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ORIGIN OF INTRARADICULAR INFECTION WITH ENTEROCOCCUS FAECALIS IN ENDODONTICALLY TREATED TEETH

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Origin of intraradicular infection with *Enterococcus faecalis* in endodontically treated teeth

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Victor (5 years old) imagines an *Enterococcus* dwelling in grass

To my family

Everything is possible if you just believe in yourself…then others will believe in you

ABSTRACT

The reported prevalence of *Enterococcus faecalis*, both a commensal of the gastro-intestinal tract and a common nosocomial pathogen, ranges from 24% to 77% in post-treatment root canal infections. To date it has not been possible to explain this prevalence, since its origin remains unknown. Its exceptional array of intrinsic and easily acquired traits, including resistance to a multitude of antibiotics, enables an adaptation to a wide variety of different environmental settings and poses a challenge in treatments.

The aim of the research was to elucidate the origin of *E. faecalis* in root canal infections to enable a means of preventing costly and time-consuming treatment failures. The potential for acquisition from the endogenous flora, a nosocomial transmission from contaminated surfaces during a root canal procedure and a food-borne route of infection were evaluated by measuring its occurrence on environmental surfaces, comparing genetic relatedness, distribution of putative virulence factors and antibiotic resistance between isolates from different sources.

DNA fingerprinting by PFGE concluded that *E. faecalis* retrieved from eight (16%) secondary root canal infections in 50 consecutively treated patients were genetically unrelated to those recovered from the patients' own intestinal tract. *E. faecalis* could not be retrieved from any of the saliva samples pertaining to the patients with the microorganism in the root canal sample, validating its transient presence in the oral cavity. Analysis of a total of 320 collected samples from 10 high-touch surfaces in six general dentistry clinics and two specialist clinics displayed a very low occurrence (0.9%) of *E. faecalis* on surfaces despite clear deficiencies in decontamination procedures. Determination of the distribution of putative virulence genes and susceptibility to clinically relevant antibiotics amongst strains isolated from root canals, foods, stool and blood culture samples by PCR and the agar dilution method, respectively, detected an association between endodontic isolates and isolates from food and stool based on a common gene pattern, consisting of *gel*E, *efa*A and *gel*E. The linkage could be corroborated by MLST analysis, demonstrating that 66.7% of the root canal isolates, 42.1% of the food strains, 34.5% of the stool isolates but only 10% of the blood isolates shared genetic lineages. Correlation of detected virulence determinants to MLST data revealed distinctive features of the resulting major genetic lineages. All isolates in CC25 were impaired to express gelatinase and all strains in CC6 lacked the gene *ace* but were enriched with antibiotic resistance and the ability to express cytolysin.

In conclusion, *E. faecalis* in root canal infections is most likely not derived from the endogenous flora or nosocomially transmitted but instead food-borne. It presumably gains access to the treated canals via micro-leakage, which stresses the need for better ways of sealing endodontically treated teeth.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-IV).

I. **Vidana R**, Billström H, Sullivan Å, Ahlquist M, Lund B (2011) *Enterococcus faecalis* infection in root canals – host-derived or exogenous source? *Letters in Applied Microbiology* **52**, 109-15.

II. **Vidana R**, Sillerström E, Ahlquist M, Lund B (2014) Potential for nosocomial transmission of *Enterococcus faecalis* from surfaces in dental operatories. *International Endodontic Journal* [Epub ahead of print; doi:10.1111/iej.12342].

III. **Vidana R**, Rashid MU, Özenci V, Weintraub A, Lund B (2015) The origin of endodontic *Enterococcus faecalis* explored by comparison of virulence factor patterns and antibiotic resistance to that of isolates from stool samples, blood cultures and food. *International Endodontic Journal* [In press].

IV. **Vidana R**, Rashid MU, Özenci V, Weintraub A, Lund B (2015) Endodontic infection with *Enterococcus faecalis* – MLST indicates a food-borne route of transmission [Manuscript].

CONTENTS

LIST OF ABBREVIATIONS

Ace	*Enterococcus faecalis* adhesin to collagen
API	Analytical profile index
Asa1	Aggregation substance
ATCC	American Type Culture Collection
bp	Base pair
CC	Clonal complex
CCUG	Culture Collection University of Gothenburg
CHEF	Contour-clamped homogenous electric field
CFU	Colony-forming units
CLSI	Clinical and Laboratory Standards Institute
CoNS	Coagulase-negative *Staphylococcus*
CylA	Cytolysin activator
Ddl	$_{D}$-alanine $_{D}$-alanine ligase
DLV	Double locus variant
DNA	Deoxyribonucleic acid
eBURST	Based upon related sequence types
EDTA	Ethylenediaminetetraacetic acid: ligand and chelator
EfaA	*Enterococcus faecalis* antigen A
Esp	Enterococcal surface protein
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
FAB	Fastidious anaerobe broth
fsr	*Enterococcus faecalis* regulator
GelE	Gelatinase
GRAS	Generally recognized as safe
h	Hours
HiRECC	High-risk enterococcal clonal complex
HLGR	High-level gentamicin-resistance
kb	Kilo base pair
M	Molar

MGE	Mobile genetic elements
MIC	Minimum inhibitory concentration
min	Minutes
MLST	Multilocus sequence typing
MRSA	Methicillin-resistant *Staphylococcus aureus*
MQ	MilliQ-water (ultrapure water)
n	Number
NaCl	Sodium chloride
ND	Not detected
PAI	Pathogenicity island
PBP	Penicillin binding protein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SLV	Single locus variant
*Sma*I	Restriction endonuclease derived from *Serratia marcescens*
spp.	Species
ST	Sequence type
TAE	Buffer solution: Tris/acetic acid/EDTA
TBE	Buffer solution: Tris/boric acid/EDTA
Tris	Tris-hydroxymethyl-aminomethane
VMG	Viable medium Gothenburg
VMGA	Viable medium Gothenburg anaerobically prepared and sterilized
VRE	Vancomycin-resistant *Enterococcus*
WGS	Whole genome sequencing

1 INTRODUCTION

1.1 HISTORICAL PERSPECTIVE

Enterococcus faecalis is a lactic acid bacterium that until 1984 was classified as a group D *Streptococcus* according to the Lancefield system of grouping based on serology (Lancefield 1933, Sherman 1937). The misclassification was based on the difficulty in distinguishing enterococci from streptococci on physical characteristics alone, since both are gram-positive cocci, which grow in pairs or chains. *E. faecalis* was thus, since its first description in 1906, termed *Streptococcus faecalis* or "*Streptococcus* of faecal origin", as it was often recovered from faecal matter or sewage (Andrewes & Horder 1906). It took almost eighty years, before the genus *Enterococcus*, with the advent of refined molecular techniques and genetic evidence, finally could be established and accepted (Schleifer & Kilpper-Bälz 1984).

1.2 THE *ENTEROCOCCUS* GENUS

The taxonomy of enterococci is subject to a continuous number of changes as the methodology of identification and differentiation evolves in combination with a growing interest in the genus. The identification of species is currently mostly based on DNA-DNA reassociation, 16S rRNA gene sequencing and whole-cell protein profiling analysis. To date, the genus is considered to comprise 49 species, listed in Table 1, according to the List of Prokaryotic Names with Standing in Nomenclature retrieved 2015-02-28 (Euzéby 1997; http://www.bacterio.cict.fr/e/enterococcus.html).

Table 1. List of species currently included in the *Enterococcus* genus.

E. alcedinis	*E. columbae*	*E. haemoperoxidus*	*E. pallens*	*E. silesiacus*
E. aquimarinus	*E. devriesei*	*E. hermanniensis*	*E. phoeniculicola*	*E. sulfureus*
E. asini	*E. diestrammenae*	*E. hirae*	*E. plantarum*	*E. termitis*
E. avium	*E. dispar*	*E. italicus*	*E. pseudoavium*	*E. thailandicus*
E. caccae	*E. durans*	*E. lactis*	*E. quebecensis*	*E. ureasiticus*
E. camelliae	*E. eurekensis*	*E. lemanii*	*E. raffinosus*	*E. ureilyticus*
E. canintestini	*E. faecalis*	*E. malodoratus*	*E. ratti*	*E. viikkiensis*
E. canis	*E. faecium*	*E. moraviensis*	*E. rivorum*	*E. villorum*
E. casseliflavus	*E. gallinarum*	*E. mundtii*	*E. rotai*	*E. xiangfangensis*
E. cecorum	*E. gilvus*	*E. olivae*	*E. saccharolyticus*	

1.3 NATURAL HABITATS AND RESERVOIRS

Enterococci are encountered in nearly everything we come in contact with. The ubiquitous presence is attributed to its robust and hardy nature in combination with an exceptional adaptive skill, since the microorganism is specialized to thrive in the harsh environment of the gastrointestinal tract of humans along with other mammals, birds, reptiles and insects (Mundt 1963, Martin & Mundt 1972). Enterococci are consequently present as faecal contaminants in fresh and marine waters, sediments, soil, aquatic and terrestrial plants as well as man-made products, including dairy products and fermented foods.

Widely distributed enterococcal species that have been implicated in human infections, mainly consist of *E. faecalis* and *E. faecium* but also *E. casseliflavus*, *E. gallinarium*, *E. durans, E. hirae, E. mundtii*, *E. avium, E. pseudoavium, E. malodoratus and E. raffinosus* (Mundy *et al.* 2000).

1.4 CHARACTERISTICS

E. faecalis is a Gram-positive spherical or ovoid cell that occurs singly, in pairs or chains of various lengths. The species is catalase-reaction negative, although it may occasionally produce a pseudo-catalase when grown on blood-containing media. The reaction is however weak and therefore easy to disregard. The microorganism is a facultative anaerobe able to catabolize a variety of energy sources with the metabolic end product always being lactic acid. It typically grows in temperatures ranging from 10°C to 45°C but exhibits optimal growth at 35°C (Sherman 1937).

Enterococci are renowned for their ruggedness and capability to endure extreme conditions. *E. faecalis* can resist oxidative stress, disinfectants, heavy metals, ethanol, sodium azide and persist desiccation for weeks or even up to months (Kearns *et al.* 1995, Bradley & Fraise 1996, Flahaut *et al.* 1998, Kramer *et al.* 2006, Howie *et al.* 2008). Furthermore it survives heating at 60°C for 30 minutes or 65°C for 10 minutes (Freeman *et al.* 1994, Bradley & Fraise 1996). Another important differentiating characteristic is that it readily grows at 6.5% NaCl concentrations and in highly acid or alkaline conditions at pH 4.0 to 9.6. *E. faecalis* also possesses the ability to hydrolyse leucin-pyrrolidonyl-β-naphthylamide (PYR) and esculin in the presence of 40% bile salts (Facklam *et al.* 2002).

1.5 VIRULENCE

E. faecalis was for a long time regarded as medically unimportant, being a constituent of the commensal intestinal flora, generally displaying low virulence. Nonetheless, *E. faecalis* commonly causes endocarditis, bacteraemias, urinary tract infections and surgical or deep wound infections, and is ranked among the leading causes of nosocomial (healthcare-associated) infections worldwide (de Kraker *et al.* 2013, Sievert *et al.* 2013). Its pathogenic potential has been ascribed to its exceptional ability to intrinsically resist antimicrobial agents, acquire and disseminate determinants of antibiotic resistance and most importantly, to adapt to changing environments (Arias & Murray 2012).

Infections with *E. faecalis* generally affect hospitalized patients on broad-spectrum antibiotics or patients mechanically compromised by catheters for instance, which underlines the opportunistic character of the bacterium. Its transition from commensal to pathogen is far from being completely understood. Although several putative virulence factors, as listed in Table 2, have been proposed and shown to be of importance for pathogenicity in murine models, they have yet not been confirmed to play a major role in human infections and mortality, possibly with the exception of cytolysin (Huycke *et al.* 1991, Vergis *et al.* 2002).

Table 2. Major putative virulence determinants proposed to be of importance for *E. faecalis* pathogenicity.

Determinant (gene)	Putative functions
Secreted factors	
Cytolysin (*cyl*A-M)	• Bacteriocidal action against a broad range of Gram-positive bacteria (Davie & Brock 1966) • Lysis of erythrocytes, polymorphonuclear neutrophils (PMN) and macrophages (Miyazaki *et al.* 1993) • Increased virulence in intraperitoneally infected mice (Ike *et al.* 1984)
Gelatinase (*gel*E)	• Biofilm formation (Hancock & Perego 2004, Mohamed *et al.* 2004) • Role in pathogenesis of apical periodontitis and experimental endocarditis, peritonitis (Singh *et al.* 1998, Thurlow *et al.* 2010, Zoletti *et al.* 2011) • Degrades gelatin, endothelin, hemoglobin, fibrinogen, fibronectin, collagen, laminin, immunoglobulins and complement proteins (Mäkinen *et al.* 1989) • Promotes translocation across intestinal wall (Zeng *et al.* 2005)

Continued on next page

Table 2 continued. Major putative virulence determinants proposed to be of importance for *E. faecalis* pathogenicity.

Determinant (gene)	Putative functions
Cell-surface associated factors	
Adhesin to collagen of *E. faecalis* (*ace*)	• Mediates adhesion to collagen (type I, IV), laminin and dentin (Nallapareddy *et al.* 2000, Hubble *et al.* 2003) • Role in conferring resistance to disinfectants (Kayaoglu *et al.* 2008) • Involved in pathogenesis of endocarditis and urinary tract infection (Koch *et al.* 2004, Singh *et al.* 2010, Nallapareddy *et al.* 2011)
Aggregation substance (*asa*1, *asp*1, *asc*10)	• Pheromone-inducible protein promoting aggregation and facilitating genetic exchange by conjugation (Clewell 1993) • Adhesion to renal, heart endothelial, intestinal endothelial, endocardial cells and collagen component of dentin (Guzman *et al.* 1989, Kreft *et al.* 1992, Sartingen *et al.* 2000, Rozdzinski *et al.* 2001) • Resistance to killing by macrophages/polymorphonuclear neutrophils despite promoted adherence and phagocytosis (Rakita *et al.* 1999, Süssmuth *et al.* 2000)
Capsular polysaccharide (*epa*, *cps*)	• Resistance to phagocytosis and evasion of host defence (Hancock & Gilmore 2002) • Biofilm formation and translocation across intestinal wall (Mohamed *et al.* 2004, Zeng *et al.* 2004, Teng *et al.* 2009)
E. faecalis antigen A (*efa*A)	• Adhesin of importance for endocarditis (Lowe *et al.* 1995) • Modulates virulence (Abrantes *et al.* 2013)
Enterococcal surface protein (*esp*)	• Adhesion, colonization and immune evasion (Shankar *et al.* 1999) • Adherence to renal cells promoting urinary tract infection (Shankar *et al.* 2001) • Biofilm formation (Toledo-Arana *et al.* 2001, Tendolkar *et al.* 2004)

1.6 ANTIBIOTIC RESISTANCE

The clinical importance of *E. faecalis* is intimately linked to its antibiotic resistance, which contributes to the risk of colonization and infection. *E. faecalis* is naturally resistant to low-levels of β-lactam antibiotics and aminoglycosides, as well as to high-levels of lincosamides (clindamycin) and the combination of streptogramins used for treatment of glycopeptide-resistant *E. faecium*, namely quinupristin-dalfopristin. In addition, the microorganism can easily acquire and disseminate resistance to a multitude of clinically relevant antimicrobial substances. Consequently, the treatment of infections with *E. faecalis* is often complicated, especially for severe conditions, which due to intrinsic resistances require a synergistic effect of combined antibiotics.

1.6.1 β-lactam resistance

β-lactam antibiotics are all antimicrobial substances that incorporate a β-lactam ring in their molecular structure. This broad class of antibiotics comprises penicillins and their derivates, carbapenems and cephalosporins. They exert a bactericidal effect by covalent binding to and blocking of proteins, termed penicillin-binding proteins (PBP), involved in the synthesis and assembly of the cell wall peptidoglycan layer (Zapun *et al.* 2008). In most cases, the subsequent disruption of cell wall production results in programmed cell death via the creation of reactive oxygen species (Kohanski *et al.* 2007).

The mechanism responsible for the intrinsic resistance in *E. faecalis* to β-lactams, resulting in minimum inhibitory concentrations (MICs) that are 10- to 100-fold higher than for streptococci, is the expression of low affinity PBPs (PBP4), which bind weakly to penicillins, carbapenems and especially cephalosporins (Murray 1990).

E. faecalis can acquire a high-level resistance, by overproduction of PBP4s, point mutations that further lessen the affinity of PBP4 or, rarely, the procurement of genes (*bla*) encoding for β-lactamase (Murray 1992, Duez *et al.* 2001, Ono *et al.* 2005). Fortunately, the occurrence of resistance to ampicillin, being the treatment of choice for enterococcal infections lacking other mechanisms for high-level resistance, is quite rare in *E. faecalis*, as opposed to *E. faecium* clinical isolates (Kristich *et al.* 2014)

1.6.2 Aminoglycoside resistance

Aminoglycosides inhibit bacterial protein synthesis by binding to the 16S rRNA of the 30S ribosomal subunit and are used for synergistic therapy of serious enterococcal infections.

E. faecalis typically exhibits an intrinsic low to moderate aminoglycoside resistance due to a low cell wall permeability. Therefore, aminoglycosides are to be combined with a cell wall-active compound, either a β-lactam or a glycopeptide, when used for treatment of complicated enterococcal infections (Moellering *et al.* 1971). High-level aminoglycoside resistance, usually conveyed on mobile genetic elements, is thus of concern.

Aminoglycoside modifying enzymes, including phosphotransferases, acetyltransferases and nucleotidyltransferases, alter the structure of the compound and thereby prevent it from binding to its target on the 30S ribosomal subunit (Ferretti *et al.* 1986, Chow 2000). Another possibility for a high-level resistance, as shown to streptomycin, is through an alteration on the ribosomal subunit itself, induced by single mutations (Chow 2000).

1.6.3 Glycopeptide resistance

The glycopeptide vancomycin is an important antibiotic and considered to be a last resort medication for treatment of infections with multi-resistant enterococci and methicillin-resistant *Staphylococcus aureus* (MRSA). The first report of resistance to glycopeptides appeared in 1986 (Leclercq *et al.* 1986). Since then, resistance to vancomycin has become

widespread and an increasing problem in clinical settings. To date, vancomycin resistance is widely prevalent in *E. faecium*, but remains relatively rare in *E. faecalis* (Sievert *et al.* 2013).

Vancomycin inhibits cell wall synthesis by interacting with the $_D$-alanyl-$_D$-alanine ($_D$-Ala-$_D$-Ala) C-terminus of late peptidoglycan precursors, thereby preventing the formation of cross-links between peptide side chains (Reynolds 1989).

Acquired resistance to glycopeptides is mediated by synthesis of altered peptidoglycan precursors to which the antibiotic is unable to bind. Consequently, the C-terminal $_D$-Ala is replaced by $_D$-lactate ($_D$-Lac) or $_D$-serine ($_D$-Ser). So far, four resistance genotypes, namely vanA, vanB, vanE and vanG, have been described for *E. faecalis* (Gholizadeh & Courvalin 2000). The genotypes differ in transferability and inducible resistance levels as shown in Table 3. The genotypes that present a serious challenge in clinical settings are vanA and vanB, and particularly vanA, since it mediates a high-level resistance to both vancomycin and teicoplanin. Both genotypes are easily transferable by transposons and their operons are located on plasmids, as opposed to vanE and vanG that thus far have only been found to be located on the chromosome (Abadía Patiño *et al.* 2002, Depardieu *et al.* 2003, Courvalin 2006).

Table 3. Acquired glycopeptide resistance in *E. faecalis.* Adapted from Courvalin 2006.

	vanA	**vanB**	**vanE**	**vanG**
Inducible resistance level	High	Variable	Low	Low
Vancomycin MIC (mg/L)	64-1000	4-1000	8-32	16
Teicoplanin MIC (mg/L)	16-512	0.5-1	0.5	0.5
Alteration of peptidoglycan precursor	$_D$-Ala-$_D$-Lac	$_D$-Ala-$_D$-Lac	$_D$-Ala-$_D$-Ser	$_D$-Ala-$_D$-Ser
Location of operon	Plasmid	Plasmid	Chromosome	Chromosome
Transferability by conjugation	Positive	Positive	Negative	Positive
Mobile element	Tn*1546*	Tn*1547* / Tn*1549*		

1.6.4 Oxazolidinone resistance

Linezolid, which is an important and fully synthetic class of antibiotics, is used for treatment of vancomycin-resistant enterococci (VRE). Although resistance to linezolid in *E. faecalis* has been documented, it still remains rare.

Oxazolidinones exert a bacteriostatic effect by interfering with the translational initiation complex and preventing the placement of the aminoacyl-tRNA at the A site of the ribosome, thereby inhibiting protein synthesis (Wilson *et al.* 2008). The most common resistance

mechanism in enterococci involves point mutations in the genes encoding domain V of 23S rRNA, resulting in a distorted target site for oxazolidinone (Leach *et al.* 2007).

1.6.5 Fluoroquinolone resistance

Ciprofloxacin is the most commonly used fluoroquinolone in clinical settings. It is mainly active against Gram-negative bacteria but also displays a moderate activity against enterococci, which is a motivation for its use in complicated urinary tract infections (Landman & Quale 1997).

Fluoroquinolones interfere with bacterial DNA replication by interacting with type II topoisomerases (topoisomerase IV and DNA gyrase) that control the supercoiling of DNA and thus results in lethal double-strand breaks (Shen *et al.* 1989). Resistance to quinolones occurs via mutations in the genes that encode gyrase and topoisomerase IV, hindering an effective bond to the antibiotic (Kanematsu *et al.* 1998).

1.7 HORIZONTAL GENE TRANSFER

A key feature that enables *E. faecalis* to quickly and successfully adapt to a plethora of different ecological niches, besides its resilient nature, is its extraordinary ability to acquire and disseminate genetic elements, encoding virulence determinants and antibiotic resistance, through horizontal gene transfer. As such, the genome of *E. faecalis* shows signs of exceptional plasticity with a remarkably high content of foreign DNA in mobile genetic elements (MGE). Extensive genomic mapping of the *E. faecalis* strain V583 for instance, revealed that that as much as 25% of the genome consisted of exogenously acquired genetic elements (Paulsen *et al.* 2003).

Genetic exchange in enterococci occurs by conjugation and in some instances by transduction involving bacteriophages (Mazaheri Nezhad Fard *et al.* 2011). Horizontal gene transfer by transformation, which is the uptake and integration of exogenous free DNA from the surroundings, has to date not been shown for enterococci. The conjugative transfer of genes and chromosomal DNA is mainly mediated by pheromone-responsive plasmids, which involve the expression of aggregation substance, and non-responsive plasmids, as well as by highly promiscuous transposons or transposable elements (TE) that are usually integrated in the chromosome (Clewell *et al.* 2014). The pheromone-independent plasmids and conjugative transposons with a broad host range, as opposed to the pheromone-responsive plasmids, have been shown to convey genes not only to other enterococcal species but also to other genera. Most alarmingly is the transfer of vancomycin resistance from *E. faecalis* to MRSA, rendering one of the few classes of agents still active against multi-resistant *Staphylococcus aureus* ineffective (Weigel *et al.* 2003).

Clinical isolates tend to harbour several MGE and specifically plasmids, which often, besides from antibiotic resistance genes, contain a pathogenicity island (PAI), believed to contribute to the evolution of non-pathogenic strains into pathogenic forms. The PAI encompasses several genes coding for virulence traits, among them cytolysin, aggregation substance,

enterococcal surface protein, stress proteins and factors altering the relationship with the host (Shankar *et al.* 2002). The genomic plasticity of *E. faecalis* is also reflected in the PAI, as it continuously evolves by addition and deletion of genes, and through recombination, resulting in a high genetic variation between strains mostly independent of clonal lineages (McBride *et al.* 2009). The pheromone-responsive exchange system, which besides conveying PAI also transfers antibiotic resistant determinants and portions of chromosomal DNA, is therefore an important driver of the genomic plasticity in *E. faecalis* (Manson *et al.* 2010).

1.8 THE DUAL NATURE OF *E. FAECALIS* – COMMENSAL AND OPPORTUNISTIC PATHOGEN

1.8.1 Commensal in the intestinal microflora

The commensal intestinal flora, constituted by a complex and diverse community of microbes in concert with each other and the host, plays a pivotal role by regulating and shaping the host's immune system, providing resistance to colonization by pathogenic or probiotic microbes, synthesizing essential vitamins and processing substrates otherwise indigestible by the host (Lozupone *et al.* 2012, Kamada & Nunez 2014). The role of *E. faecalis* in the intestinal consortium remains to be determined. The species has been implicated in colonization resistance due to its production of potent bacteriocins and superoxide, and is believed to be of importance for metabolism (Giraffa 1995, Huycke & Moore 2002). However, its part in these functions is probably minute, since *E. faecalis* together with other enterococcal species constitute a minority in the vast intestinal population, specifically less than 1% of the intestinal flora (Sghir *et al.* 2000).

The predominant enterococcal species in the human normal flora is *E. faecalis* (Qin *et al.* 2010). However, a shift in the normal intestinal flora to a predominance of *E. faecium* over *E. faecalis* has been observed in some individuals and some countries (Ruoff 1990, Devriese & Pot 1995, Layton *et al.* 2010). The most commonly encountered enterococcal species in the gut of animals, bird and insects are also *E. faecalis* and *E. faecium* (Martin & Mundt 1972, Lebreton *et al.* 2014). As such, the intestinal tract of both humans and animals constitute important reservoirs for *E. faecalis*. The prevailing previous belief was that infections with *E. faecalis* were endogenously acquired from the resident commensal flora (Kaye 1982, Wells *et al.* 1988, Alexander *et al.* 1990). The advent of clinical studies that demonstrated a transfer of enterococcal strains, often clones, and interchangeable genetic elements amongst strains between patients in hospital settings changed this assumption (Jett *et al.* 1994, Weinstein *et al.* 1996). Nowadays, there is compelling evidence supporting the idea that exogenously acquired strains, differing from commensal strains in the capabilities to persist, adapt and invade, are the cause for enterococcal infections, either directly or indirectly by a dissemination of virulence determinants and antibiotic resistance to the resident population (Arias & Murray 2012). Hence, commensal *E. faecalis* strains are generally regarded to possess low virulence and antibiotic resistance, although its genomic plasticity allows it to rapidly obtain and circulate virulence determinants and antibiotic resistance (Mundy *et al.* 2000, Lempiäinen *et al.* 2005).

The established commensal flora in an individual is assumed to remain stable over time, unless the balance is disturbed, most often by antibiotic therapy, severe underlying disease or dietary changes (Turnbaugh *et al.* 2009, Wu *et al.* 2011, Faith *et al.* 2013). The disruption of homeostasis as a result of antibiotic treatment may have a profound and long lasting effect on the composition of the flora and subsequently the host, often detrimental since it enables the colonization of pathogens or possibly the transformation of commensals to pathogens (Berg 1996, Donskey 2004, Jernberg *et al.* 2007). It is in this scenario that the traits of *E. faecalis* become useful taking precedence over less adaptive microorganisms, resulting in overgrowth and the potential for subsequent systemic infection after translocation across the intestinal barrier (Ubeda *et al.* 2010).

1.8.2 Nosocomial pathogen

E. faecalis accounts together with *E. faecium* for approximately 90% to 95% of all enterococcal infections, with a ratio of nearly 3:1. Previously, the ratio was 10:1 but this changed in the last decade due to the frequent resistance to vancomycin, ampicillin and high levels of aminoglycosides in *E. faecium* (Top *et al.* 2007). Thus it appears that *E. faecium* is highly reliant on antibiotic resistance to cause infections, whereas *E. faecalis* has a greater potential to do the same regardless of resistance (Huycke *et al.* 1998, Mundy *et al.* 2000). Consequently, the impressive intrinsic traits, in combination with a high potential to rapidly acquire virulence determinants and antibiotic resistance have enabled *E. faecalis* to successfully adapt and survive in various environments, including hospital settings.

E. faecalis is capable of dwelling on dry environmental surfaces, such as bed rails and door handles for up to four months (Kramer *et al.* 2006). Additionally, it is able to endure extreme temperatures and suboptimal concentrations of chemical disinfectants, such as chlorine, glutaraldehyde and ethanol (Freeman *et al.* 1994, Kearns *et al.* 1995, Bradley & Fraise 1996). *E. faecalis* is therefore likely to be transmitted from patient to patient via the hands of healthcare workers or insufficiently disinfected medical equipment (Hayden 2000). The use and likely overuse of antimicrobial substances in debilitated patients gives the species an additional advantage and facilitates colonization and subsequent infection. *E. faecalis* has accordingly since the late 1970s, emerged as one of the four most commonly isolated nosocomial pathogens worldwide, coinciding with the introduction of third-generation cephalosporins to which the microorganism is intrinsically resistant (Murray 1990, de Kraker *et al.* 2013, Sievert *et al.* 2013).

Epidemiological typing of nosocomially transmitted *E. faecalis*, implicated in infections of the bloodstream, urinary tract, endocardium, abdomen, pelvic, skin and soft tissues, have revealed highly hospital-adapted clones causing global outbreaks by replacing the previously dominating heterogeneous and antibiotic-susceptible populations. The reported most prevalent clonal complex (CC) among hospitalized patients is CC6 (formerly known as CC2), followed by CC9 and CC28-ST87 (Kuch *et al.* 2012). These high-risk enterococcal clonal complexes (HiRECCs) are characterized by resistance to multiple antibiotics and high levels

of genetic recombination, presumably resulting in an increased number of virulence traits and transmissibility (Leavis *et al.* 2006).

1.8.3 Root canal infections

Root canal treatment or endodontic treatment is performed when the dental pulp is severely inflamed, necrotic and infected or the root canal space is needed for retention of a coronal restoration when too little tooth structure remains. The time-consuming and therefore costly procedure consists of the meticulous cleaning and shaping of the root canal system with flexible files, aided by irrigation with disinfectants and dentin conditioners. When the treatment is not finalized in one session, an inter-appointment dressing with calcium hydroxide, which exerts an antibacterial and proteolytic effect, is placed in the root canals and sealed off with a temporary filling in the coronal portion of the tooth. The treatment is subsequently completed with the filling or obturation of the treated root-canal space to prevent reinfection, where after a restoration of the tooth crown takes place, involving additional costs. Moreover, endodontic treatment frequently forms the foundation for more elaborate prosthodontic constructions, restoring or replacing several damaged or lost teeth. Hence, the consequences of a failed endodontic treatment are often detrimental for the patient.

The aetiology behind endodontic treatment failures is mainly a persisting infection in the root canal system, as a result of microorganisms from a primary infection surviving the chemo-mechanical treatment, or the establishment of a new infection, possibly during a treatment session or after the treatment has been completed. A microorganism that has been intimately associated with treatment failures is *E. faecalis*, since it is frequently recovered from infected endodontically treated teeth and is supposedly able to resist an endodontic treatment, including the high pH exercised by a inter-appointment dressing containing calcium hydroxide (Molander *et al.* 1998). Its prevalence in root-filled teeth ranges between 24% and 70% in studies utilizing culture-based techniques of detection (Engström 1964, Möller 1966, Molander *et al.* 1998, Sundqvist *et al.* 1998, Peciuliene *et al.* 2000, Peciuliene *et al.* 2001, Hancock *et al.* 2001, Pinheiro *et al.* 2003) and between 66% and 77% when molecular methods were applied (Rôças *et al.* 2004, Siqueira & Rôças 2004). At the same time, *E. faecalis* is only occasionally retrieved from untreated teeth with an endodontic infection or saliva samples. Microbiological studies on primary endodontic infections, using both culture and molecular methods for detection, report a median prevalence of 9% (range 4-40%) for *E. faecalis* (Rôças *et al.* 2004). The prevalence in oral rinse samples is equally low, at most 11%, which is in line with the recent understanding that *E. faecalis* is not a commensal in the oral cavity but merely transient in the oral flora (Sedgley *et al.* 2004, Aas *et al.* 2005). As such, the origin of enterococci in endodontic infections has remained unanswered.

Several traits have been proposed to enhance the ability of *E. faecalis* to persist in the nutrition and oxygen-depraved milieu of the root canal, and elicit inflammatory reactions around the root end, termed apical periodontitis (Table 2). Adherence factors, such as *Enterococcus faecalis* antigen A (*efa*A), aggregation substance and adhesin to collagen (*ace*)

are probably of importance in the colonization of the root canal space (Sedgley *et al.* 2005). Particularly, the putative virulence factors enterococcal surface protein (*esp*) and gelatinase (*gel*E) have been emphasized in the pathogenesis of apical periodontitis by promoting biofilm formation and aggravating tissue damage and bone resorption around the root end (Ramamurthy *et al.* 2002, Wang *et al.* 2011, Zoletti *et al.* 2011). Interestingly, cytolysin does not seem to be of great importance in endodontic infections with *E. faecalis* (Sedgley *et al.* 2005, Penas *et al.* 2013). The most important characteristics though, needed for survival in root filled canals for long periods of time, is likely the intrinsic resistance to disinfectants and endodontic medicaments in combination with the ability to down-regulate metabolic activity, entering a dormant or even viable but non-cultivable (VBNC) state (Lleò *et al.* 2001, Castellani *et al.* 2013).

1.8.4 Adjunct in food production

Enterococci are considered to be a part of the usual microflora in dairy products, such as different types of cheeses, and fermented foods, ranging from charcuteries and sausages to olives (Foulquié Moreno *et al.* 2006). They are an important adjunct in the fermentation process of certain products, where they contribute to the ripening and organoleptic properties, possibly through lipolytic activities, casein degradation, citrate utilization and production of aromatic volatile compounds (Giraffa 2003). Furthermore, enterococci have been accredited biopreservative properties by the production of bacteriocins, which are secreted antibacterial peptides with activity against phylogenetically related bacteria, such as *Listeria monocytogenes* (Giraffa 1995, Franz *et al.* 1999). Enterococci are thus occasionally deliberately added as adjuncts in starter cultures or are, more commonly, present in food items as contaminants, either in raw foods as faecal contamination from animals, or in later stages of production as contamination resulting from human handling (Gelsomino *et al.* 2002). Consequently, fermented food products usually contain species such as *E. faecalis*, *E. faecium*, and occasionally also *E. durans*, *E. casseliflavus* and *E. hirae* (Franz *et al.* 1999).

Besides their assumed beneficial roles, enterococci pose a huge problem for the food industry. Being among the most thermo-tolerant of the non-sporulating bacteria, enterococci have been implicated in spoilage of cooked and processed foods, specifically when "reworking" has occurred, meaning that material from faulty products is reused in the manufacturing process (Franz *et al.* 1999). More importantly, enterococci, in contrary to other lactic acid bacteria, have not attained the status of "Generally Recognized As Safe" (GRAS). Although *E. faecalis* and *E. faecium* present a higher incidence of putative virulence factors and antibiotic resistance among clinical strains, these traits have also been detected in food-associated isolates (Eaton & Gasson 2001, Franz *et al.* 2001, Mannu *et al.* 2003, Semedo *et al.* 2003, Creti *et al.* 2004, Lepage *et al.* 2006). In this context, a reported high incidence of aggregation substance in *E. faecalis* food isolates, has evoked great concern, since it may enable a conjugative transfer of antibiotic resistance and virulence determinants to commensals in the digestive tract (Eaton & Gasson 2001, Huycke 1992, Licht *et al.* 2002). As such, the permanent or transient human carriage of food-borne and potentially pathogenic

strains of animal or environmental origin seriously questions the safety of food products containing enterococci (Franz *et al.* 2003). Nevertheless, the dissemination of multi-resistant and pathogenic enterococci among humans via the food chain has yet to be clearly proven (Ogier & Serror 2008).

1.9 EPIDEMIOLOGICAL TYPING METHODS

The need for novel and more accurate methods for epidemiological surveillance, to prevent and control infection, followed the increasing number of reports on hospital outbreaks with *E. faecalis* and *E. faecium* in the late 1970s. Until then, distinction between different isolates was heavily dependent on phenotypic characteristics, such as serotype, biotype or phagetype. A method to separate large DNA fragments by electrophoresis utilizing an alternating voltage gradient, known as a pulsed-field gel electrophoresis (PFGE), was therefore developed at Columbia University in 1984 (Schwartz & Cantor 1984). The method enabled the detection of genetic variations, and thus genetic relatedness, between strains by analysis of fragment patterns or macro-restriction profiles obtained by digestion of the genome with restriction endonucleases, such as *Sma*I. The method rapidly gained popularity and became "the gold standard" for subspecies typing, allowing for tracing of geographical dissemination and source. PFGE was thus decisive in revealing that hospital-adapted enterococcal clones were transmitted between patients via health-care workers or contaminated equipment, causing hospital outbreaks, not only within but also between hospitals (Bonten *et al.* 1996, Austin *et al.* 1996, Chow *et al.* 1993). The method also aided in determining the lack of host-specificity and wide spread of *E. faecalis*, as identically or closely related strains could be found not only in the intestinal tract of humans and animals alike but also in food products (van den Bogaard *et al.* 1997, Jensen *et al.* 1999, Gelsomino *et al.* 2002). With the advent of sequence-based typing methods coupled with the ability to unambiguously compare the results to a curated database on the Internet, long-term epidemiology was made possible and clones could easily be traced back to clonal lineages or complexes spread around the globe (Ruiz-Garbajosa *et al.* 2006). A sequence-based method that has come to surpass PFGE in epidemiological typing of *E. faecalis* is multilocus sequence typing (MLST), since it compensates for interference introduced by horizontal genetic transfer that could render related strains unrelated by PFGE (Maynard Smith *et al.* 1993, Maiden *et al.* 2013). MLST is assumed to overcome this problem by detecting allelic variations at seven genomic loci, consisting of internal regions in housekeeping genes that exhibit slow genetical evolution but still high enough levels of polymorphism to allow for investigations on relatedness and population structure (Maiden 2006).

The discriminatory ability of MLST has been shown to be at least equal to that of PFGE (Nallapareddy *et al.* 2002). Furthermore MLST, in contrast to PFGE, offers easy inter-laboratory comparison and is not affected by bias arising when interpreting banding patterns (Singer *et al.* 2004). PFGE is however a less costly method and is better suited for epidemiological typing of a small set of isolates.

In the light of genetic recombination, one limitation with MLST, could be that it simplifies the complex relationships between strains by only analysing a limited number of alleles to extrapolate descendence and population structure. Consequently, the method is unable to differentiate very closely related strains. This limitation can be overcome by comparing full genome sequences and it is thus likely that whole genome sequencing (WGS) will come to replace MLST in a near future to allow for an even more precise characterization of isolates, including mapping of virulence genes and antibiotic resistance (Sabat *et al.* 2013).

2 AIMS OF THE THESIS

The overall aim with this thesis was to shed light on the origin and characteristics of *E. faecalis* in root canal-treated teeth, thereby aiding in the prevention of these prevalent and often treatment-resistant infections. The specific aims for each paper were thus:

I. To evaluate whether *E. faecalis* in root canal infections were endogenously derived from the commensal flora or had an exogenous source.

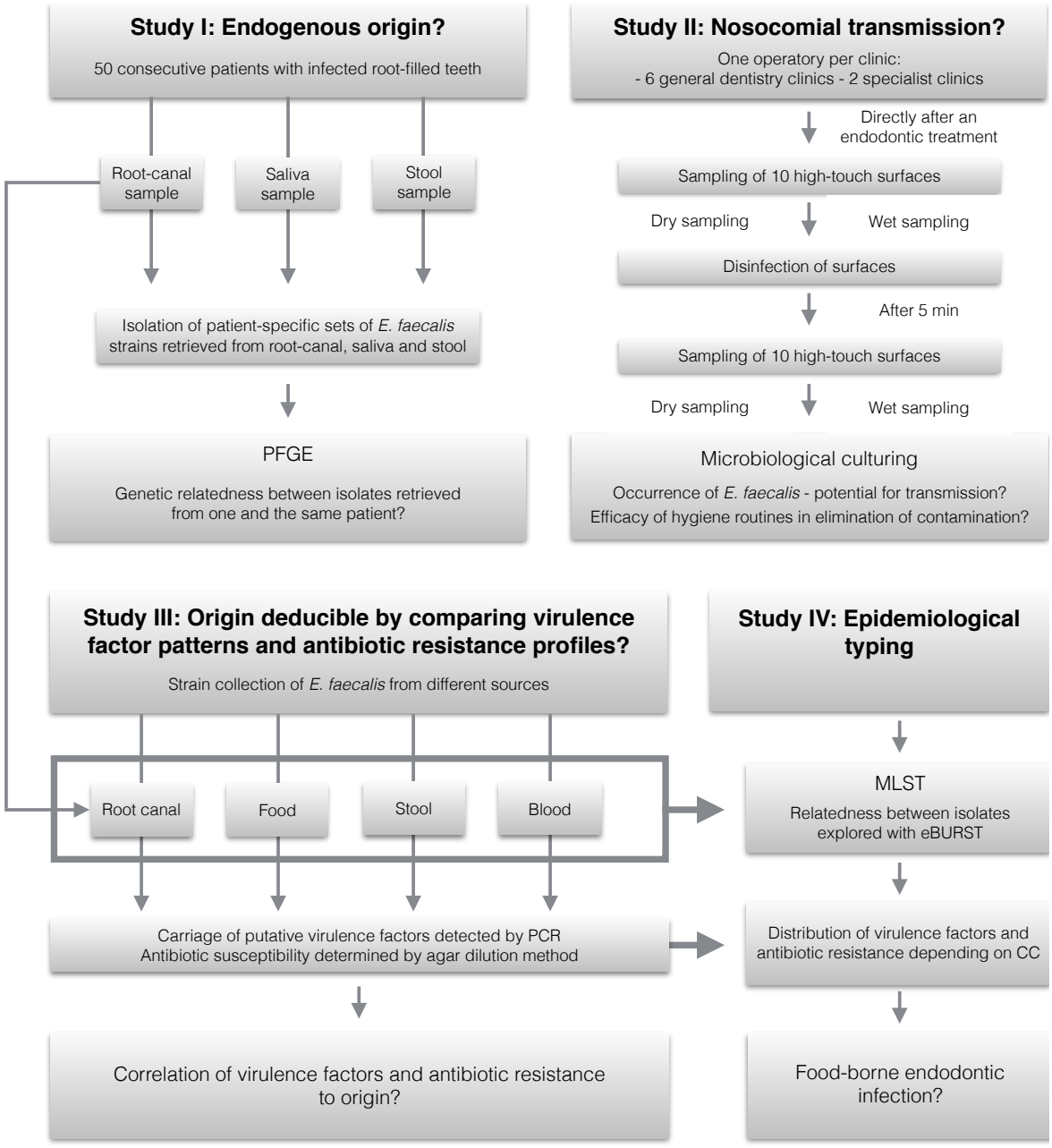
II. To assess the potential for a nosocomial transmission of *E. faecalis* in conjunction with a root canal treatment by measuring its occurrence on high-touch environmental surfaces in dental operatories in relation to the efficacy of disinfection routines.

III. To elucidate the origin of *E. faecalis* isolated from infected root-filled teeth by comparing them to strains recovered from fermented food products, positive blood cultures and stool regarding putative virulence determinants and antibiotic susceptibility profiles, where strains from common origin were hypothesized to harbour similar characteristics.

IV. To explore the possibility for a food-borne endodontic infection by establishing the phylogenetic relationships between *E. faecalis* isolates characterized in paper III.

3 MATERIAL AND METHODS

3.1 OVERVIEW OF STUDY DESIGN



3.2 PARTICIPATING PATIENTS (PAPER I)

A total of 50 patients, referred to a private specialist clinic limited to endodontics in Stockholm, Sweden, and the endodontic specialist clinic at the Department of Dental Medicine, Karolinska Institutet, Stockholm, for non-surgical re-treatment of infected root-filled teeth, were consecutively included during March 2007 and April 2009. To be eligible for inclusion, the patients needed to be over 18 years of age and give a written consent, in accordance to the ethical approval provided by the Regional Ethics Committee at Karolinska Institutet, Stockholm. One root-filled tooth per patient was considered for sampling and it had to render an aseptic working field possible. Consequently, teeth exhibiting extensive tooth loss complicating rubber dam application, or teeth needing disassembling of post-retained crowns, were excluded. Moreover, all teeth had to display clear radiographic signs of apical periodontitis.

All enrolled patients agreed to the sampling of root canals and saliva, and to deliver a stool sample if *E. faecalis* were to be recovered from the root canals. *E. faecalis* isolates recovered from these stool samples were not intended to be included in the other studies, since antibiotic treatment prior to referral was not uncommon.

3.3 PARTICIPATING DENTAL CLINICS (PAPER II)

Environmental surface samples were collected between May 2011 and February 2012 in six general dentistry clinics and two major specialist clinics limited to endodontics in the county of Stockholm. The encompassed general dentistry clinics had been selected from a simple random collection of 15 clinics out of a total of 52 clinics, as to represent both middle and low socio-economic areas, and include medium to large sized clinics. The participating clinics gave their informed consent to an environmental sampling in one operatory per clinic and in conjunction with a root canal treatment; both before and after disinfection routines were applied.

3.3.1 Selection of clinical high-touch surfaces

High-touch surfaces that dental personnel most likely would come in contact with in the course of a root canal treatment were selected for sampling. They consisted of four surfaces expected to be regularly disinfected and six surfaces expected to be disinfected more seldom (Table 4). The portion to be sampled in larger sized surfaces was determined visually and included the area most likely to be touched.

Table 4. Clinical high-touch surfaces targeted for bacteriological sampling according to an estimated frequency of disinfection. The surfaces were sampled in duplicate, utilizing a wet and dry method, and before and after disinfection. Only a portion of larger sized surfaces was sampled. The visually determined area is indicated in brackets.

Surfaces expected to be disinfected frequently	Surfaces expected to be disinfected infrequently to seldom
Work bench closest to operator (10 x 10 cm)	Exposure button for X-ray
Tray holder (10 x 10 cm)	Lead collar (thyroid shield) – strap area (5 x 5 cm)
Suction hose holder	Glove dispenser (5 x 5 cm)
Handle on drawer containing material and equipment for endodontic treatment	Cartridge with gutta-percha points used for root-filling
	Caps on bottles with irrigation solution used during endodontic treatment
	Left mouse-button

3.4 SCREENED FOOD PRODUCTS (PAPERS III AND IV)

A total of 30 food items, consisting of dairy products based on both pasteurized and raw milk, charcuteries, sausages, olives and fresh vegetables and herbs were purchased in a regular grocery store in the Stockholm area and screened for *E. faecalis* (Table 5). The sampling aimed at collecting two *E. faecalis* isolates differing in phenotype when possible.

Table 5. Screened food products purchased in a regular grocery store between November and December 2012.

Type of food	Country of origin	Animal source	Preservation technique
Charcuteries and sausages			
Danish Salami (pre-sliced)	Denmark	Pig	Air-dried, smoked
Hot amigo	Germany	Pig	Air-dried, smoked
Jalapeno sausage	Germany	Pig	Smoked
Kolbász Csabai	Hungary	Pig	Air-dried, smoked
Kolbász Csabahús	Hungary	Pig	Air-dried, smoked
Salsiccia Napoli Piccante	Italy	Pig	Air-dried
Tapas salami	Spain	Pig	Air-dried, smoked

Continued on next page

Table 5 continued. Screened food products purchased in a regular grocery store between November and December 2012.

Type of food (fat content)	Country of origin	Animal source	Preservation technique
Dairy products			
Danablu 30%	Denmark	Cow	Pasteurized
Danablu 37%	Denmark	Cow	Pasteurized
Havarti	Denmark	Cow	Pasteurized
Appenzeller 29%	Switzerland	Cow	Non-pasteurized
Appenzeller 32%	Switzerland	Cow	Non-pasteurized
Gruyere	Switzerland	Cow	Non-pasteurized
Grana Padano	Italy	Cow	Non-pasteurized
Parmigiano Reggiano (Ecological)	Italy	Cow	Non-pasteurized
Tallegio	Italy	Cow	Pasteurized
Basajo	Italy	Sheep	Non-pasteurized
Manchego	Spain	Sheep	Pasteurized
Manchego Valdehierro	Spain	Sheep	Non-pasteurized
Tomme de Savoie	France	Cow	Non-pasteurized
Brie du Grand Pére	France	Cow	Pasteurized
Brie Moulin	France	Cow	Pasteurized
Brie de Meaux	France	Cow	Non-pasteurized
Camembert	France	Cow	Pasteurized
Greek salad-cheese	Greece	Cow	Pasteurized
Feta	Greece	Sheep/goat	Pasteurized
Olives, herbs and vegetables			
Ecological Kalamata olives	Greece		
Green olives filled with Roquefort-cheese	Spain		
Basil in pot	Sweden		
Ruccola pre-washed in sealed bag	Sweden		

3.5 SAMPLING

3.5.1 Endodontically treated teeth (PAPERS I, III and IV)

The sampling of root-filled canals was performed according the protocol proposed by Möller in 1966, with some modifications (Möller 1966).

Initially, an access preparation through the tooth crown, including removal of caries and restorations with defective margins was made without exposing root-filling material. Next, the tooth was isolated using a rubber dam secured to the tooth by a clamp, enabling a

meticulous disinfection of the operating field and tooth crown with 30% hydrogen peroxide followed by a 0.5% chlorhexidine–ethanol solution. The access preparation could then be completed with sterile burs without water-cooling. Root-filling material was extracted from the root canals with Profile rotary instruments (Dentsply Maillefer, Ballaigues, Switzerland) and Hedström files (Sendoline, Täby, Sweden). The removal of gutta-percha was conducted without the use of chemical solvents as previously advised, to prevent negative effects on the microorganisms to be retrieved (Molander *et al.* 1998). At the same time, the produced frictional heat was kept to a minimum by utilizing sparse amounts of VMG I (viable transport medium Gothenburg) sampling fluid as lubrication when needed (Möller 1966). The canals were when possible instrumented to within 0.5–1 mm of the radiographical apex and to an ISO file size apically of 25. Prior to sampling, VMG I sampling fluid was introduced into the canal to a level just below the canal orifice and agitated with a sterile ISO size 20 Hedström file. The solution inside the canal was then completely absorbed into sterile charcoal impregnated paper points taken to the full working length. The paper points were then immediately transferred into 3 ml of VMGA III (Viability Medium Gothenburg anaerobically prepared and sterilized) transport medium and sent for prompt microbiological analysis (Dahlén *et al.* 1993).

3.5.2 Stimulated saliva (PAPER I)

Saliva samples were collected immediately after termination of the endodontic sampling procedure. Chewing on a small piece of paraffin for 5 min stimulated salivary secretion, which could subsequently be collected in a plastic container. A total of 2 ml saliva was transferred to 4 ml of VMG II and stored at -70°C prior to analysis (Jordan *et al.* 1968).

3.5.3 Environmental surfaces (PAPER II)

The environmental surfaces listed in Table 4 were sampled in duplicate, by utilizing both a dry and wet technique in one operatory per participating clinic. Dental personnel were kept unaware of which surfaces that would be sampled and were therefore asked to leave the operatory before sampling was initiated. The first duplicate of samples was taken succeeding a root canal treatment but before disinfection of the surfaces in the operatory. Subsequently, disinfection of the surfaces was performed by dental personnel utilized a 45% v/v isopropyl alcohol-based disinfection solution with a surfactant additive (LIV+45; Lahega Kemi AB, Helsingborg, Sweden). After 5 min, so as to allow for sufficient time of action and evaporation of the disinfection solution, a second set of samples were taken.

The sampling was on all occasions conducted aseptically by the same person, aided by an assistant. Samples were first collected using dry collection swabs with regular sized cotton swab tips (Copan Transystem® Amies charcoal plastic swab 114C; Copan Diagnostics, Corona, CA, USA), which after each sampling were placed in a marked transport tube included in the sterile sampling kit. A duplicate of samples was then collected by swabbing each surface with a sterile small cotton compress (15 x 15 mm), soaked in FAB-medium (Fastidious Anaerobe Broth; Lab M, Heywood, UK) and held with a sterile locking forceps

(Figure 1). In addition, each moistened surface was swabbed with a dry cotton compress to absorb remaining liquid and increase the recovery of microorganisms. Each inoculated compress was successively dropped down in a corresponding marked tube containing fresh FAB-medium. Positive and negative controls, consisting of a buccal swab sample collected from one of the operators and an unused swab, respectively, were included to each set of samples. The samples were kept dark and cool and hastily delivered to the microbiological laboratory for culture and analysis.

Figure 1. Sampling performed aseptically on a suction hose holder with a cotton swab soaked in FAB-medium. The sampling targeted the area most likely to be touched by dental personnel.

3.5.4 Food products (PAPERS III and IV) and stool (PAPERS I, III and IV)

The intact layer of protective packaging or casing around the food product to be sampled was dissected with sterile scalpels and forceps, which were changed for each product. When possible, a small sample was then cut out from both the inner and outer part of the product and subsequently sliced very thin or scraped to create flakes. Approximately 1 g of the sample was next transferred to a sterile vial containing 4.5 ml PBS (0.01M, pH 7.4) and vortexed thoroughly before a ten-fold serial dilution up to 10^{-5} and subsequent culture analysis was performed.

The stool samples were screened for *E. faecalis* following a ten-fold serial dilution up to 10^{-6} of 0.5 g of faecal matter suspended in 4.5 ml PBS (0.01M, pH 7.4).

3.6 MICROBIOLOGICAL CULTURING AND IDENTIFICATION

3.6.1 Samples from root canals, saliva, food and stool (PAPERS I, III and IV)

Samples, presumably containing high concentrations of enterococci, were subjected to serial dilutions and plated on selective and non-selective agar by spreading 100 µl of bacterial suspension. The plates were then incubated aerobically in 37°C for 48 h. Identification of isolates as enterococci was based on Gram-staining appearance, colony morphology, catalase activity and growth on Enterococcus selective agar (Acumedia; Lansing, MI, USA). Moreover, isolates readily growing in the presence of 6.5% NaCl, bile-esculin and sorbitol after aerobic incubation in 37°C overnight were identified on species level as *E. faecalis* (Teixeira *et al.* 2011). Figure 2 shows differentiating agar plates containing arabinose and sorbitol used to identify *E. faecalis*. Isolates in pure culture were next suspended in freezing medium (0.5 ml LB broth with 30% glycerol) and stored in -70°C till further analysis.

Figure 2. Agar plates containing arabinose and sorbitol used to differentiate between *Enterococcus* species after incubation at 37°C overnight. *E. faecalis* is capable of fermenting sorbitol but not arabinose, thereby inducing a change in colour from red to yellow on the plate containing sorbitol, whereas the agar with arabinose remains red.

3.6.2 Environmental samples (PAPER II)

Cotton swabs in FAB-medium were vortexed thoroughly and subsequently incubated in 37°C overnight. The enriched bacterial suspensions and the Copan collection swabs, contained in Amies transport medium supplemented with charcoal, were then streaked on selective, non-selective and differentiating agar and incubated under both aerobic and anaerobic conditions. Anaerobic culture conditions were achieved by using tightly sealed jars containing disposable envelopes generating hydrogen and carbon dioxide (BBL®, GasPak®; Becton Dickinson

Microbiology Systems, Cockeysville, MD, USA). Identification of bacteria to genus level, and when possible to species level, was performed in accordance to the Manual of Clinical Microbiology (Versalovic *et al.* 2011). A simplified flow chart depicting the procedure is presented in Table 6.

Table 6. Overview of the procedure used for identifying main bacterial groups.

Gram-stain and morphology	**Biochemical testing and species identification**			
Gr+ cocci	Catalase positive →	Sleifer	Novobiocin	DNAse
		↓	↓	↓
	Micrococcus spp.	–	Sensitive	–
	Staphylococcus aureus	+	Sensitive	+
	Staphylococcus epidermidis	+	Sensitive	–
	Catalase negative → NaCl 6.5% –, α-haemolysis → viridans streptococci			
	→ NaCl 6.5% +, bile esculin + → *Enterococcus* spp.			
			↓	
			Arabinose	Sorbitol
	Enterococcus faecalis		–	+
	Enterococcus faecium		+	+/–
	Enterococcus durans / hirae		–	–
Gr+ rods	Positive spore test → *Bacillus* spp.			
	Negative spore test → *Corynebacterium* spp. and other Gr+ rods			
Gr– cocci	Oxidase positive & DNAse* negative → *Neisseria* spp.			
	Oxidase positive & DNAse* positive → *Moraxella* spp.			
	*DNAse activity was assessed after 48 h incubation in 10% CO_2			
Gr– rods	BioMérieux API 20E → *Enterobacteriaceae*			

3.7 COLLECTION OF STRAINS AND STOOL SAMPLES (PAPERS III AND IV)

3.7.1 Isolates from treated root canals

In order to provide a total of 30 endodontic *E. faecalis* strains (one isolate from each patient) for analysis, isolates from patients included in paper I were supplemented with strains from a collection stored in -70°C at the Department of Laboratory Medicine, Division of Clinical Microbiology, Karolinska Institutet, Stockholm. The strain collection comprised isolates collected during the years 2006 to 2014 from patients treated for apical periodontitis at the student and specialist clinic at the Department of Dental Medicine at Karolinska Institutet in Stockholm. All isolates had been retrieved from teeth that had previously been root-filled or chemo-mechanically treated.

Root-filled teeth had been sampled as described earlier in section 3.5.1. Isolates in the strain collection from infected teeth, which were not root-filled and earlier had been shaped, irrigated with Dakin's solution (0.5% buffered sodium hypochlorite; Apotek Produktion & Laboratorier AB, Huddinge, Sweden) and medicated with a calcium-hydroxide dressing (DT Dressing; Dental Therapeutics AB, Nacka, Sweden) after a rinse with 3% EDTA (Tubulicid Plus; Dental Therapeutics AB, Nacka, Sweden) and occasionally 2% chlorhexidine gluconate (Consepsis; Ultradent Products, South Jordan, UT, USA) had been sampled accordingly.

The provisional filling was removed aseptically after rubber dam application and disinfection of the tooth crown. The calcium hydroxide dressing in the root canals was then carefully flushed out with sparse amounts of VMG I sampling fluid after agitation with a sterile ISO files size 25 taken to the full working length. Next, the canals were anew filled to the level of the orifices with VMG I and, after additional agitation and scraping against the canal walls, the fluid was completely absorbed into charcoal-impregnated paper points and transferred to VMGA III or FAB-medium (Fastidious Anaerobe Broth; Lab M, Heywood, UK).

3.7.2 Isolates from positive blood cultures

The blood isolates encompassed 30 *E. faecalis* strains (one isolate from each patient) recovered from positive blood cultures in the years from 2010 to 2013 from patients admitted to Karolinska University Hospital Huddinge, one of Stockholm's major hospitals, for suspected sepsis. The isolates had been collected from routine samples sent to the hospital's clinical microbiological laboratory and were stored in -70°C.

3.7.3 Collection of stool samples from healthy individuals

A total of 30 commensal strains (one isolate from each individual) were gathered from stool samples from healthy Swedish volunteers participating in clinical studies during 2010-2014 (Rashid *et al.* 2013, Rashid *et al.* 2014, Rashid *et al.* 2015a, Rashid *et al.* 2015b). All included participants were determined healthy after being subjected to necessary physical examinations and reported a normal frequency of defecation (five or more times a week). Furthermore, only individuals that had refrained from the use of antibiotics and other medication except contraceptives during a minimum of three months were considered for

inclusion. The stool samples, which had been stored in -70°C, were screened for commensal *E. faecalis* strains by culture analysis.

3.8 MICROBIOLOGICAL ASSAYS AND ANALYSIS

3.8.1 DNA extraction

Genomic DNA was obtained by "crude extraction" after aerobic culturing of all isolates on Columbia blood agar (Acumedia; Lansing, MI, USA) for two passages. One loopful of bacteria in pure culture was suspended in 1 ml MQ and boiled at 95°C for 15 min. Following placement on ice for 5 min, the lysate was centrifuged at 12 000 rpm for 2 min, where after the DNA-containing supernatant could be carefully extracted and stored at -20°C.

3.8.2 Detection of cytolysin expression (PAPER IV)

Cytolysin expression resulting in β-haemolysis, that is zones of clearing around colonies as depicted in Figure 3, was recorded after aerobic incubation at 37°C overnight on agar plates containing Colombia blood agar supplemented with 5% citrated horse blood.

Figure 3. *E. faecalis* isolated from food presenting β-haemolysis on blood agar.

3.8.3 Polymerase chain reaction

3.8.3.1 Molecular verification of species identity (PAPERS I, II and III)

The biochemical typing of isolates as *E. faecalis* was verified by PCR using species specific primer pairs targeting the gene *ddl* encoding ddl-$_D$-alanine:$_D$-alanine ligase (Dutka-Malen *et al.* 1995). Primer sequences and PCR conditions are presented in Table 7.

3.8.3.2 Determination of putative virulence factors (PAPER III)

Virulence genes, detected by multiplex-PCR assays, and a chromosomal deletion in the *fsr* gene cluster region (*ef1841*/*fsr*C), resulting in a gelatinase-negative phenotype (Nakayama *et al.* 2002), that was targeted in a single reaction are presented in Table 7 along with utilized primer sequences, reference strains and PCR conditions.

PCR reactions were prepared by adding 1.5 µl of DNA-template to a 23.5 µl reaction mixture with illustra™ puReTaq Ready-To-Go PCR Beads (GE Healthcare UK Limited, Little Chalfont, UK) containing a concentration of 200 mM of each dNTP, 2.5U of puReTaq DNA Polymerase, 0.2 mM of each primer in 10 mM Tris-HCl (pH 9.0) with 50 mM KCl and 1.5 mM $MgCl_2$. PCR was run on a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA, USA) thermal cycler. Afterwards, the PCR products were loaded on a 1.5% UltraPure™ Agarose gel (Invitrogen Corporation, Carlsbad, CA, USA) by mixing 10 µl of each PCR product with 2 µl of 6x loading dye (Thermo Fisher Scientific, Waltham, MA, USA). GeneRuler™ 1kb Plus DNA ladder (Thermo Fisher Scientific) was used as a molecular marker. Electrophoresis was performed for 55 min at 110 V in 1xTAE and the amplicons were visualized under ultraviolet light after staining with ethidium bromide.

Table 7. PCR primers and conditions used to verify the identity of *E. faecalis* isolates and detect putative virulence determinants. Oligonucleotide sequences utilized were according to the stated references.

Amplified gene	Sequence of primers (5' – 3')	PCR conditions	Amplicon size (bp)	Reference
$ddl_{E.faecalis}$	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	94°C–2 min; 30 cycles (94°C–60 s; 54°C–60 s; 72°C–60 s) and 72°C–10 min	941	Dutka-Malen *et al.* 1995
ef1841/*fsr*C	GATCAAGAAGGGAAGCCACC CCAACCGTGCTCTTCTGGA	94°C–2 min; 35 cycles (92°C–30 s; 56°C–60 s; 72°C–2 min) and 72°C–5 min	1050	Nakayama *et al.* 2002
esp	TTGCTAATGCTAGTCCACGACC GCGTCAACACTTGCATTGCCGAA	Multiplex PCR for *esp*, *cyl*A and *gel*E: 95°C–10 min; 30 cycles (94°C–60 s; 56°C–60 s; 72°C–60 s) and 72°C–10 min	933	Eaton & Gasson 2001
*cyl*A	GACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTTAC		688	Creti *et al.* 2004
*gel*E	ACCCCGTATCATTGGTTT ACGCATTGCTTTTCCATC		419	Eaton & Gasson 2001
*efa*A	GCCAATTGGGACAGACCCTC CGCCTTCTGTTCCTTCTTTGGC	Multiplex PCR for *efa*A, *ace* and *asa*1: 95°C–5 min; 30 cycles (95°C–60 s; 58°C–60 s; 72°C–60 s) and 72°C–10 min	688	Creti *et al.* 2004
ace	GGAATGACCGAGAACGATGGC GCTTGATGTTGGCCTGCTTCCG		616	Creti *et al.* 2004
*asa*1	CCAGCCAACTATGGCGGAATC CCTGTCGCAAGATCGACTGTA		529	Creti *et al.* 2004

Reference strains used as controls:

E. faecalis ATCC 29212: *ddl* + ; *esp* –; *cyl*A +; *gel*E +; *ef1841*/*fsr*C +; *efa*A +; *ace* +, *asa*1 +

E. faecalis MMH594: *ddl* + ; *esp* +; *cylA* +; *gel*E +; *ef1841*/*fsr*C –; *efa*A +; *ace* –; *asa*1 +

E. faecium HS2 (Billström *et al.* 2009): negative control

3.8.4 Pulsed-field gel electrophoresis (PAPER I)

PFGE was performed according to a protocol originally proposed by de Lencastre *et al.* 1999 and modified by Lund *et al.* 2002a.

E. faecalis strains, isolated from root canals and stool from patients included in paper I, were cultured overnight on Columbia blood agar. The isolates were subsequently embedded in low-melting agarose (SeaPlaque® agarose; FMC BioProducts, Rockland, ME, USA) and subjected to lysis *in situ* using a solution consisting of 6.0 mM Tris pH 8.0, 1.0 M NaCl, 0.1 M EDTA pH 8.0, 0.2% Na-deoxycholate, 0.5% sarkosyl, 0.5% Brij-58, 500 µg/ml RNase and 1 mg/ml lysozyme. All reagents were purchased from Sigma (Saint Louis, MO, USA). Next, proteins were degraded by incubating the discs overnight at 50°C in a buffer containing 0.5% EDTA pH 9.0, 1% sarkosyl and 2 mg/ml proteinase K (Promega Corporation, Madison, WI, USA). The discs were repeatedly washed in 1xTE (10 mM Tris pH 7.5, 1 mM EDTA pH 7.5) before the DNA was digested with the oligonuclease *Sma*I (Promega Corporation, Madison) overnight at 37°C. The discs were subsequently loaded in a 1.2% agarose gel (SeaKem® LE agarose; FMC BioProducts) and placed in a CHEF apparatus (Bio-Rad GenePath® System; Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis was run for 20 h at 14°C with a pulse time that was linearly ramped from a switch time of 5.3 s to 34.9 s at a voltage of 6.0 (V/Cm). The gels were stained in ethidium bromide enabling visualization of DNA bands under ultraviolet illumination.

3.8.4.1 Interpretation of banding patterns - criteria for determining genetic relatedness

The banding patterns of isolates obtained from one and the same patient were compared visually. The relatedness between the isolates were interpreted according to the following criteria: isolates were considered identical when no band differed between the isolates, a difference of three bands or less rendered the strains as genetically related and at band differences greater than three, the strains were regarded as unrelated (Tenover *et al.* 1995).

3.8.5 Minimum inhibitory concentration of antibiotics (PAPER III)

The lowest concentrations of clinically relevant antibiotics, required to completely inhibit bacterial growth (MICs), was determined using the agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (M07-A9 2012; M100-S22 2012). Reference strains used were *E. faecalis* ATCC 29212, *E. faecalis* CCUG 34062, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922.

In short, final inoculums of 10^4 CFU were spotted on Mueller-Hinton agar plates containing the following antibiotics (Sigma-Aldrich, St. Louis, MO, USA), which were dissolved and diluted according to the provided specifications: the penicillins ampicillin and piperacillin-tazobactam, the carbapenem imipenem, the aminoglycoside gentamicin, the glycopeptide vancomycin, the fluoroquinolone ciprofloxacin and the oxazolidinone linezolid. Inoculated plates were incubated overnight at 37°C before antibiotic resistance was assessed using

breakpoints provided by the European Committee on Antimicrobial Susceptibility Testing (Table 8).

Table 8. EUCAST's clinical breakpoint table v. 4.0 valid from 2014-01-01 used to determine antibiotic susceptibility of *E. faecalis* strains.

Antimicrobial compound	Sensitive (MIC ≤)	Resistant (MIC >)
Ampicillin	4 mg/L	8 mg/L
Piperacillin-tazobactam	4 mg/L	8 mg/L
Imipenem	4 mg/L	8 mg/L
Gentamicin	128 mg/L	128 mg/L
Vancomycin	4 mg/L	4 mg/L
Ciprofloxacin	4 mg/L	4 mg/L
Linezolid	4 mg/L	4 mg/L

3.8.6 Multilocus sequence typing (PAPER IV)

Extracted DNA was sent to ID Genomics Inc. (Seattle, WA, USA) for MLST using the *E. faecalis* strain MMH594, known to belong to the hospital-associated clone ST6, as control (Ruiz-Garbajosa *et al.* 2006, McBride *et al.* 2007).

Portions of the housekeeping genes *gdh*, *gyd*, *pstS*, *gki*, *aroE*, *xpt* and *yiq*L were amplified as described in Table 9. Sequencing was subsequently performed with forward and reverse primers after purification of the PCR products. A corresponding sequence type (ST) was assigned to each allelic profile after matching of the generated sequences to those present in the MLST database (http://efaecalis.mlst.net). Unmatched sequences were analysed for accuracy, before being sent to the database curator to be designated novel allelic numbers, thus comprising a new ST.

The relatedness between the different STs and their most likely parsimonious pattern of descent from a predicted founder was thereafter explored with eBURST v3 (http://eburst.mlst.net, Feil *et al.* 2004), incorporating the complete dataset of profiles stored in the PubMLST database (http://pubmlst.org/efaecalis) as a reference (Jolley & Maiden 2010). Single-locus variants (SLVs), being STs sharing at least six identical alleles with another ST, clustered into clonal complexes (CCs) and were thus determined closely related. The founding genotype of each CC was predicted to be the ST with the most SLVs within the group, thereby designating the CC. STs not grouping into CCs constituted singletons.

Table 9. Primer sequences used to target *E. faecalis* housekeeping genes for amplification and later sequencing to obtain allelic profiles (Ruiz-Garbajosa *et al.* 2006).

Targeted gene	Primer sequence (5' – 3')	Product size (bp)
Glucose-6-phosphate dehydrogenase		530
gdh-1	GGCGCACTAAAAGATATGGT	
gdh-2	CCAAGATTGGGCAACTTCGTCCCA	
Glyceraldehyde-3-phosphate dehydrogenase		395
gyd-1	CAAACTGCTTAGCTCCAATGGC	
gyd-2	CATTTCGTTGTCATACCAAGC	
Phosphate ATP binding cassette transporter		583
pstS-1	CGGAACAGGACTTTCGC	
pstS-2	ATTTACATCACGTTCTACTTGC	
Glucokinase		438
gki-1	GATTTTGTGGGAATTGGTATGG	
gki-2	ACCATTAAAGCAAAATGATCGC	
Shikimate-5-dehydrogenase		459
aroE-1	TGGAAAACTTTACGGAGACAGC	
aroE-2	GTCCTGTCCATTGTTCAAAAGC	
Xanthine phosphoribosyltransferase		456
xpt-1	AAAATGATGGCCGTGTATTAGG	
xpt-2	AACGTCACCGTTCCTTCACTTA	
Acetyl-CoA acetyltransferase		436
yiqL-1	CAGCTTAAGTCAAGTAAGTGCCG	
yiqL-2	GAATATCCCTTCTGCTTGTGCT	

Conditions for PCR: 94°C–5 min; 30 cycles (94°C–30 s; 52°C–30 s; 72°C–60 s) and 72°C–7 min

3.9 STATISTICAL CALCULATIONS (PAPER III)

Fisher's exact test (two-tailed) was applied to analyse the difference in prevalence of the various virulence factors between the strains of different sources. GraphPad QuickCalcs software set to summing small p values was utilized for the calculations (Website accessed the 14th of August 2014: www.graphpad.com/quickcalcs/contingency1). Significance was established at $p \leq 0.05$.

4 RESULTS

4.1 GENETIC RELATIONSHIP BETWEEN STRAINS ISOLATED FROM ROOT CANALS AND THE ENDOGENOUS FLORA (PAPER I)

4.1.1 Patients and patient-specific sets of *E. faecalis* isolates

The 50 consecutively included patients constituted a population encompassing 23 men and 27 women aged between 23 and 76 years, with a mean age of 52. Root canal samples from 50 infected teeth were collected and microbiologically analysed. Of the sampled 50 teeth, 33 were molars (66%), 10 were premolars (20%) and seven were incisors (14%). Although, samples of secreted saliva were collected from all patients, only samples from patients with *E. faecalis* in the root canals were processed. In addition, stool samples were only requested from patients exhibiting *E. faecalis* in the endodontic sample.

In total, *E. faecalis* was isolated in eight of the 50 teeth (16%). In six root canal samples, *E. faecalis* was present in pure culture. In the other two samples, it was found together with *Enterobacter* spp. or *Actinomyces* spp. Consequently eight stool and saliva samples were screened for *E. faecalis*. The microorganism could not be retrieved from any of the serially diluted saliva samples. Only six of the eight stool samples (75%) produced retrievable *E. faecalis*. In the two stool samples devoid of *E. faecalis*, *E. faecium* was the abundant species. When possible, at least six random colonies with *E. faecalis* were harvested from every positive sample (Table 10). Thus, microbiological analyses rendered six patient-specific sets of isolates from root canals and stool that could be further analysed with PFGE.

4.1.2 Genotypic analyses with PFGE

Evaluation of the PFGE-derived macro-restriction profiles, using the criteria recommended by Tenover *et al.* 1995, clearly disclosed a lack of genetic relatedness between strains retrieved from root canals and stool from the one and same patient (Figure 4). This was true for all six cases analysed. Moreover, there was no genetic relationship on an inter-individual level between the different endodontic isolates from the eight patients. However, the strains retrieved from infected teeth were identical or related on an intra-individual level, in comparison to the isolates from the commensal intestinal flora that could display a greater genetic polymorphism (Table 10).

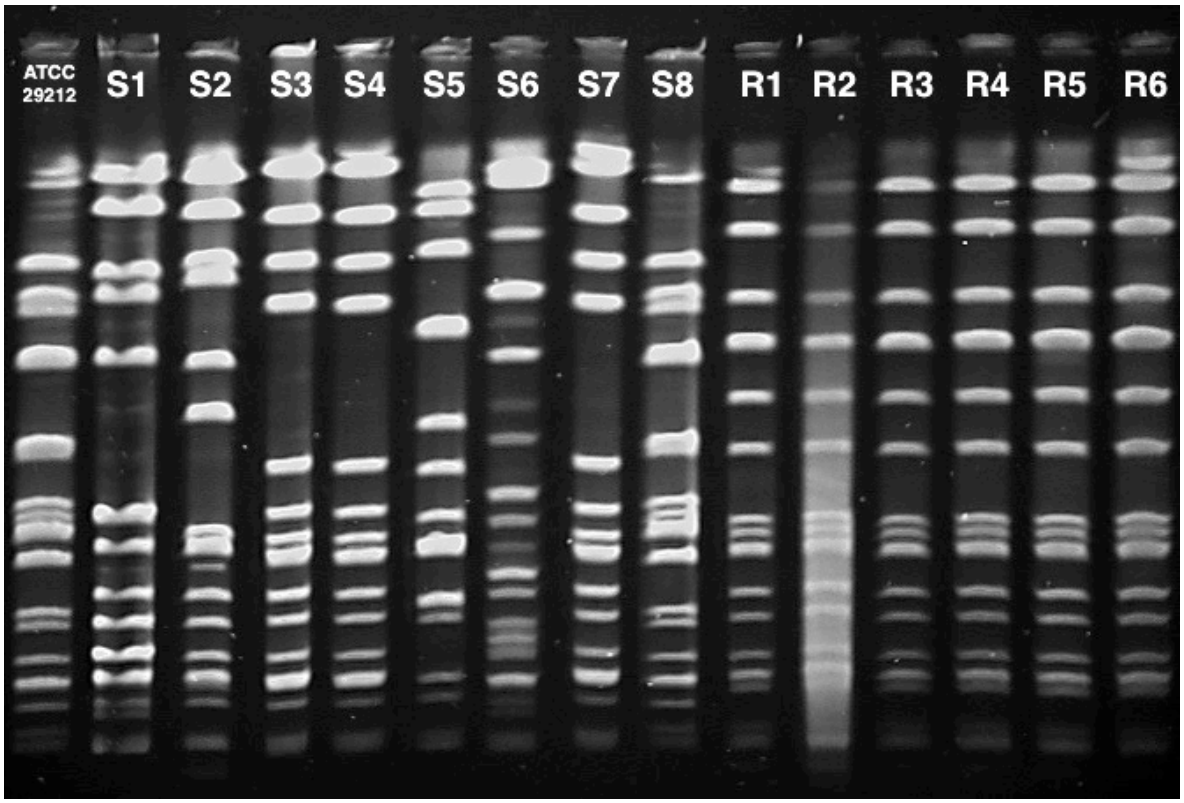


Figure 4. Macro-restriction profile for isolates recovered from the stool sample (S1-S8) and root canal sample (R1-R6) belonging to patient 1. Isolates R1 to R6 were closely related, as they at most differed with one band from each other. In the stool sample, only isolates S3, S4 and S7 were identical. The endodontic isolates differed with more than seven bands from those retrieved from stool and were thus not related genetically. These results were representative for the other five patients, from which complete sets of isolates could be obtained for comparison with PFGE. *E. faecalis* ATCC 29212 was used as reference.

Table 10. *E. faecalis* strains isolated from root canal and stool samples collected from patients in paper I and analysed with PFGE according to criteria by Tenover *et al.* 1995

Patient sample	Analysed colonies	Number of strains	Genetic relationship between strains
Root canal			
Patient 1	6	2	Related
Patient 10	12	3	Related
Patient 12	6	2	Related
Patient 15	6	1	Identical
Patient 19	6	2	Related
Patient 23	6	1	Identical
Patient 32	6	1	Identical
Patient 34	6	1	Identical
Stool			
Patient 1	8	5	Not related
Patient 10	12	6	Not related
Patient 12	6	1	Identical
Patient 15	ND	ND	
Patient 19	6	1	Identical
Patient 23	1	1	
Patient 32	ND	ND	
Patient 34	6	1	Identical

4.2 POTENTIAL FOR NOSOCOMIAL TRANSMISSION FROM CONTAMINATED SURFACES IN DENTAL OPERATORIES (PAPER II)

4.2.1 Bacterial contamination and occurrence of *E. faecalis* on high-touch clinical surfaces

Out of 320 collected environmental samples, resulting from sampling of 80 clinical high-touch surfaces in duplicate and on two occasions, 130 samples (40.6%) exhibited bacterial growth. The most commonly isolated microorganisms (36.3%) were environmental bacteria, such as *Bacillus* spp., and bacteria from normal skin flora, consisting of coagulase-negative staphylococci (CoNS), *Micrococcus* spp., and occasionally coagulase-positive staphylococci and *Corynebacterium* spp. Salivary contamination of surfaces, typically with *Streptococcus* spp., was just found in 11 samples (3.4%). *E. faecalis* was only retrieved from three samples (0.9%) together with CoNS, *Micrococcus* spp., *Enterobacteriaceae* and *Bacillus* spp. The samples were collected in two clinics situated in low-socioeconomic parts of Stockholm County and from surfaces expected to be disinfected less frequently to seldom, specifically

the strap of the lead collar, the caps on the bottles containing irrigation solutions for endodontic treatment and the glove dispenser. Two of the samples had been taken before disinfection and one after disinfection routines had been applied.

Sampling with swabs moistened in FAB-medium was superior to the dry technique, as it resulted in an 11.25% (n = 18/160) increased bacterial recovery from sampled surfaces. The microbiological results for the two methods were pooled together, since the results coincided in 46 of the 80 surfaces (57.5%) sampled before disinfection and 43 of the 80 surfaces (53.75%) examined after disinfection.

4.2.2 Efficacy of disinfection routines

One of the six general dentistry clinics was excluded from the evaluation of the efficacy of applied disinfection routines, as some of the samples collected from that clinic had been marked erroneously.

The disinfection measures in the general dentistry clinics were, in comparison to the specialist clinics, deficient, as the contamination levels were generally higher before disinfection routines were undertaken. Moreover, applied disinfection routines resulted in a 6% increased contamination in the general dentistry clinics, whereas a 10% reduction in contamination level was achieved in the specialist clinics (Figure 5).

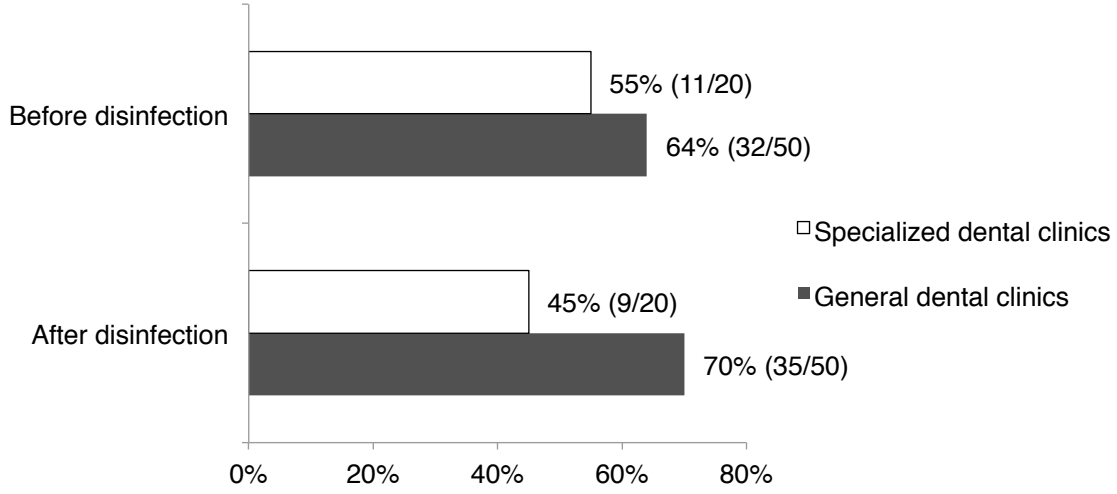


Figure 5. Contamination levels before and after disinfection.

The microbial composition before and after disinfection measures had been undertaken, remained practically unchanged in the general dentistry clinics (Figure 6). In the specialist clinics, some uncontaminated surfaces exhibited contamination with CoNS after disinfection measures had been applied, indicating that the surfaces had been touched with bare hands.

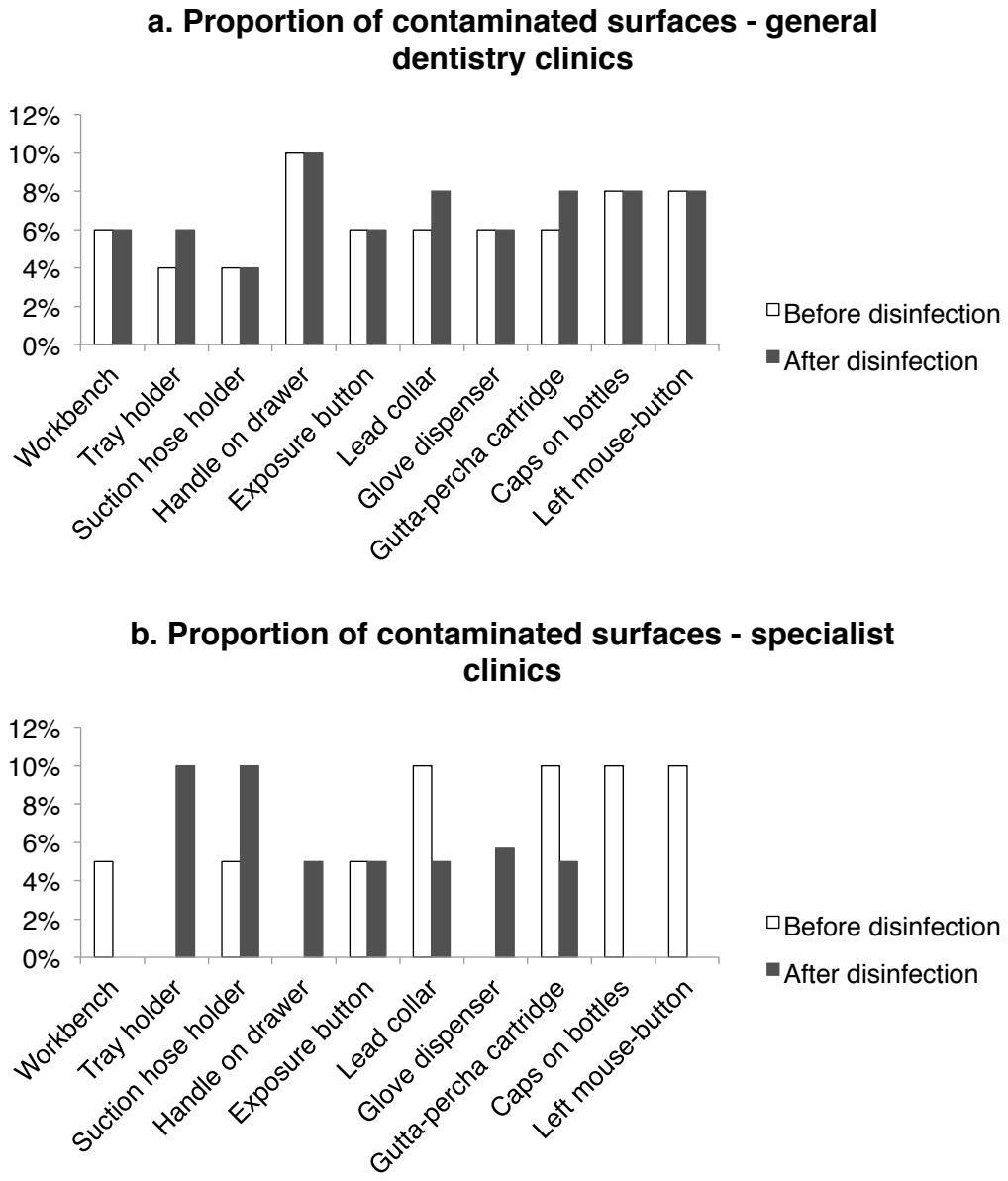


Figure 6. Graph depicting contamination levels before and after disinfection measures had been undertaken in general dentistry clinics (a) and specialist clinics (b). The proportions shown are based on pooled microbiological results using two different sampling methods. The most contaminated surfaces after an endodontic treatment were the caps on bottles with irrigation fluids, the left mouse button, the drawer handle, cartridge with gutta-percha points and lead collar. Following disinfection measures, the most contaminated surfaces were found again on the drawer handle, the lead collar, the gutta-percha cartridge and tray holder.

4.3 TRACING THE ORIGIN OF ENDODONTIC *E. FAECALIS* BY COMPARING VIRULENCE FACTOR PATTERNS, ANTIBIOTIC SUSCEPTIBILITY PROFILES AND GENETIC LINEAGES

4.3.1 Analysed collection of strains (PAPERS III and IV)

The 30 endodontic strains analysed encompassed the eight isolates recovered from patients in paper I together with 22 isolates from a stored strain collection. The screening of 30 food items resulted in 19 isolates from 10 food items. The stool samples from healthy individuals generated 30 isolates, which after verification with PCR targeting *ddl* were reduced to 29, since one isolate mistakenly had been identified as *E. faecalis* by biochemical typing. The 30 isolates recovered from positive blood cultures were confirmed *E. faecalis* strains.

The collection of analysed isolates is presented in Table 11 along with the results obtained by PCR, MIC determination and MLST.

Table 11. Collection and designation of isolates analysed in papers III and IV. Antibiotic resistance determined by the agar dilution method is denoted as follows: A=Gentamicin MIC > 256 mg/L; B=Ciprofloxacin MIC 256 mg/L; C=Ciprofloxacin MIC 128 mg/L; D=Ciprofloxacin 64 mg/L; E=Ciprofloxacin 32 mg/L. Clustering of sequence types (ST) in clonal complexes is indicated in bold style by the ST of the predicted founder according to eBURST analysis. Isolates associated with other STs but lacking a known predicted founder, are indicated by "*Assoc*" in the column for CCs. Isolates not grouping are represented by "*Single*", meaning singleton. The presence of *gel*E in combination with the chromosomal deletion in the region *ef*1841/*fsr*C (fsrC), resulting in a gelatinase negative phenotype is indicated by (+). Equally, the inability to demonstrate β-haemolysis on blood agar while carrying the gene *cyl*A is indicated by (+).

Food	**Desig nation**	**Antibiotic resistance**	**ST**	**CC**	**Detected putative virulence genes**						
Source of isolation					***esp***	***cyl*A**	***gel*E**	***fsr*C**	***efa*A**	***ace***	***asa*1**
Kolbasz Csabai	F1a		40	**40**			+		+	+	
	F1b		141	**141**	+		+		+	+	
Kolbasz Csabahus	F2a	A	249	*Single*			+		+	+	
	F2b		140	*Single*			+		+	+	+
Hot amigo	F3a		624	*Single*		(+)	+		+	+	
	F3b		613	*Single*					+	+	+
Tomme de Savoie	F4a		97	**25**		+	(+)	+	+	+	+
	F4b		25	**25**	+	+	(+)	+	+	+	+
Manchego	F5a		72	**72**		+	+		+	+	+
	F5b		206	**206**					+	+	+
Manchego Valdehierro	F6a		594	*Single*			+		+	+	
	F6b		206	**206**					+	+	+
Brie de Meaux	F7a		40	**40**	+		+		+	+	+
	F7b		595	*Single*			+		+	+	+
Camembert	F8		168	**72**			+		+	+	+
Danablu 30%	F9a		97	**25**			(+)	+	+	+	+
	F9b		19	**19**	+	+	+		+	+	
Basajo	F10a		599	*Single*			+		+	+	+
	F10b		97	**25**		+	(+)	+	+	+	+

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Root canal	**Desig nation**	**Antibiotic resistance**	**ST**	**CC**	**Detected putative virulence genes**						
Source (year of isolation)					***esp***	***cyl*A**	***gel*E**	***fsr*C**	***efa*A**	***ace***	***asa*1**
Patient 1 (2007)	R1		72	**72**			+		+	+	
Patient 10 (2007)	R2		72	**72**			+		+	+	+
Patient 12 (2007)	R3		72	**72**			+		+	+	
Patient 15 (2007)	R4		73	**25**					+	+	
Patient 19 (2008)	R5		25	**25**	+		(+)	+	+	+	
Patient 23 (2008)	R6		74	**25**					+	+	
Patient 32 (2008)	R7		608	**206**					+	+	+
Patient 34 (2008)	R8		21	**21**	+				+	+	
Collection (2006)	R9		133	**25**					+	+	+
Collection (2007)	R10	A, B	64	**8**	+		(+)	+	+	+	
Collection (2007)	R11		607	**25**	+		(+)	+	+	+	
Collection (2007)	R12		209	**8**	+		(+)	+	+	+	
Collection (2007)	R13		72	**72**			+		+	+	+
Collection (2007)	R14		606	**95**			+		+	+	+
Collection (2007)	R15		326	**25**	+		(+)	+	+	+	
Collection (2008)	R16		21	**21**	+				+	+	
Collection (2010)	R17		40	**40**			+		+	+	
Collection (2011)	R18		97	**25**			(+)	+	+	+	+
Collection (2011)	R19		97	**25**			(+)	+	+	+	+
Collection (2011)	R20		97	**25**			(+)	+	+	+	+
Collection (2012)	R21		273	*Assoc*			+		+	+	+
Collection (2012)	R22		596	*Assoc*			+		+	+	
Collection (2012)	R23		72	**72**			+		+	+	
Collection (2012)	R24		25	**25**	+		(+)	+	+	+	
Collection (2012)	R25		25	**25**	+		(+)	+	+	+	
Collection (2012)	R26		165	**165**			+		+	+	
Collection (2012)	R27		40	**40**			+		+	+	+
Collection (2013)	R28		117	**21**			(+)	+	+	+	+
Collection (2014)	R29		602	**25**			(+)	+	+	+	+
Collection (2014)	R30		268	**40**	+		+		+	+	

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Stool Source (year of sample collection)	Designation	Antibiotic resistance	ST	CC	Detected putative virulence genes						
					esp	*cylA*	*gelE*	*fsrC*	*efaA*	*ace*	*asa1*
Collection (2011)	F1		173	*Assoc*			+		+	+	+
Collection (2011)	F2		147	*Single*					+	+	+
Collection (2011)	F3		79	*Assoc*			+		+		
Collection (2011)	F4		40	**40**	+	(+)	+		+	+	+
Collection (2011)	F5		609	*Assoc*			+		+	+	
Collection (2011)	F6		387	*Single*			+		+	+	
Collection (2011)	F7		236	**236**			+		+	+	
Collection (2011)	F8		611	*Single*			+		+	+	+
Collection (2011)	F9		602	**25**			(+)	+	+	+	+
Collection (2011)	F10		593	*Single*			+		+	+	
Collection (2011)	F11		22	**21**		(+)	+		+	+	+
Collection (2011)	F12		597	*Single*			+		+	+	
Collection (2011)	F13		598	**228**			+		+		+
Collection (2011)	F14		168	**72**		(+)	+		+	+	+
Collection (2011)	F15		91	*Single*	+		+		+	+	
Collection (2011)	F16		603	*Single*			+		+	+	
Collection (2011)	F17		34	**34**			+		+	+	
Collection (2011)	F18		72	**72**			+		+	+	
Collection (2011)	F19		602	**25**			(+)	+	+	+	+
Collection (2011)	F20		16	**16**			+		+	+	
Collection (2012)	F21		514	**191**			(+)	+	+	+	+
Collection (2012)	F22		612	**72**			+		+	+	
Collection (2012)	F23		100	**100**			+		+	+	+
Collection (2012)	F24		604	**25**					+	+	
Collection (2010)	F25		605	*Assoc*			+		+	+	
Collection (2010)	F26		40	**40**	+		+		+	+	
Collection (2010)	F27		40	**40**	+		+		+	+	
Collection (2010)	F28		179	**16**	+	(+)	(+)	+	+	+	+
Collection (2013)	F29		97	**25**			(+)	+	+	+	+

Continued on next page

Blood	**Desig nation**	**Antibiotic resistance**	**ST**	**CC**	**Detected putative virulence genes**						
Source (isolated 2010-2013)					***esp***	***cyl*A**	***gel*E**	***fsr*C**	***efa*A**	***ace***	***asa*1**
Strain collection	B1	A,B	6	**6**	+	(+)	+		+		
Strain collection	B2	E	6	**6**			(+)	+	+		+
Strain collection	B3		27	*Assoc*			+		+	+	+
Strain collection	B4		21	**21**	+				+	+	+
Strain collection	B5		145	**21**	+				+	+	+
Strain collection	B6	A,D	6	**6**		(+)	+		+		
Strain collection	B7	A,C	6	**6**		+	+		+		+
Strain collection	B8		30	**30**	+		(+)	+	+	+	
Strain collection	B9		323	*Assoc*					+	+	
Strain collection	B10		16	**16**	+	+			+	+	+
Strain collection	B11	A,E	6	**6**		(+)	+		+		
Strain collection	B12		40	**40**	+	(+)	+		+	+	
Strain collection	B13		116	**116**			+		+	+	
Strain collection	B14		21	**21**			+		+	+	
Strain collection	B15		16	**16**	+	+			+	+	+
Strain collection	B16		16	**16**	+	+			+	+	+
Strain collection	B17		41	**41**	+		(+)	+	+		+
Strain collection	B18	E	6	**6**	+	(+)	+		+		
Strain collection	B19		610	**165**			+		+	+	
Strain collection	B20		624	*Single*			+		+		
Strain collection	B21		81	**81**		+	+		+	+	
Strain collection	B22	A,B	6	**6**		+	+		+		
Strain collection	B23	A,B	6	**6**		+	+		+		
Strain collection	B24		376	*Single*			+		+	+	
Strain collection	B25		79	*Assoc*			+		+	+	
Strain collection	B26	A,B	6	**6**		+	+		+		+
Strain collection	B27		179	**16**	+	+	(+)	+	+	+	+
Strain collection	B28		19	**19**			+		+	+	+
Strain collection	B29		220	**40**	+	+	+		+	+	
Strain collection	B30		25	**25**	+		(+)	+	+	+	

4.3.2 Number of putative virulence genes depending on source of isolation (PAPER III)

The isolates from food products carried in average the highest number of putative virulence factors, followed by strains recovered from blood, stool and root canals (Table 12).

Table 12. Descriptive data on virulence determinants per source, not counting *gel*E whilst present together with the deletion in the region *ef*1841/*fsr*C.

Source of isolation	Average number of putative virulence factors	Min-max	Median number of putative virulence factors
Root canal	3.1	2-4	3
Stool	3.4	2-6	3
Blood	3.6	2-6	3.5
Food	3.8	3-5	4

4.3.3 Distribution of virulence genes (PAPER III)

The distribution of virulence genes depending on the source of isolation is depicted in Figure 7, based on the results in Table 11.

All root canal isolates lacked the gene *cyl*A, which was a significant distinction compared to isolates from food and blood (p=0.0019 and p=0.0002, respectively). Half of the root canal strains carrying *gel*E also harboured the deletion in the region *ef*1841/*fsr*C. Consequently, 60% of the root canal strains were not capable to express gelatinase. The low ability of the endodontic strains to express gelatinase greatly contrasted to the strains from stool, which had a greater capacity to produce gelatinase ((75.9%)(p=0.0082)).

The highest prevalence for *esp* and *cyl*A was recorded for the strains recovered from blood. The difference in the presence of these two genes was significantly higher when compared to the isolates from stool (p=0.047 for *esp*; p=0.048 for *cyl*A). Conversely, *ace* was significantly less prevalent in the strains recovered from blood compared to the isolates from other groups (p=0.0003 vs. root canals; p=0.0034 vs. food and p=0.0102 vs. stool).

Although not significant, *asa*1 was most prevalent in food strains (68.4%). The gene *efa*A was detected in all isolates.

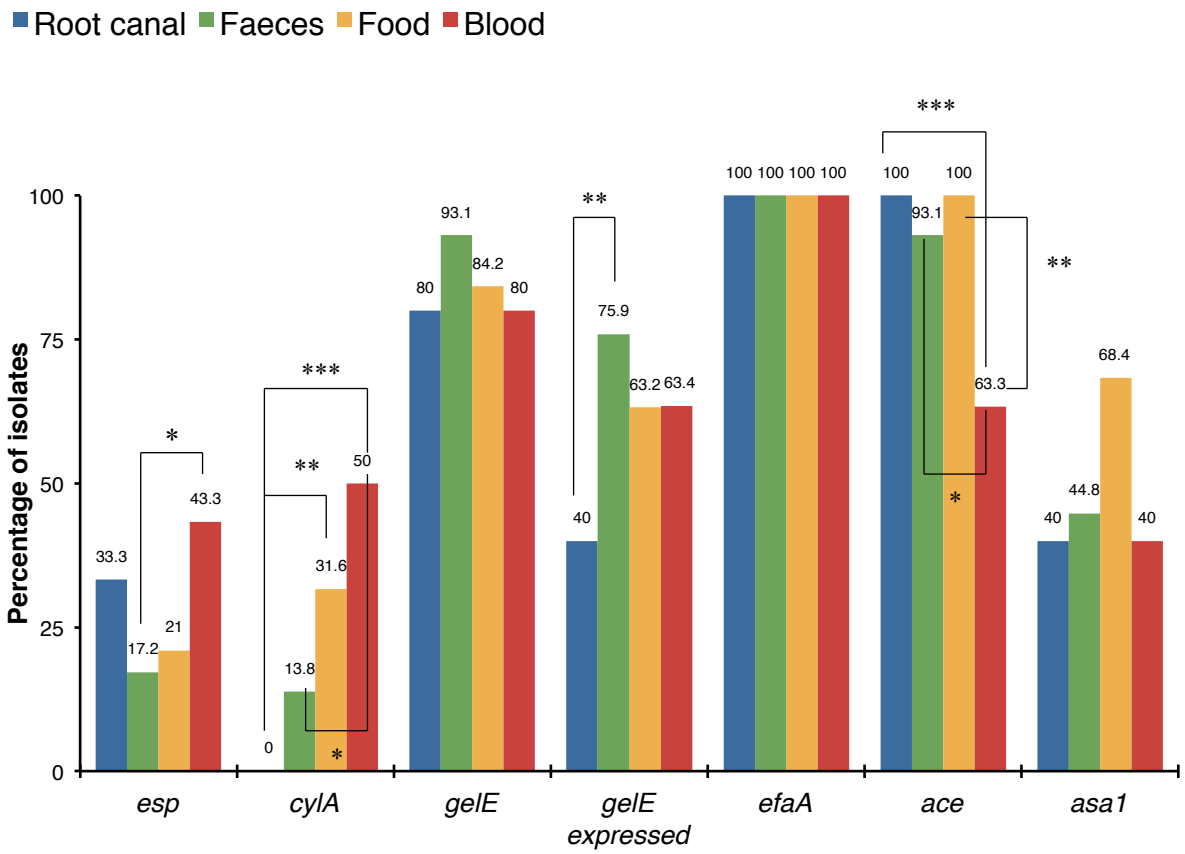

Figure 7. Distribution of putative virulence factors according to the site of isolation and based on the number of isolates carrying the respective gene/total number of isolates included in the group. Significant differences ($p \leq 0.05$) between the fractions for each gene studied are marked according to level of significance: * p value ranging from 0.05 to 0.01; ** p value ranging from 0.01 to 0.001; *** p value ranging from 0.001 to 0.0001.

4.3.4 Virulence gene patterns (PAPER III)

The virulence factor patterns observed are shown in Table 13. The pattern consisting of the combination of genes *gel*E, *efa*A and *ace* was prevalent in all groups but significantly greater in strains from root canals (p=0.0073), stool (p=0.0009) and food (p=0.0069) compared to the blood isolates. Altogether, the blood isolates displayed a greater diversity in gene patterns and were more often enriched with *esp* and/or *cyl*A compared to the other groups.

Table 13. Prevalence of virulence gene patterns according to source of isolation and in total. The most prevalent gene combination in each group is highlighted in bold and italic. The combination of genes *gel*E, *efa*A and *ace*, which was prevalent in all groups, is marked in black. The number of different gene patterns in each group reflects the degree of diversity between isolates from the same source. The presence of the deletion in the region *ef*1841/*fsr*C is denoted *fsr*C.

Virulence factor pattern	Root canal	Stool	Food	Blood	Prevalence in all samples (108)
*gel*E *efa*A *ace*	20% (6/30)	***38% (11/29)***	15.8% (3/19)	***16.7% (5/30)***	23.1%
*gel*E *efa*A *ace* *asa*1	16.6% (5/30)	10.3% (3/29)	***21% (4/19)***	6.7% (2/30)	13%
*gel*E *fsr*C *efa*A *ace* *asa*1	16.6% (5/30)	13.8% (4/29)	5.3% (1/19)		9.3%
esp *gel*E *fsr*C *efa*A *ace*	***23.3% (7/30)***			6.7% (2/30)	8.3%
*efa*A *ace* *asa*1	6% (2/30)	3.4% (1/29)	15.8% (3/19)		5.5%
esp *gel*E *efa*A *ace*	3.3% (1/30)	10.3% (3/29)	5.3% (1/19)		4.6%
*efa*A *ace*	6% (2/30)	3.4% (1/29)		3.3% (1/30)	3.7%
*cyl*A *gel*E *efa*A				13.3% (4/30)	3.7%
esp *cyl*A *gel*E *fsr*C *efa*A *ace* *asa*1		3.4% (1/29)	5.3% (1/19)	3.3% (1/30)	2.8%
esp *cyl*A *gel*E *efa*A *ace*			5.3% (1/19)	6.7% (2/30)	2.8%
*cyl*A *gel*E *efa*A *ace* *asa*1		6.9% (2/29)	5.3% (1/19)		2.8%
esp *cyl*A *efa*A *ace* *asa*1				10% (3/30)	2.8%
*cyl*A *gel*E *fsr*C *efa*A *ace* *asa*1			10.5% (2/19)		1.8%
*cyl*A *gel*E *efa*A *ace*			5.3% (1/19)	3.3% (1/30)	1.8%
*cyl*A *gel*E *efa*A *asa*1				6.7% (2/30)	1.8%
esp *cyl*A *gel*E *efa*A				6.7% (2/30)	1.8%
esp *efa*A *ace* *asa*1				6.7% (2/30)	1.8%
esp *efa*A *ace*	6% (2/30)				1.8%
*gel*E *efa*A		3.4% (1/29)		3.3% (1/30)	1.8%
esp *cyl*A *gel*E *efa*A *ace* *asa*1		3.4% (1/29)			0.9%
esp *gel*E *efa*A *ace* *asa*1			5.3% (1/19)		0.9%
esp *gel*E *fsr*C *efa*A *asa*1				3.3% (1/30)	0.9%
*gel*E *efa*A *asa*1		3.4% (1/29)			0.9%
*gel*E *fsr*C *efa*A *asa*1				3.3% (1/30)	0.9%
Diversity (Number of patterns)	8	11	11	15	
Prevalence of *gel*E, *efa*A, *ace*	80% (24/30)	86.2% (25/29)	84.2% (16/19)	43.3% (13/30)	

4.3.5 Resolution of the strain collection into genetic lineages by MLST (PAPER IV)

MLST resolved the strain collection, encompassing 108 isolates, into 43 STs already present in the PubMLST database (http://pubmlst.org/efaecalis) and 20 novel STs, specifically ST593 to ST599, ST 602 to ST613 and ST624 (Figure 8).

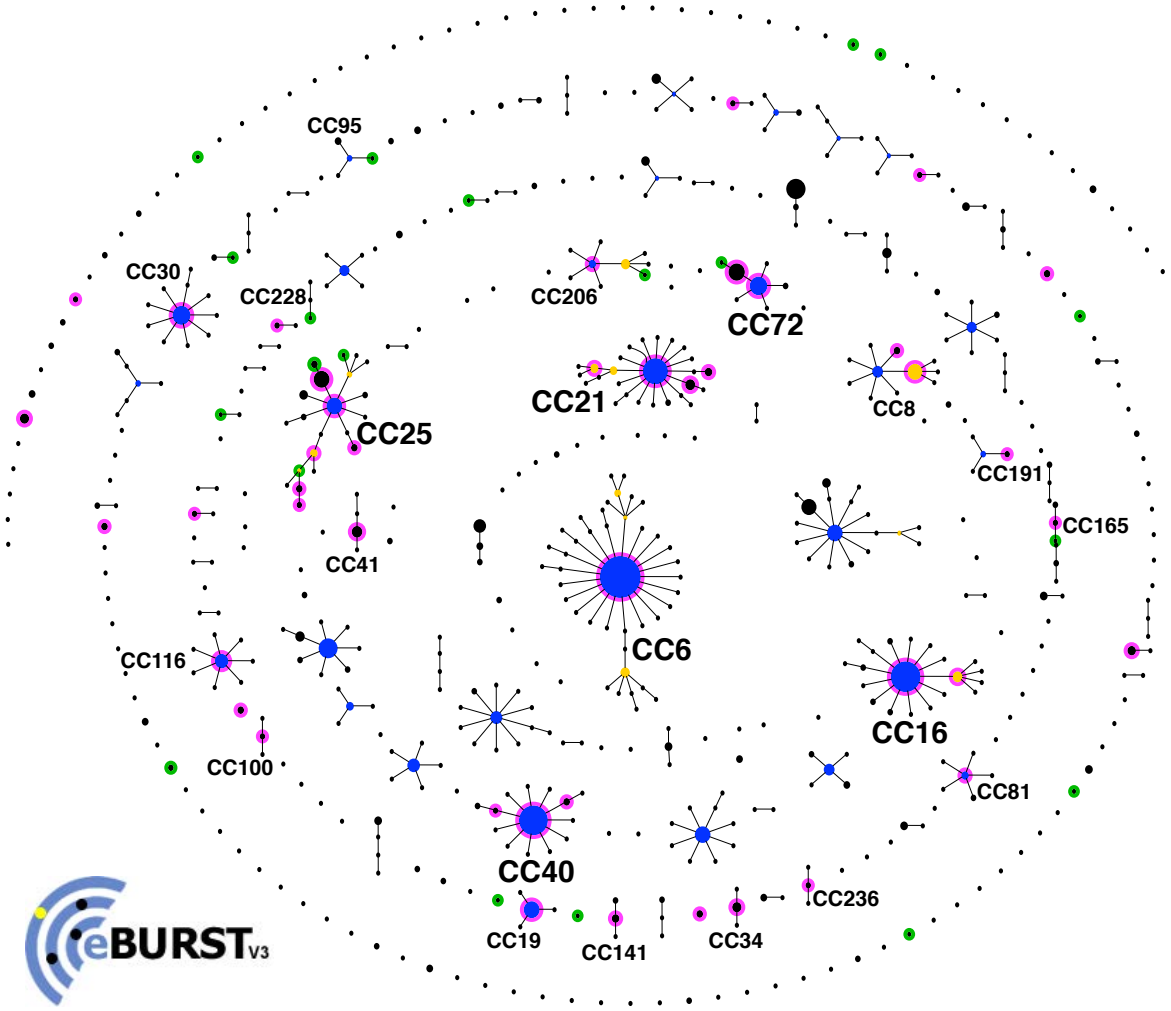

Figure 8. Allele-based population snapshot generated using eBURST. The strain collection was compared to a reference dataset, consisting of all 1321 isolates contained in the PubMLST database as per date of acquisition 2015-02-05. STs present in both the strain collection and the reference dataset are highlighted in pink, whereas novel STs existent in the strain collection are marked in green. The CCs, to which the collection of strains mainly associated, are designated with the number corresponding to the ST of the predicted founding genotype, highlighted in blue. The size of the nodes corresponds to number of isolates. The yellow nodes indicate the presence of subgroup founders.

Analysis of population structure and genetic relatedness using eBURST in conjunction with the reference dataset, comprised of 1321 isolates forming 78 groups, revealed that 76.9% (83/108) of the investigated isolates grouped into 21 CCs (Table 14). A total of 66.7% of the root canal isolates clustered, together with 42.1% of the food strains, 34.5% of the stool isolates and 10% of the blood strains into CC25, CC40 and CC72. The blood isolates mostly associated with CC6, CC16 and CC21 (53.3%).

Table 14. Clustering of the 108 *E. faecalis* isolates into 21 clonal clusters (CCs) according to location of isolation. Isolates assigned to the same CC shared identical alleles at ≥ 6 of 7 loci. The majority of isolates are shaded in grey and the resulting major CCs are marked in bold.

Assignment according to eBURST	**Source of isolation**				**Prevalence of assignment among all 108 isolates**
	Root canal	**Food**	**Stool**	**Blood**	
CC25	40% (12/30)	21% (4/19)	13.8% (4/29)	3.3% (1/30)	19.4%
CC40	10% (3/30)	10.5% (2/19)	10.3% (3/29)	6.7% (2/30)	9.3%
CC72	16.7% (5/30)	10.5% (2/19)	10.3% (3/29)		9.3%
CC6				30% (9/30)	8.3%
CC21	10% (3/30)		3.4% (1/29)	10% (3/30)	6.5%
CC16			6.9% (2/29)	13.3% (4/30)	5.6%
CC206	3.3% (1/30)	10.5% (2/19)			2.8%
CC8	6.7% (2/30)				1.8%
CC165	3.3% (1/30)			3.3% (1/30)	1.8%
CC19		5.3% (1/19)		3.3% (1/30)	1.8%
CC141		5.3% (1/19)			0.9%
CC30				3.3% (1/30)	0.9%
CC41				3.3% (1/30)	0.9%
CC81				3.3% (1/30)	0.9%
CC116				3.3% (1/30)	0.9%
CC34			3.4% (1/29)		0.9%
CC100			3.4% (1/29)		0.9%
CC191			3.4% (1/29)		0.9%
CC228			3.4% (1/29)		0.9%
CC236			3.4% (1/29)		0.9%
CC95	3.3% (1/30)				0.9%
Group without founder	6.6% (2/30)		13.8% (4/29)	10% (3/30)	8.3%
Singletons		36.8% (7/19)	24.1% (7/29)	6.6% (2/30)	14.8%

An exploration of the linkage between double-locus variants (DLVs) in eBURST, using a more relaxed group definition consisting of identical alleles at 5 of 7 loci as presented by blue connecting lines in Figure 9, suggests a connection between CC25, CC206 and CC72 via CC41 to CC21, CC8 and CC191. Furthermore, CC6 and CC16 were linked, whereas CC40 comprised an own group including CC9 among others.

Taking the linkages to CC25 into account, 76.7% of the root canal isolates grouped together with 42% of the food strains, 30.9% of the stool isolates and 16.6% of the blood isolates. The isolates encompassed in CC6 and CC16 accounted for 43.3% of the blood isolates and 6.9% of the strains retrieved from stool. CC40 was comprised of an almost equal proportion of isolates from the four sources.

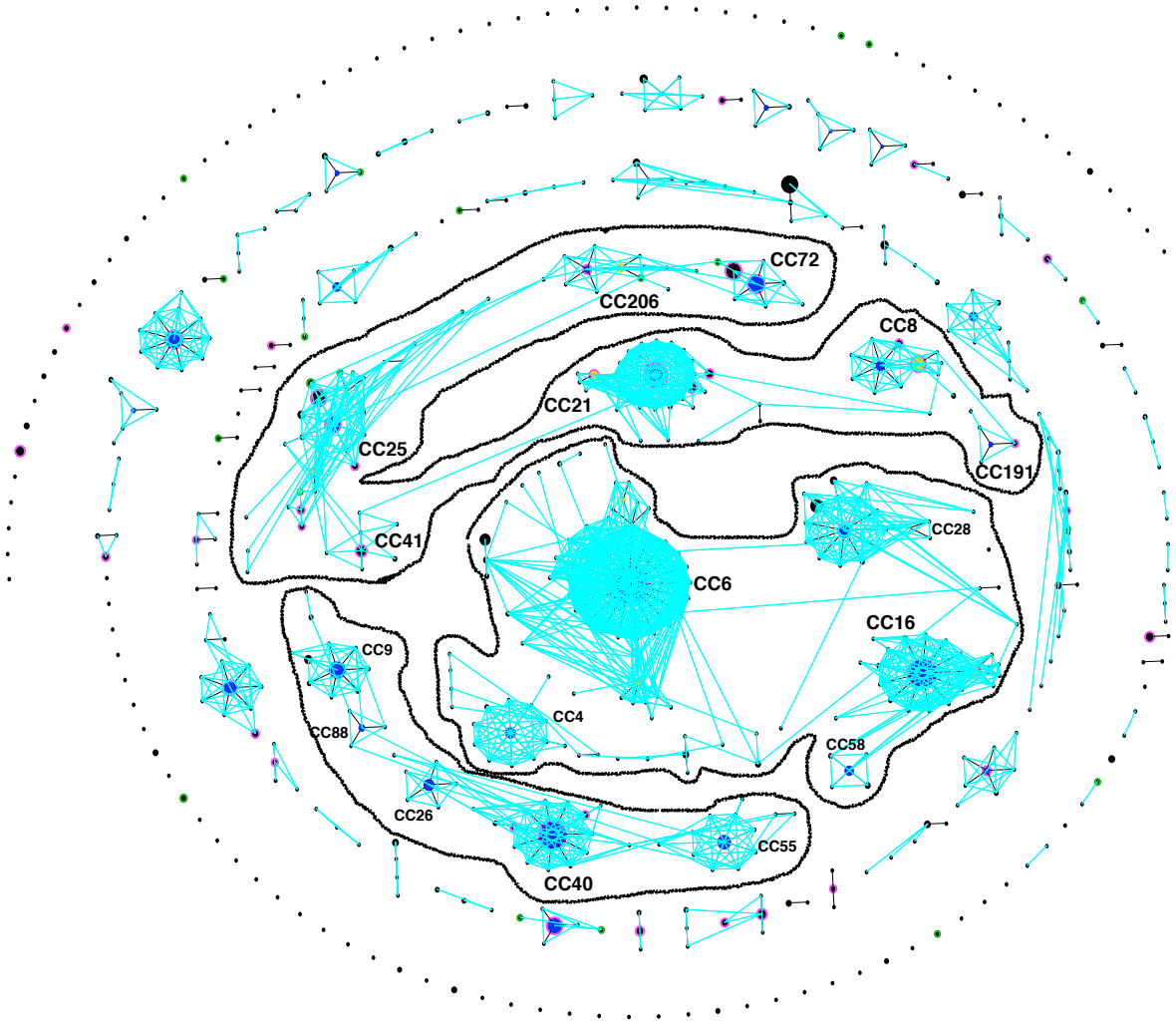

Figure 9. Population snapshot showing the relationship between STs, analysed in eBURST by interconnecting DLVs, presented as blue lines. Black lines encircle the groups resolved by using the more relaxed 5/7-group definition.

4.3.6 Distribution of virulence determinants in the identified major CCs (PAPER IV)

The putative virulence factors detected were, in addition to the recorded expression of β-haemolysis, correlated to the six most prevalent CCs, thereby revealing distinctive genotypic features of the CCs (Figure 10). The blood strains encompassed in CC6 all lacked the gene *ace.* All of the isolates in CC25 had the chromosomal deletion in the region encoding for the gelatinase regulator. Conversely, the deletion was not detected at all in the isolates associated to CC40 and CC72. The highest prevalence of the putative virulence genes *esp*, *cyl*A, *ace* and *asa*1 was demonstrated in strains associated with CC16. Isolates in CC40 were often enriched with *esp*, *gel*E and *ace*. The strains clustering into CC6 and CC16 and expressing cytolysin were all isolated from bloodstream infections. The strains expressing cytolysin in CC25 and CC72 were recovered from food.

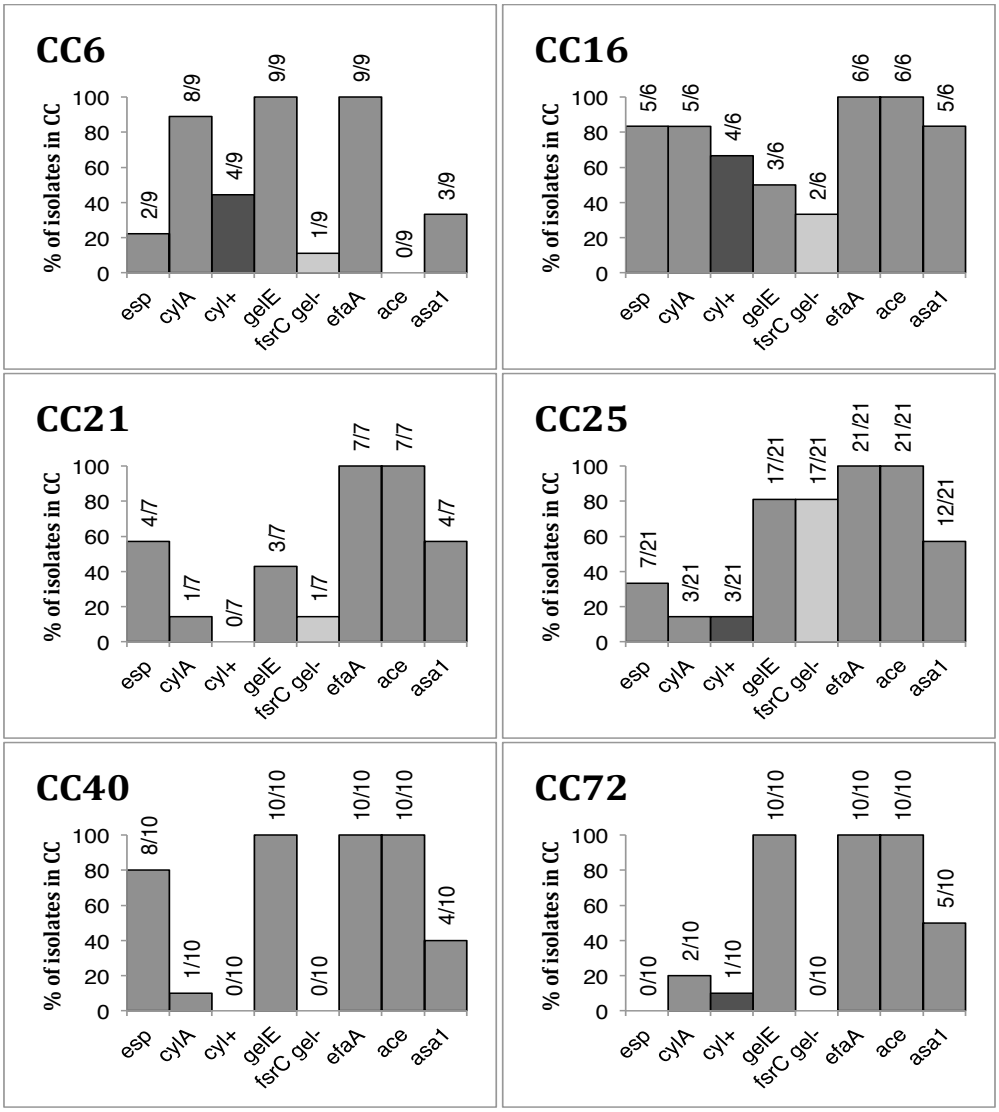

Figure 10. Correlation of identified putative virulence genes and cytolysin expression to the prevalent CCs. The bars in dark grey represent the proportion of isolates that demonstrated expression of cytolysin by showing β-haemolysis on blood agar. The bars in light grey denote the proportion of isolates displaying the deletion in ef1841/fsrC.

4.3.7 Antibiotic resistance (PAPER III and IV)

Resistance to ciprofloxacin in the range of 32 mg/L to 256 mg/L and often in combination with high-level gentamicin resistance (HLGR; MIC >256 mg/L) was detected in nine (30%) of the analysed blood isolates and in one (3.3%) of the root canal strains (Table 11). HLGR was present in one (5.3%) of the assessed food strains. Otherwise, the majority of isolates evaluated were susceptible to all tested antibiotics. MICs needed to inhibit growth of 50% and 90% of the analysed strains, along with range and median MIC values, are presented in Table 15.

All of the blood isolates exhibiting antibiotic resistance had the allelic combination of housekeeping genes corresponding to ST6, forming CC6, which is a known cluster comprised of hospital-adapted strains (Ruiz-Garbajosa *et al.* 2006). The single root-canal isolate demonstrating HLGR and resistance to high levels of ciprofloxacin belonged to another high-risk clonal complex, CC8, known to encompass strains with resistance to multiple antibiotics (McBride *et al.* 2007). The food isolate presenting antibiotic resistance was a singleton according to eBURST analysis.

Table 15. Descriptive statistics for the antibiotic susceptibility testing performed on the 108 *E. faecalis* strains.

MIC values in mg/L	Ampicillin	Piperacillin-tazobactam	Imipenem	Gentamicin	Ciprofloxacin	Linezolid	Vancomycin
Min	0.5	1	0.5	1	0.25	1	0.5
Max	2	8	2	>256	256	2	2
MIC_{50}	1	4	1	8	1	2	1
MIC_{90}	1	8	2	16	4	2	2

5 DISCUSSION

DNA fingerprinting of root canal isolates in comparison to strains recovered from stool, representing the commensal intestinal flora, failed to provide evidence for an endogenous source for *E. faecalis* in infected root-filled teeth (PAPER I). The inability to establish a genetic relatedness between isolates from root canals and the patient's own commensal intestinal flora could be attributed to bias as a result of a low number of isolates. However, precluding an endogenous route of infection, the microorganism was not to be found in the stool samples from two of the patients with *E. faecalis* present in the root canals. In a third patient, only one single bacterial colony could be harvested subsequent to enrichment. The dominating enterococcal species in the stool samples from these three patients was *E. faecium*, which was in line with an observed shift in the intestinal flora towards *E. faecium* in some individuals and some countries (Devriese & Pot 1995). Concurrently, such a shift has not been reported in root canal infections. *E. faecalis* is the still the most commonly isolated enterococcal species, with *E. faecium* being rarely encountered. In addition, the microorganism could not be detected in any of the screened saliva samples, which was in accordance with the now established transient presence of *E. faecalis* in the oral cavity (Aas *et al.* 2005). Taken together, the results consequently pointed to an exogenous origin for the endodontic isolates, greatly contrasting to the previous assumption that *E. faecalis* originated from the commensal oral flora (Engström 1964, Stuart *et al.* 2006), an implication of the incorrect classification of enterococci as streptococci, since streptococci comprises a large proportion of the cultivable commensal oral flora.

A potential exogenous source for *E. faecalis* that needed to be investigated was environmental surfaces in dental clinics, since those in analogy to environmental surfaces in hospitals could enable a nosocomial transmission. Supporting this notion was the paradox presented by the frequent recovery of the microorganism from previously root-filled teeth but rarely from untreated root canal infections, which could be explained by a possible recontamination occurring during or after a root canal treatment (Siren *et al.* 1997). Thus it seemed possible that *E. faecalis* dwelling on environmental surfaces could be transferred to infect root canals in the course of a treatment via contaminated hands or instruments as showed to occur in hospital settings (Hayden 2000). The potential for such a transmission was however estimated to be very low, since environmental sampling of high-touch surfaces in both general dentistry clinics and specialist clinics, directly following a root canal treatment only detected a very low occurrence (0.9%) of *E. faecalis* (PAPER II). The results in this study could have been skewed by only including clinics that were willing to participate, thereby excluding clinics with a perceived low hygienic standard. The participating clinics, knowing that the environmental sampling targeted the efficacy of disinfection measures could have also surely adapted an increased level and awareness of hygiene. Moreover, the inherent errors when using culture-based techniques for sampling may have resulted in an inability to detect the microorganism present because of numbers below the detection limit. For instance, the recovery of microorganisms from dry surfaces using a swabbing technique could certainly have resulted in an inability to mirror the true

bacterial composition of the sampled site, since it has been shown to at best capture a quarter of the original inoculum (Moore & Griffith 2007). It is therefore possible that the actual occurrence of *E. faecalis* on high-touch surfaces in dental operatories was higher than demonstrated. Contradicting this however, are the clear deficiencies in disinfection measures observed among the majority of the participating general dentistry clinics as opposed to the specialist clinics and the circumstance that *E. faecalis* was not a difficult organism to cultivate or detect in samples, especially when using selective growth media. Furthermore, the aim was to test the potential for transmission by hands or instruments touching a contaminated surface and in this respect, the inability to capture or transfer a contaminant by chance was an integral component in the equation. A molecular technique, such as a 16S rRNA based approach, would otherwise have been better suited, although potentially confounded by its inability to distinguish between viable and non-viable bacteria or DNA-contamination. Alas, at present there are at no other studies, measuring contamination with *E. faecalis* in the dental setting, to compare the obtained results with. Hence, it is so far likely that the occurrence of *E. faecalis* on surfaces in dental operatories, in comparison to hospital settings, where a contamination with faecal matter is more likely to occur, is too low to regard a nosocomial transmission of *E. faecalis* as a credible explanation for the high prevalence in secondary root canal infections.

The concept of coronal leakage, meaning the leakage of bacteria and their by-products via restorations, voids, caries lesions or cracks in the tooth crown, as a cause for reinfection of root canal-treated teeth has been suspected in previous studies, which recorded culture reversals with a sudden occurrence of *E. faecalis* in teeth treated in multiple visits (Sjögren *et al.* 1991, Sundqvist *et al.* 1998). Supporting this notion was a study that reported a correlation between the presence of *E. faecalis* in root canal infections and the occurrence of a lost or compromised coronal seal (Siren *et al.* 1997). The reason for the frequent presence of *E. faecalis* in root-filled canals but not in untreated ones or the oral cavity for that matter would thus seem to be the eradication of a plethora of different genera, comprising the flora in the two latter, offering colonization resistance. Hence, only endodontically treated canals, stripped of competing microorganism, appeared to constitute a niche that *E. faecalis* strains, opportunistic in nature, were capable of colonizing and overtaking. However plausible, the origin of *E. faecalis* in root canal infections remained unknown to provide a complete picture. An *in vitro* study, showing that *E. faecalis* contained in food items might leak through temporary fillings, led to the proposal of food-borne route of transmission (Kampfer *et al.* 2007). An explanation for the reported discrepancy in prevalence of *E. faecalis* in primary and secondary root canal infections that remained to be explored was thus the likelihood for endodontic *E. faecalis* to originate from foods.

Determination of putative virulence genes in *E. faecalis* has been applied extensively in molecular epidemiological surveys throughout the years. The results have been used in an attempt to determine the origin of isolates based on the assumption that isolates of common origin also share the same prevalence of various virulence genes. As such, a high prevalence of potential virulence determinants was more often associated with isolates originating from

hospitals and clinical infections than with strains from food, followed by the intestinal tract and the environment (Eaton & Gasson 2001, Creti *et al.* 2004, Abriouel *et al.* 2008). A lack of molecular epidemiological surveys targeting the distribution of virulence determinants amongst endodontic *E. faecalis* isolates in direct comparison to strains retrieved from food products was the incentive behind the third study.

The screening of *E. faecalis* isolates from root canals, food, stool and blood for putative virulence factors however failed to provide clear evidence for a food-borne transmission based on a congruence of harboured genes (PAPER III). The food strains were in average equipped with far more genes encoding potential virulence determinants than strains retrieved from root canal infections. The only similarity detected when comparing the distribution of virulence genes, suggesting a possible relatedness, was a common genotypic pattern consisting of a combination of the genes *gel*E, *efa*A and *ace*, which was shared among over 80% of the isolates from root canals, food and stool, in comparison to 43.3% of the isolates from blood (Table 13). The found association in genotypic pattern appears plausible, considering that ingestion of food items containing *E. faecalis* has been shown to result in a transient presence of food strains in the oral flora (Razavi *et al.* 2007). Consequently, a repeated ingestion of foods containing *E. faecalis* would certainly increase the likelihood for food strains ending up in a root-filled tooth via coronal leakage. Furthermore, a linkage between food and stool was probable, since food strains after oral transit have been shown to transiently blend in and interact with the commensal flora, with a potential to become permanent in the intestinal tract by colonization or replacement of resident strains in times of ecological disturbances, for instance evoked by antibiotic therapy (Dever & Handwerger 1996, Lund *et al.* 2002b, Gelsomino *et al.* 2003). It is even possible, that the *E. faecalis* strains retrieved from food items, in fact were contaminants derived from the environment or human and animal commensal flora, since they were present in the majority of the screened food items together with *E. faecium*, *E. durans* and on occasions also *E. hirae* (data not shown). Hence, the food chain may act as an important vector in the dissemination of *E. faecalis* from different compartments, enabling the circulation of strains between animals, environment and humans.

In relation to the analysed strains from stool and blood, the food strains demonstrated the highest number of detected virulence genes and the root canal strains the lowest. Previous studies have shown, in agreement with the results presented herein, that root canal isolates possess a low number of putative virulence factors and completely lack the ability to express cytolysin (Sedgley *et al.* 2005, Penas *et al.* 2013). The previously proposed correlation between gelatinase expression and the pathogenesis of apical periodontitis (Wang *et al.* 2011, Zoletti *et al.* 2011) could however not be verified. In accordance with the study by Zoletti *et al.*, 50% of the endodontic isolates investigated had the deletion in the *fsr*C locus whilst harbouring the gene encoding gelatinase. Hence, only 40% of the strains recovered from root canals of teeth displaying clear radiographic signs of bone resorption around the root-end were actually able to express gelatinase. In comparison, a significantly larger proportion of strains isolated from stool were able to produce gelatinase. The inability of the majority of

endodontic isolates to express gelatinase does not seem to support the assumption that gelatinase-expression would be coupled with biofilm formation, assumed to be of great importance in treatment-resistant root canal infections (Hancock & Perego 2004, Mohamed *et al.* 2004, Distel *et al.* 2002). This notion is corroborated by studies demonstrating the ability to form biofilms is extremely common among *E. faecalis* isolates irrespective of source of isolation and the presence of the genes *esp* and *gel*E (Creti *et al.* 2004, Di Rosa *et al.* 2006). Instead other genes, such as *bop*D (biofilm on plastic surfaces), locus *epa* (enterococcal polysaccharide antigen) and the *bee* gene cluster (biofilm enhancer in *Enterococcus*), are more likely to be implicated in biofilm formation (Hufnagel *et al.* 2004, Tendolkar *et al.* 2006, Teng *et al.* 2009). Gelatinase and cytolysin therefore seem to be of little importance for *E. faecalis* strains in endodontic infections. This conclusion appears reasonable bearing in mind that *E. faecalis* in root canal infections are confined to the treated root canal, which in most cases is devoid of substantial amounts of degradable tissue components or a plethora of competing microorganisms. Hence, the need for proteolytic and cytolytic enzymes appears limited. Further, the commonly reported low prevalence of putative virulence factors in food isolates could not be ascertained by the results in this thesis. Instead, the findings are a source of concern, since 68.4% of the food strains harboured the pheromone-inducible aggregation factor *asa*1. Aggregation substance has been shown to facilitate conjugation by aggregating *E. faecalis* strains, thereby increasing the likelihood for an exchange of potential virulence determinants and antibiotic resistance to occur (Eaton & Gasson 2001, Waters & Dunny 2001). The food strains in the present collection, possessing several of the investigated putative virulence genes, thus harboured a disturbing potential to adapt to various environments, induce disease and, most importantly, transfer virulence genes onwards to the resident flora during intestinal passage. These results are corroborated by other studies that underpin food safety issues and rule out *E. faecalis* achieving the GRAS status (Eaton & Gasson 2001, Franz *et al.* 2001, Templer *et al.* 2008).

Antibiotic susceptibility testing could not provide indications on relatedness. The vast majority of strains, except a proportion encompassing 30% of the blood isolates, were susceptible to the tested clinically relevant antimicrobial compounds. Although the strains exhibiting antibiotic resistance were believed to comprise a hospital-adapted clone, this could not be verified by the analysis of virulence determinants. The difficulty in drawing conclusions on origin based on the prevalence of potential virulence genes has also been perceived in numerous studies (Coque *et al.* 1995, Duprè *et al.* 2003, Bittencourt de Marques & Suzart 2004, Johansson & Rasmussen 2013). A reason for this is most likely the extraordinary genomic plasticity of *E. faecalis*, which enables it to continuously improve adaptation to different situations and environments by incorporating and sharing virulence determinants and antibiotic resistance genes (Coburn *et al.* 2007). The combination of factors, enhancing infection and colonization potential when expressed together in the right background shaped by the interaction with the host or ecological niche, is in the light of genomic plasticity most probably not static. The ability of *E. faecalis* to successfully adapt has been attributed to an assumed propensity to constantly acquire new traits and resistances.

Research on *Escherichia coli*, another commensal with pathogenic potential, has however demonstrated that a capability to reduce the genomic content was equally important for adaptation and fitness (Dobrindt *et al.* 2010). The low prevalence of virulence factors amongst the root canal strains might therefore be explained by a deletion of genes in response to the barren conditions of the root-filled canal. Genome reduction as a survival strategy, although yet not shown for *E. faecalis*, is a reasonable key mechanism to conserve resources and energy and could provide a justification for the occurrence of different virulence gene profiles according to site of isolation although a clonal relationship exists. In this context, it may be plausible to believe that the environmental conditions in endodontically treated canals select for *E. faecalis* strains able to successfully adapt. PFGE analysis concluded that the enterococcal strains recovered from one and the same tooth were identical or at least closely genetically related (Table 10). Assuming that endodontic *E. faecalis* were food-borne, one would expect a greater genetic heterogeneity than actually observed, since the food items generally contained a mixture of different enterococcal strains and species. A plausible explanation for this finding, besides the occurrence of yet unknown factors crucial for colonization and survival, might be that only strains capable of genome reduction as a mechanism of adaption were able to survive in the root canal. Future studies are needed to address this observation, as well as, to explain the predisposition for *E. faecalis* in contrast to other enterococcal species to inhabit the root-filled canal space.

Altogether, considering the inherent genomic malleability of *E. faecalis* by recombination, epidemiological surveys aiming at elucidating the origin of strains should be conducted using molecular techniques establishing relatedness by comparing less variable regions of the genome. MLST, being such a technique, was able to provide a clearer picture of population structure and genetic relatedness amongst the analysed strain collection and strengthen the observed association between isolates from root canals, food and stool (PAPER IV). The linkage was most likely made possible by the fairly sized collection of strains, retrieved from a limited geographical location, in combination with the MLST database now finally containing a sufficient number of STs forming CCs. Previous epidemiological typing of *E. faecalis* were certainly hampered by an inadequate number of strains included in the database, thus resulting in a high proportion of STs not grouping and therefore being defined as singletons. In the present strain collection, 36.8% of the food isolates and 24.1% of strains from stool were categorized as singletons. It is possible that a continuous addition of strains to the MLST database in the future will reduce these proportions and further confirm a plausible relatedness between strains isolated from these two loci, thereby extending our knowledge on the impact of food microflora on the commensal flora and the risks associated with foods containing *E. faecalis*.

Analysis of MLST data using the algorithm eBURST demonstrated that 66.7% of the endodontic strains clustered into the same CCs as 42.1% of the food isolates and 34.5% of stool isolates, namely CC25, CC40 and CC72 (Table 14). A proportion entailing 40% of the endodontic strains associated to CC25 along with 21% of the strains isolated from the screened food items. These results are in agreement with previous studies, demonstrating that

42.1% of root canal isolates and 20% of isolates from French cheeses grouped into CC25 (Jamet *et al.* 2012, Penas *et al.* 2013). Strains associating to CC40 and CC21 have been commonly isolated in Europe and the USA from animals, foods and hospitalized and non-hospitalized humans (Ruiz-Garbajosa *et al.* 2006, McBride *et al.* 2007, Solheim *et al.* 2011, Jamet *et al.* 2012). Specifically, CC40 has been connected with hospital-acquired strains in Poland and thus presumed to belong to the HiRECCs, an acronym for high-risk enterococcal clonal complexes (Kawalec *et al.* 2007). A mixed origin for strains in these two CCs was substantiated by the observed distribution into CCs, displayed in Table 14, and the linkage of DLVs by eBURST that revealed a connection between CC21 and CC25 among others. The suspected hospital-adaptation of CC40 was acknowledged, as CC40 seemed to form an own entity together with the known HiRECC CC9 (Figure 9). The most renowned hospital-adapted CC, namely CC6, was interconnected with CC16 and encompassed, without exceptions, all blood isolates presenting high-level resistance to gentamicin and ciprofloxacin.

Distinctive features of the CCs, to which a majority of investigated strains associated to, became evident when correlating the distribution of putative virulence factors to the resolved CCs (Figure 10). The ability to express cytolysin was, in accordance with previous studies, most prevalent in strains associating with CC6 and CC16 (McBride *et al.* 2007, Freitas *et al.* 2009). Moreover and in line with the result reported by McBride *et al*. 2007, a higher prevalence of genes coding for potential virulence traits was detected among strains in CC16, whereas the blood isolates clustering into CC6 were highly enriched with antibiotic resistance, indicative of a hospital-adaptation. Remarkably, all isolates in CC6 lacked the gene *ace*, implicated in virulence and the pathogenesis of endocarditis according to murine models (Lebreton *et al.* 2009, Singh *et al.* 2010), suggesting that the gene is not of importance in human bloodstream infections that potentially could result in endocarditis. An inability to express gelatinase due the chromosomal deletion in the region of *fsr*C was shared by all isolates that belonged to CC25 and harboured the gene for gelatinase. This characteristic of CC25 was supported by the results from a study that correlated the deletion to STs obtained by MLST (Galloway-Peña *et al.* 2011). In contrast, none of the strains in CC40 and CC72 had the deletion although all carried the gene encoding gelatinase. The inhibited expression of gelatinase has been suggested to facilitate pheromone-induced conjugation and subsequent horizontal gene transfer by increasing the level of active pheromones, which would otherwise have been degraded by gelatinase (Waters *et al.* 2003). As such, CC25 might comprise strains with an enhanced ability for horizontal gene transfer, especially since over half of the strains analysed also harboured a gene coding for aggregation substance. Future studies corroborating these findings would indeed aggravate the concerns with foods containing *E. faecalis*, since food strains reportedly seem to cluster into CC25.

The correlation of distribution of putative virulence determinants and antibiotic resistance to the results obtained from MLST analysed by eBURST demonstrated interesting and possibly distinctive features of the major CCs, not reported elsewhere, that could improve our

understanding of *E. faecalis* virulence potential and how it is related to population structure and heritage. Furthermore, such a correlation could explain the prevalence of observed gene deletions amongst CCs and specifically STs and in what way they relate to certain sites of isolation to address the question if a genome reduction occurs as an adaptive response to a specific environment or is an distinctive inherited hallmark of a particular CC. Unfortunately, the MLST database currently does not contain information on genotypic and phenotypic profiles that would enable a correlation to STs or CCs. Future efforts should thus be made to incorporate this data when available.

Epidemiological typing by MLST was able to provide support for an actual association between strains retrieved from root canals and food, suggesting that *E. faecalis* in root canal infections are most likely food-borne and gain entry to the treated root canals via micro-leakage through the tooth crown, thereby contributing to the concept of coronal leakage and emphasising the need for better ways to seal the root-filled canal. The concept of coronal leakage however has been long debated. A high long-term success rate for root canal treatment has been reported in numerous clinical studies (Friedman & Mor 2004). Hence, the three-dimensional seal provided by the root filling was regarded as "hermetic" and imperative to prevent infection. Igniting the dispute and contradicting the implied impermeable nature of the seal were laboratory dye-leakage studies on teeth demonstrating that a root filling, regardless of technique or materials used, leaks over time if exposed. A corroborating retrospective cross-sectional study based on radiographic data soon followed, showing that the technical quality of the coronal restoration was more important than the technical quality of the root canal treatment (Ray & Trope 1995). It is now, after an argument back and forth, generally accepted that the outcome can be correlated to both the quality of the root canal treatment and the subsequent coronal restoration (Gillen *et al.* 2011). Yet, coronal leakage is not emphasised equally in the dental universities. The custom to place a restoration directly over the root filling still prevails, although the method of additionally sealing off the root filling by removing a part of it at the canal orifice and placing a plug with a restorative material appears more effective in preventing a reinfection (Yamauchi *et al.* 2006). Nevertheless, more research is needed to prove which method and what material is better for preventing coronal leakage in a clinical situation, not only after a completed root canal treatment but also in-between treatment sessions. Root canal treatment is time consuming and costly and often forms the foundation for restorative efforts and prosthodontic replacement of lost teeth. The gain on a both personal and economic level is therefore tremendous if treatment failures can be avoided by taking relative simple measures, such as to place an additional seal over the root filling.

6 GENERAL SUMMARY AND CONCLUSIONS

Enterococcus faecalis has long been acknowledged as the "star" in secondary root canal infection, since it has the ability to withstand a root canal treatment and, most importantly, survive in the barren environment that root-filled canals pose. It owes its exceptional resilience to various intrinsic resistance mechanisms in combination with a highly malleable genome, which enables it to adapt to a wide variety of milieus by acquiring new traits, including antibiotic resistance. Hence, *E. faecalis* has also emerged as a clinical challenge in hospital settings, were it ranks as one of the most common nosocomial pathogens worldwide. It was for a long time assumed that *E. faecalis* in root canal infections were derived from the endogenous flora, since *E. faecalis* are natural inhabitants of gastro-intestinal tract. It was thus assumed that the microorganism would also pertain to the commensal oral flora. Recently though, the microorganisms has been established as being merely transient in the oral flora. Since, the origin of *E. faecalis* in root canal infections has remained unknown. Different explanations for the frequent encounter of the microorganism in secondary infections have been proposed, ranging from a nosocomial transmission occurring during a root canal procedure to a food-borne route of infection. In order to devise preventive measure to avoid supposedly treatment-resistant root canal infections with *E. faecalis* or other implicated microorganisms with the same mode of transmission, its source must first be identified. Hence, this became the overall aim of the conducted research.

The major conclusions that can be drawn from the results in this thesis are the following:

- Endodontic *E. faecalis* strains are likely not derived from the endogenous commensal flora of the gastro-intestinal tract.
- The potential for a nosocomial transmission during a root canal treatment from contaminated high-touch surfaces in dental operatories is probably small, in spite of deficiencies in desinfection measures.
- Decontamination procedures in general dentistry clinics, need urgent revision and monitoring, since the procedures resulted in an increased contamination.
- Food strains displayed in average the highest number of putative virulence factors, amongst them aggregation substance, thus raising concerns regarding food-safety.
- *E. faecalis* in root canal infections are most likely food-borne since strains from root canals and food items shared a common genotypic pattern and to non-negligible proportions belonged to the same genetic lineages, specifically CC25, CC40 and CC72. Still the root canal strains displayed the lowest prevalence of potential virulence determinants, presumably due to gene deletions as an adaptive response.
- The putative virulence determinants gelatinase and cytolysin are probably of less importance for endodontic *E. faecalis*.
- Antibiotic resistance to gentamicin and ciprofloxacin was mainly detected in a proportion of blood isolates, which all belonged to a renowned cluster of hospital-adapted clones, namely CC6, typically causing nosocomial infections.

- Distinctive features of the major identified CCs were observed. All isolates associated with CC6 lacked *ace* and those clustering into CC25 were unable to express gelatinase although they harboured *gel*E. Strains pertaining to CC16 were highly enriched with the virulence genes examined and all the isolates in CC40 and CC72 had the potential to express gelatinase.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING (SUMMARY IN SWEDISH)

Det övergripande syftet med forskningen var att söka efter ursprunget för tarmbakterien *Enterococcus faecalis* som i hög utsträckning påträffas i tidigare rotfyllda tänder som uppvisar tecken på infektion. Kunskap om dess ursprung och via vilka vägar den etableras i rotkanaler kan förhoppningsvis möjliggöra för riktade insatser att förhindra uppkomsten av rotkanalsinfektioner med enterokocker och möjligen även andra mikroorganismer som följer samma spridningsvägar.

Enterococcus faecalis är utrustad med egenskaper eller virulensfaktorer som gör att den kan motstå den kemo-mekaniska rotbehandlingen bättre än andra vanligt i rotkanalsinfektioner förekommande bakterier. Således uppfattas enterokocker som terapiresistenta och genererar en stor ekonomisk förlust i och med att kostsamma och tidskrävande rotbehandlingar misslyckas. Tidigare studier rapporterar en prevalens av bakterien på ungefär 40 % i tidigare rotbehandlade tänder samtidigt som mikroorganismen sällan påträffas i munhålan.

Den allmänna uppfattningen har varit och är fortfarande i hög grad att enterokocker associerade med rotkanalsinfektioner har ett endogent ursprung, det vill säga är patientegen. Enterokocker hör till den normala tarmfloran hos nästan alla däggdjur, inkluderandes människa, och fåglar. De är därmed vanligt förekommande i vår omgivning som ett resultat av kontamination från avföring. Samtidigt är det väl känt att infektioner med *E. faecalis*, som är ett växande medicinskt problem främst då de kan överföra antibiotikaresistens till andra bakteriearter, kan överföras nosokomialt, det vill säga som en sjukhusöverförd smitta. Enterokocker är i USA den andra eller tredje vanligaste nosokomialt överförda smittan och leder årligen till tusentals dödsfall i blodförgiftning. Trots att enterokocker har dubbla roller, både i hälsa och sjukdom, har de historiskt sett och fortfarande ett viktigt användningsområde i fermentationsprocessen av diverse livsmedel för att erhålla rätt konsistens, textur och arom. Dessa enterokocker är i regel utrustade med färre sjukdomsframkallande egenskaper eller virulensfaktorer i motsats till de som figurerar i sjukhussmittor och som har förvärvat en ökad virulens. Intag av livsmedel innehållandes enterokocker har föreslagits kunna utgöra en källa till enterokocker i rotkanalsinfektioner, eftersom läckage via otäta fyllningar eller sprickor i tänder kan möjliggöra en väg in till de behandlade rotkanalerna.

Frågeställningar som vi därmed ämnade besvara var om enterokocker i rotkanaler är patientegna (Studie I), nosokomialt överförda (Studie II) eller födoämnesrelaterade (Studie III och IV).

I avhandlingens första studie jämfördes *E. faecalis* stammar isolerade från rotbehandlade tänder med de från respektive patients egen normala tarmflora. Den genetiska släktskapsanalysen visade att enterokocker från rotkanalerna på patienterna inte var besläktade med de som återfanns i avföringen och slutsatsen blev att bakterien orsakandes svårbehandlade rotkanalsinfektioner sannolikt inte är patientegen.

Den andra studien undersökte om enterokocker som påträffas i rotbehandlade tänder överförts nosokomialt från ytor i behandlingsrummet via kontaminerade instrument eller operatörens händer i samband med en rotbehandling. Resultaten från denna studie visade att enterokocker inte var vanligt förekommande på ytor i behandlingsrummet, vilket kan antyda att rotkanalsinfektioner med enterokocker sannolikt inte är nosokomialt överförda. En annan iakttagelse som gjordes, var att hygienrutiner på undersökta allmäntandvårdskliniker, i motsats till specialistkliniker, inte var tillräckliga för att eliminera kontamination med bakterier på ytorna i behandlingsrummet. Rengöringsproceduren verkade snarare kontaminera ytor som tidigare inte uppvisade bakteriepåväxt. Denna studie visar således på ett behov av fler mikrobiologiska undersökningar på tandvårdskliniker, för att möjliggöra förbättringar i nuvarande hygienrutiner.

Målsättningen med forskningsprojektets tredje arbete var att försöka härleda ursprunget för enterokockerna från rotkanalerna genom att jämföra de mot de ofta högvirulenta enterokockerna från blodförgiftningar samt de vanligen lågvirulenta från normal tarmflora och fermenterade livsmedel, såsom ostar och charkuterier. Gener som kodar för tänkta virulensfaktorer kartlades och resistens mot kliniskt relevanta antibiotika undersöktes. Det visade sig att enterokocker isolerade från livsmedel, i motsats till tidigare studier, bar på i genomsnitt flest virulensfaktorer, vilket ifrågasätter säkerheten med livsmedel innehållandes bakterien. Blodisolaten var också bestyckade med ett stort antal möjliga virulensfaktorer. De särskilde sig dock markant från de andra isolaten, eftersom de med få undantag var de enda som uppvisade antibiotikaresistens, något som skulle kunna tyda på en anpassning till en sjukhusmiljö. En kombination av gener som antas vara av vikt för nedbrytning av gelatin, bindning till cellulära ytproteiner och kollagen var förekommande i lika hög grad bland bakteriestammar från rotkanaler, avföring och livsmedel, men inte bland isolat från blodförgiftningar. De studerade genprofilerna verkade således gruppera stammar från rotkanaler, avföring och livsmedel, vilket skulle kunna stärka antagandet att enterokocker i rotkanaler är livsmedelsburna. Skillnader i antalet virulensfaktorer skulle kunna bero på enterokockernas exceptionella förmåga till anpassning till föränderliga levnadsvillkor genom att plocka upp, men sannolikt även göra sig av med genetiskt material.

Den möjliga associationen mellan stammar från rotkanaler och livsmedel prövades i den fjärde studien genom en genetiskt baserad epidemiologisk typning för att möjliggöra identifiering av ursprung och geografisk spridning. Kollektionen med enterokockisolat indelades i klonala komplex, det vill säga grupper innehållandes besläktade stammar eller stammar från samma klon, och jämfördes mot en internationell databas med enterokocker från diverse härkomst, bland annat födoämnesrelaterade isolat och isolat från människa och djurbesättningar. Metoden visade att en betydande andel av isolaten från rotkanaler, livsmedel och avföring grupperade sig i samma klonala komplex. Blodisolaten som uppvisade antibiotikaresistens hörde som misstänkt till ett välkänt sjukhusanpassat klonalt komplex som orsakat utbrott i flera länder.

Sammanfattningsvis, verkar det sannolikt att enterokocker orsakandes kostsamma och ofta svårbehandlade rotkanalsinfektioner är livsmedelsburna och får möjlighet att infektera den rotbehandlade tanden genom läckage via tandkronan. Vidare forskning bör därför fokusera på utvecklingen av nya material eller metoder för att förhindra läckage och försegla de rotbehandlade kanalerna bättre.

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