotics restore bowel flora and improve liver enzymes in human alcohol-induced liver injury: a pilot study.** *Alcohol* 2008, **42:**675–682.
41. Leber B, Spindelboeck W, Stadlbauer V: **Infectious complications of acute and chronic liver disease.** *Semin Respir Crit Care Med* 2012, **33:**80–95.
42. Hartmann P, Chen WC, Schnabl B: **The intestinal microbiome and the leaky gut as therapeutic targets in alcoholic liver disease.** *Front Physiol* 2012, **3:**402.
43. Hayashi H, Sakamoto M, Benno Y: **Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods.** *Microbiol Immunol* 2002, **46:**535–548.
44. Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA: **Diversity of the human intestinal microbial flora.** *Science* 2005, **308:**1635–1638.
45. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI: **A core gut microbiome in obese and lean twins.** *Nature* 2009, **457:**480–484.

doi:10.1186/1471-230X-14-40
Cite this article as: Tuomisto *et al.*: Changes in gut bacterial populations and their translocation into liver and ascites in alcoholic liver cirrhotics. *BMC Gastroenterology* 2014 **14**:40.