TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Technische Mikrobiologie

Distribution of virulence factors in *Enterococcus faecalis* and its adaption to conditions in the intestinal tract

Angela Gerda Lindenstrauß

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. S. Scherer

Prüfer der Dissertation: 1. Univ.-Prof. Dr. R. F. Vogel

2. Univ.-Prof. Dr. D. Haller

Die Dissertation wurde am 28.03.2012 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 01.08.2012 angenommen.

Danksagung

Diese Arbeit entstand im Rahmen des Graduiertenkollegs 1482 "Mittlerfuktion des Darms zwischen luminalen Faktoren und Signalen des Wirts", welches durch die Deutsche Forschungsgemeinschaft finanziert wurde.

Ich möchte mich besonders bei Herrn Prof. Dr. Rudi F. Vogel bedanken, der mir das Thema zur Verfügung stellte. Ich danke für die zahlreichen wissenschaftlichen Anregungen, das Interesse an meiner Arbeit und die stete Bereitschaft zur fachlichen Diskussion.

Bei Herrn Prof. Dr. Dirk Haller bedanke ich mich für die Übernahme des Koreferats und für die Möglichkeit an seinem Lehrstuhl die Zellkulturversuche durchführen zu können.

Herrn Prof. Dr. Siegfried Scherer danke ich für die Übernahme des Prüfungsvorsitzes.

Prof. R. Balfour Sartor und seinem Team danke ich für die großzügige Zusammenarbeit und Gastfreundschaft in Chapel Hill.

Dr. Roger Vogelmann danke ich für die Bereitstellung der klinischen Isolate.

Für die unkomplizierte Hilfe mit den Transkriptomdaten danke ich Richard Landstorfer und Svenja Simon.

Dem gesamten Graduiertenkolleg, der eheml. Vorsitzenden Frau Prof. Dr. Hannelore Daniel und der Koordinatorin Dorothea Wörner danke ich sehr für die besondere Zeit.

Ich danke allen Mitarbeitern des Lehrstuhls für Biofunktionalität der Lebensmittel, besonders Sigrid Kisling, Micha Hoffmann, Natalie Steck, Marie-Ann von Schillde, Emanuel Berger, Nadine Waldschmitt und Nico Gebhardt.

Des Weiteren gebührt eine großer Dank apl. Prof. Dr. Matthias A. Ehrmann für seine Unterstützung bei manch unerwartetem Ereignis während der Laborarbeit und für die vielen hilfreichen Diskussionen.

Darüber hinaus gilt mein Dank natürlich allen Mitarbeiterinnen und Mitarbeitern des Lehrstuhls für Technische Mikrobiologie für die kollegiale Zusammenarbeit und das angenehme Arbeitsklima. Ich danke den TAs und Angela Seppeur für die vielen nützlichen Tipps und ihre Hilfsbereitschaft. Ebenso bedanke ich mich bei Agnes Bringmann, deren Screening-Ergebnisse im Rahmen ihrer Studienarbeit hier eingeflossen sind. Ein großer Dank gilt Georg Lutterschmid und Christian Lenz für die IT-Betreuung. Patrick Preissler und Jürgen Behr danke ich sehr für die Einarbeitung und die Hilfe mit der 2D-Anlage. Insbesondere geht ein Dank an Marina Lamparter, Simone Freiding, Sina Punke und Matthias Stübner.

Meiner Familie und Fabian danke ich ganz besonders für die Unterstützung während der Promotionszeit und vor allem in den letzten Wochen. Erst durch eure Unterstützung war diese Arbeit möglich.

List of Contents

Abbreviations

°C	degree Celcius
µ	micro (10-6)
µg	micorgram
µl	microlitre
%	percent
2D	two dimensional
APS	ammonium persulfate
ATCC	American type culture collection, Manassas, Virginia, USA
B.	*Bacillus*
BHI	brain heart infusion broth
BLAST	basic local alignment search tool
bp	base pair(s)
BSA	bovine serum albumin
Chaps	3-((3-Cholamidopropyl)dimethylammonium)-1-propanesulfonic acid
CoA	coenzyme A
Da	Dalton
DEPC	Diethylpyrocarbonate
DNA	desoxyribonucleic acid
dNTP	desoxy nucleotide triphosphate
DSMZ	German Collection of Microorganisms and Cell Cultures
DTT	dithiothreitol
E.	*Enterococcus*
EDTA	ethylendiaminetetraacetic acid
g	gram, g-force
GRAS	generally regarded as safe
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IEF	isoelectric focusing
k	kilo (103)
l	liter
LAB	lactic acid bacteria
LB	lysogeny broth
LC-ESI MS/MS	Liquid chromatography electrospray ionization tandem mass spectrometry
M	mega (106)molar, mol per litre
m	milli (10-3)
mA	milliampere
mg	milligram
min	minutes
ml	milliliter
mM	millimolar, millimol per litre
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time of flight mass spectrometry
mRNA	messenger ribonucleic acid
n	nano (10-9)
nm	nano meter
NCBI	National center for Biotechnology Information
OD	optical density

OD590	optical density at 590 nm wavelength
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PTS	phosphoenolpyruvate-dependent sugar phosphotransferase systems
RAPD	random amplification polymorphic DANN
RNA	ribonucleic acid
RPKM	reads per kilobase per million mapped reads
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute medium
S.	*Streptococcus*
Staph.	*Staphylococcus*
sec	second
SDS	sodium dodecyl sulfate
SOLiD	sequencing by oligonucleotide ligation and detection
Strep.	*Streptococcus*
TBE	Tris-Borat-EDTA
TE	Tris, EDTA
TEMED	N,N,N´,N´-tetramethyl-ethylendiamine
TMW	Technische Mikrobiologie Weihenstephan
Tris	tris (hydroxymethyl) aminomethan
U	units
UPGMA	unweighted pair group method using arithmetic mean
UV	ultra violet
V	volt
v	volume

List of Figures

List of Tables

1 Introduction

1.1 About *Enterococcus faecalis*

Enterococcus, a genus of lactic acid bacteria of the phylum *Firmicutes*, was first described by Thiercelin *et al*. (1899) and Thiercelin and Jouhad (1903). Initially, these microorganisms were classified within the genus *Streptococcus* (*S*.) (Andrewes and Horder, 1906). In 1933, Lancefield classification of streptococci belonging to serological group D included the species *S. faecalis* and *S. faecium*. In 1984, Schleifer and Kilpper-Bälz proposed to transfer *S. faecalis* and *S. faecium* to the genus *Enterococcus* based on their results from DNA-DNA hybridization and 16S rRNA sequencing in consideration of a suggestion of Kalina (1970). In the last decades more species have been described within the genus *Enterococcus,* which to date includes over 40 species (http://www.bacterio.cict.fr/e/enterococcus.html; http://www.ncbi.nlm.nih.gov/Taxonomy, January 2012).

Enterococci possess a Gram-positive cell wall structure and belong to lactic acid bacteria (LAB) with a cell arrangement that occurs singly, in pairs or as short chains. They are capable of aerobic respiration if heme is available for incorporation and metabolize glucose by homolactic fermentation (Bryan-Jones and Whittenbury, 1969). Enterococci are heat tolerant and able to grow in a wide range of temperature (from 10 to 45 °C). They also tolerate different pH values in the range between 4.0 to 9.6 and high salt concentrations (6.5 % NaCl) (Hardie and Whiley, 1997; Sherman,1937).

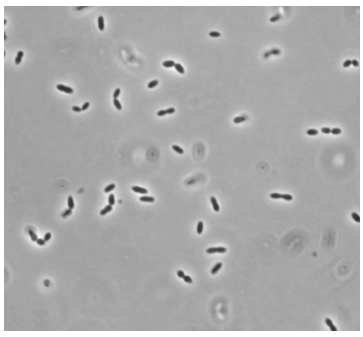


Figure 1 *Enterococcus faecalis* strain OG1RF, visualized using an oil immersion objective (1000x magnification) in an Axiolab microscope (Carl Zeiss MicroImaging GmbH, Germany).

1.2 Habitat and host specificity of *E. faecalis*

Enterococci are widely distributed in the environment and can occur ubiquitous, in soil,

surface waters, on plants and in the gastrointestinal tract of mammalians, as well as in the digestive tract of several bird, insect, reptile and fish species (Deibel and Silliker, 1963; Devriese *et al*., 1987; Macovei and Zurek, 2006; Martin-Platero *et al*., 2006). The detection of *Enterococcus* (*E.*) species in water is considered to be from fecal contamination (Ahmed *et al*., 2005) indicating that all isolates are of intestinal origin and the persistent nature of this genus enables them to survive in diverse environments. Beside the ability to endure a wide range of growth temperature (10 – 45 °C) and pH value (4.0 - 9.6) they can even survive heating at 62.8 °C for 30 min and tolerate environments with 40 % bile salt and 6.5 % NaCl (Sherman, 1937). These important characteristics allow this microorganism to compete efficiently for nutrients and living space. Another reason for this widespread appearance is its broad spectrum of energy sources including diverse carbohydrates, citrate, lactate, glycerol, malate, many α-keto acids and the diamino acids arginine and agmatine (Bizzini *et al*., 2010; Marquis *et al.*, 1987; Sarantinopoulos *et al*., 2001; Ward *et al*., 1999).

Species of this genus are distributed differently according to their physiological ability as well as adaptability to corresponding physicochemical factors of the environment and conditions within the host (see Table 1).

Table 1: Important *Enterococcus* species and their habitat (modified from Portenier *et al.*, 2003).

***Enterococcus* (*E.*) species**	**Habitat**
E. faecalis	oral cavity, gastrointestinal tract, animals, water, food
E. faecium	oral cavity, gastro-intestinal tract, animals, water, food
E. gallinarum	foods, human (infrequently)
E. casseliflavus	soil, plants, food, human (infrequently)
E. hirae	animals
E. durans	humans, animals, foods

Some enterococci are commensals of the oral cavity and normal human gastrointestinal microbiota. The human stool consists of 10^{11} bacteria per gram and harbors a huge number of various bacteria species. The predominant genera are, *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacteria* and *Ruminococcus* (Kurokawa *et al.*, 2007; Lay *et al.*, 2005; Matsuki *et al.*, 2004; Matsuki *et al.*, 2002). More subdominant but also frequently found

genera are *Enterococcus* and *Lactobacillus*. The common concentration of *Enterococcus* cells ranges between 10^2-10^8 CFU per gram of digestive content (Gelsomino *et al*., 2003; Kleessen *et al*., 2000). The most prevalent species of *Enterococcus* isolated from humans are *E. faecalis* and *E. faecium* (Devriese and Pot, 1995; Protenier *et al.*, 2003, see Table 1). In the intestine *E. faecalis* uses non-absorbed sugars as an energy source and even mucins that are heavily glycosylated produced by goblet cells can be catabolized (Corfield *et al.*, 1992). A study about biochemical and phenotypical characterization of enterococcal isolates from surface water could trace the source of contamination and they were able to distinguish between isolates from human and animal origin in a limited region (Ahmed *et al.*, 2005). Some strains of *E. faecalis* showed the ability to produce surface pili that are involved in the formation of biofilm. Strains of *E. faecalis* causing endocarditis showed a significantly intensified biofilm formation than non-endorcarditis isolates (Nallapareddy *et al.*, 2006). These findings indicate a tendency of specific *E. faecalis* strains to prefer specific host ecologies.

1.3 Dual nature of *E. faecalis*

E. faecalis is a human commensal and appears to be widely distributed in the human surrounding. Notably, there is an ambivalent behavior of this organism in relation to humans. Today it is not ascertained if there are some rare representatives with a fatal impact to the human health or if any apparently harmless commensal can change into a pathogen. In this sense, the role of the diversity within different strains of *E. faecalis* as compared to the difference in hosts and their physical condition remains unclear. These two sides that have been described for *E. faecalis* are elucidated in the following sections.

1.3.1 Beneficial traits of *E. faecalis*

The genus *Enterococcus* is widely distributed in the human food environment. Generally, *E. faecalis* is associated with traditionally produced food, mainly in food products from animal origin such as fermented meat and milk products (Giraffa *et al.*, 2003), but also found in brined vegetables (Adegoke and Babalola, 1988; Alemu *et al*., 2006; Xiong *et al.*, 2012).

1.3.1.1 Presence *E. faecalis* in cheeses

The presence of *E. faecalis* in raw foods could be mediated via environmental contamination or inadequate facility hygiene during food processing. Due to its persistent

nature it is able to survive and convey the fermentation process (Giraffa, 2003). Together with *E. faecium* and *E. durans*, *E. faecalis* is mainly present in cheeses, in e.g. in Spanish Cebreiro cheese (Centeno *et al.*, 1999), Manchego (Nieto-Arribas *et al.*, 2011), Kefalotyri (Litopoulou-Tzanetaki, 1990), Feta (Litopoulou-Tzanetaki *et al.*, 1993, Sarantinopoulos *et al.* 2002), farmhouse Cheddar type cheese (Gelsomino *et al.*, 2002) and Appenzeller cheese (Templer and Baumgartner, 2007). In these artisan (raw milk) cheeses enterococci have been designated "nonstarter lactic acid bacteria" (NSLAB). In particular, they found in late fermentation/ripening stages of cheeses produced in the Mediterranean area from bovine, ewe's, goat, or water buffalo milk (Freitas *et al.*, 1996; Mannu *et al.*, 2002; Litpoulou-Tzenataki,1990). Due to the fact that some enterococci are thermotolerant (e.g. survival of an exposure of 60 °C for 30 min), they are even present in cheeses produced from pasteurized milk. This way they can be true contaminants, but they are also applied as artisan cheese starter preparations or occur in the house flora in which they act more as adjunct cultures to the starter cultures. In such artisan cheeses the sensory profile, color, aroma and taste of fully ripened cheeses is positively affected by the presence of the enterococcal flora. During and after ripening enterococci affect the concentration of total free amino acids, long-chain free fatty acids, diacetyl and acetoin and therefore enhance the sensory profile (Centeno *et al.*, 1999, Sarantinopoulos *et al.*, 2002). The concentration of enterococci is dependent on the composition of food. In fully ripened cheeses enterococci can be detected between 10^5-10^7 CFU/g, whereas in fermented sausages 10^5 CFU/g is already a comparatively high level of enterococci (Gelsomino *et al.*, 2003; Marchesini *et al.* 1992).

1.3.1.2 Presence *E. faecalis* in meat products

The presence of enterococci in fermented meat products has a different background than in milk products because of the non-pasteurized, raw material at the beginning of the fermentation process. Normally, deliberately added, highly competitive starter cultures or endogenous lactobacilli originating from the animal and its environment or from slaughterhouses and production facilities are responsible for the induction of the fermentation process. During this process the number of enterococci differs and can lead to their complete disappearance within the microbiota at the end of the ripening process (Metaxopoulos *et al.* 2001; Marchesini *et al.*, 1992). However, the persistence of enterococci during ripening can be attributed to their range of growth temperatures and

their high tolerance to salt. The function of enterococci in meat fermentation is rather adjacent than predominant as compared with starter cultures in cheese fermentation. Nevertheless, there are some types of artisan sausages in which *E. faecalis* is present, e.g. chorizo (Laukova *et al.*, 2011). Some strains can produce bacteriocins, such as cytolysin or AS-48, and may play an important role to prevent the growth of spoilage bacteria or even pathogens, such as *Listeria* (Ananou *et al.*, 2005; Foulquié Moreno *et al.*, 2002; Gilmore *et al.*, 1994; Laukova *et al.*, 2011).

1.3.1.3 Probiotic potential *E. faecalis*

According to Guarner and Schaafsma (1998) and FAO-WHO (2002) probiotic microorganisms are defined as "live microorganisms, which when consumed in adequate amounts, confer a health effect on the host". Today, the application of a probiotic microorganism in foods is in high demand, e.g. in fermented milk products, infant formulas, as well as pharmaceutical preparations. The exigencies to classify a microorganism as a probiotic are as follows: they must have the ability (i) to adhere and attach to mucosal or epithelial cells, (ii) to persist within the gastrointestinal tract, (iii) to be non-invasive, non-pathogenic and non-carcinogenic, (iv) to reduce the adherence of pathogens, and (v) to prevent the growth of pathogens via the production of acids, hydrogen peroxide, and bacteriocins (Salminen *et al.* 1996; FAO-WHO, 2002). Among probiotic microorganisms primarily but not exclusively two genera are found, *Lactobacillus* and *Bifidobacterium* (Klein *et al.*, 1998). Strains of further genera that have already been in use as probiotics are other representatives of lactic acid bacteria (LAB), such as *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides*, *E. faecium* and *E. faecalis*. The use of *Enterococcus* strains as probiotics has been discussed controversially. In animal feeding the application of the probiotic *E. faecium* strain NCIMB 10415(SF68) in Cylactin® (Hoffmann-La Roche Ltd., Basel, Switzerland) is commonly propagated to maintain the health status of the animals. In spite of concerns on the safe use of some strains of *E. faecium* or *E. faecalis*, studies have shown beneficial effects in the treatment of diarrhea in adults and children, and some strains have been used in pharmaceutical products for decades without reports of adverse effects, e.g. *E. faecalis* Symbioflor1® (SymbioPharm, Herborn, Germany) (Wunderlich *et al.*, 1989; Jansen *et al.*, 1993; Bhardwaj *et al*., 2010). This strongly indicates that members of *Enterococcus* are more than commensals of the host microbiota and stand in a mutualistic relationship.

Nevertheless, the world heath organization (WHO) does not recommend the use of enterococci as probiotics because of the possible acquirement of virulence characteristics and antibiotic resistances by means of mobile genetic elements, which could elicit a pathogenic behavior of the microorganism. The WHO leaves the onus on the producer to prove that a probiotic *Enterococcus* strain is incapable to acquire certain resistant or virulence genes (WHO food safety, 2001). In accordance, the American Food and Drug Administration (FDA) does not classify enterococci to be generally recognized as safe (GRAS). These statements are based on the current status of research and leave *E. faecalis* in applications as a probiotic to a contended status.

1.3.2 Pathogenic behavior of *E. faecalis*

As described above *E. faecalis* and *E. faecium* belong to the normal microbiota in the gastrointestinal tract of humans and can even show beneficial effects. The fact that these microorganisms are as such closely related to humans enhances the possibility of an unintended contamination, and many infective strains originate from the persons intestinal flora. In the 1980s the perspective on enterococci started to change when high-level of resistance to ampicillin was found (Coudron *et al.*, 1992; Grayson *et al.*, 1991; Murray and Mederski-Samaroj, 1983). To date, the species *E. faecalis* and *E. faecium* have further emerged as nosocomial pathogens associated with serious infections in humans, even rare cases of infections caused by *E. avium*, *E. durans*, *E. casseliflavus/flavescens* and *E. gallinarum* have been observed (Hidron *et al.*, 2008; Centers for Disease Conrol and Prevention, U.S., 2004; Reid *et al.*, 2001). *Enterococcus* species are on the third place of the most common pathogens associated with cases of healthcare-associated infections reported to the national healthcare safety network of the United States from January 2006 - October 2007; see Figure 2 (Hidron *et al.*, 2008). In Germany, enterococcal infections account for 8.61 % of nosocomial infections as reported from January 2006 through December 2010 to the national nosocomial infection surveillance (Gastmeier *et al.*, 2010).
E. faecalis can cause a wide variety of infections in parts with severe onset. Commonly caused infections by *E. faecalis* are wound infections, urinary tract infections, bacteremia, endocarditis, meningitis, and endodontic infections (Hancock *et al.*, 2001; Molander *et al.*, 1998; Nallapareddy *et al.*, 2006; Vergis *et al.*, 2002). The bacterium is able to produce extracellular superoxide and derivative reactive oxygen species (e.g. H_2O_2), which have been identified to play an important role in causing chromosomal instability in colonic epithelial cells (Huycke *et al.*, 1996; Wang and Huycke, 2007). These reactive oxidants

with their damaging impact are assumed to be responsible for the formation of sporadic adenomatous polyps and colorectal cancer (Huycke *et al.*, 2002).

As described above the pathogenicity of *E. faecalis* can range from less serious infections to severe and life-threatening illnesses. Even though the progression of an enterococcal infection depends on the health and consequently on the immune status of the affected patient, strains of this species carry certain traits to survive and adapt to diverse environments.

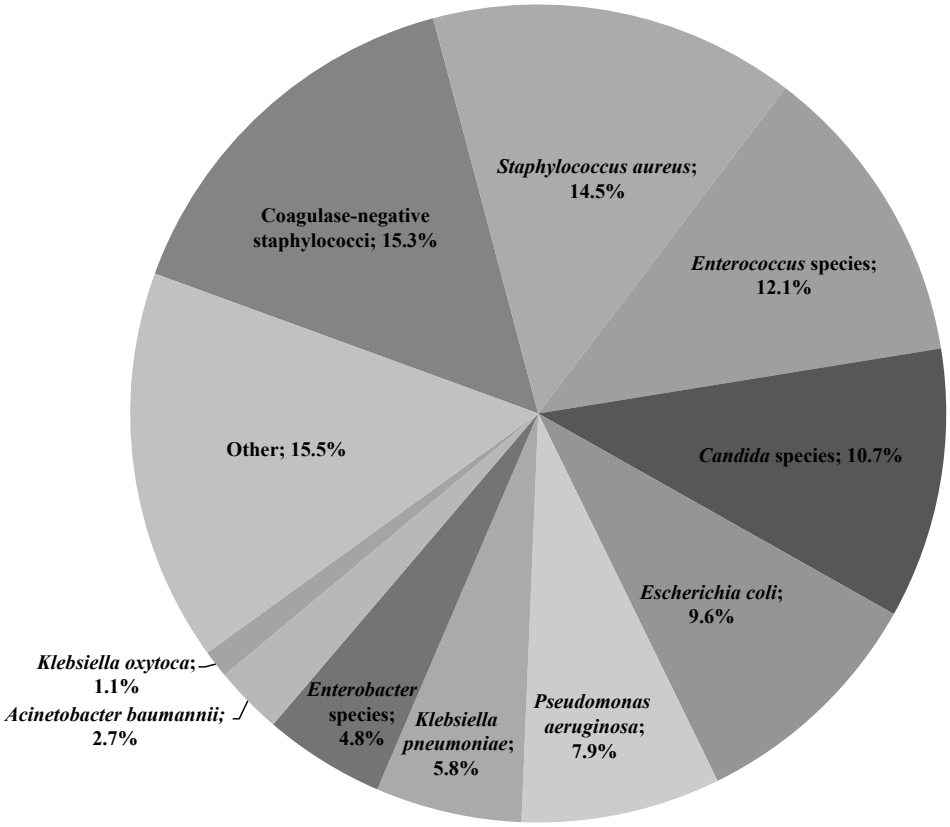


Figure 2: *Enterococcus* spp. among the 10 most common pathogens accounting for over 84 % of any healthcare-associated infections reported by hospitals to the national healthcare safety network of the United States from January 2006 - October 2007 (Hidron *et al.*, 2008).

1.3.2.1 Antibiotic resistances in *E. faecalis*

Another reason of concern is the increasing number of antibiotic resistances among *Enterococcus* isolates what makes them recognized as feared nosocomial pathogens that can be challenging to treat. Enterococci intrinsically tolerate β-lactam antibiotics because they express penicillin-binding proteins. They are resistant to cephalosporins, nalidixic acid, macrolides and even low levels of clindamycin and aminoglycosides (Engel *et al.*,

1980; Portillo *et al.*, 2000; Zervos *et al.*, 1987). Besides their intrinsic antibiotic resistances there has been an increase in acquired resistances to a broad spectrum of antibiotics that is developed coevally through the augmented use of antibiotics in hospitals and animal breeding in the last decades. The acquired resistances are against chloramphenicol, tetracyclines, rifampin, fluoroquinolones, aminoglycosides and vancomycin (Hegstad *et al.*, 2010; Klare *et al.*, 2003). Especially, the resistance to vancomycin has been related to the use of avoparcin a growth promoter in animal feeds (Bager *et al.*, 1997). Vancomycin resistant enterococci (VRE) were firstly isolated in Europe and United States in the late 1980s in clinical infections (Leclercq *et al.*, 1988; Sahm *et al.*, 1989; Uttley *et al.*, 1988). Avoparcin is closely related to vancomycin a glycopeptide antimicrobial agent and was prohibited in 1997 (Heuer *et al.*, 2002). The spread of resistances within this species occurs through the acquisition of plasmids or transposons of other microorganisms harboring resistant genes or could be generated by spontaneous mutations (Hegstad *et al.*, 2010). Studies have also shown that enterococci possess an increased tendency for an interexchange of genetic elements, including genes for antibiotic resistances with a conjugation rate of about 10^{-2} transconjugants per donor cell (Huycke *et al.*, 1992). As described in section 1.4, some enterococci possess transferable genetic elements, conjugative plasmids and transposons, which can be exchanged with other Gram-positive species and even with Gram-negative bacteria (Clewell *et al.*, 2002; Cocconcelli *et al.*, 2003; Doucet-Populaire, *et al.* 1992).

1.3.2.2 Virulence traits in *E. faecalis*

Some strains of *E. faecalis* show all relevant characteristics to adhere to, colonize and translocate through host tissues, and can even spread these traits to other strains for example by horizontal gene transfer (Krueger *et al.*, 2004; Noble *et al.*, 1992; Wells *et al*., 1990). In the following section important virulence traits according to their function of disease pathology are described (see Figure 3): surface factors that provide adherence, e.g. collagen binding adhesin (Ace), aggregation substance (As), endocarditis antigen (EfaAfs/fm), enterococcal surface protein (Esp) and secreted factors such as cytolysin (Cyl), gelatinase (GelE), and serine protease (SprE), which enable invasion through host tissues.

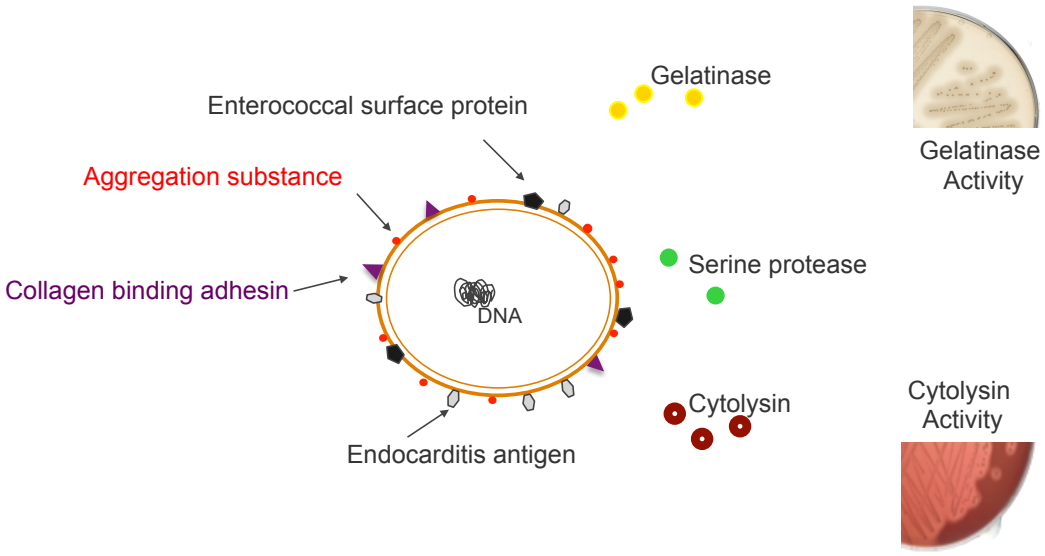


Figure 3: *E. faecalis* and its virulence determinants on the cell surface and secreted factors.

Collagen binding adhesin

The adhesin for collagen from *E. faecalis* (Ace) is a member of the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) and was identified by significant domain homologies with the collagen binding protein (Cna) of *Staphylococcus* (*Staph.*) *aureus* (Rich *et al.*,1999). Besides the structural similarity of Ace to Cna of *Staph. aureus* it also shows a functional analogy because of the binding to extracellular matrix proteins collagen types I and IV, and laminin (Foster and Höök, 1998; Nallapareddy *et al.,* 2000). The *ace* gene appears to be frequently present in *E. faecalis* strains. The ability to bind to such matrix proteins is favored to the surface hydrophobicity of the bacterial cells evoked by the adhesin protein (Zareba *et al.*, 1997).

A study by Xiao *et al.* (1998) indicated that the expression of Ace is enhanced at growth temperature of 46°C. On the other hand, Nallapareddy *et al.* (2000) identified Ace-specific antibodies in sera obtained from patients suffering from *E. faecalis* endocarditis. This finding indicates that Ace is expressed upon heat shock as well as *in vivo* during human infections.

Aggregation substance

The aggregation substance (AS, encoded by the *agg* gene) is a surface-bound protein that induces the formation of bacterial cell accumulations and mediates conjugative transfer of plasmids between aggregated donor and recipient cells (Olmsted *et al.*, 1991). Furthermore

AS promotes *in vitro* the binding of *E. faecalis* to eukaryotic cells such as renal tubular cells (Kreft *et al.,* 1992), intestinal epithelial cells (Sartingen *et al.,* 2000; Wells *et al.*, 2000) and macrophages (Rakita *et al.*, 1999). Even the survival of *E. faecalis* within macrophages has been reported (Süssmuth *et al.*, 2000). A study by Chow *et al.* (1993) has shown that AS contributes to a higher mortality in a rabbit endocarditis model when *E. faecalis* harbours additionally the genetic information for the expression of hemolysin, which is another virulence trait. Although some studies could not confirm this finding with other *in vivo* models such as mouse models (Johnson *et al.*, 2004) and a rabbit endophthalmitis model (Jett *et al.*, 1998), AS does play a role within the infection process, which typically starts with a settlement and colonization of bacteria on various tissues. However, a detailed mechanism on the specific contribution of AS within an infection process of *E. faecalis* is still not known.

Cell wall adhesin

The adhesin-like *E. faecalis* and *E. faecium* antigens (EfaAfs and EfaAfm respectively) were first identified through antigen analysis of sera from patients suffering from endocarditis caused by enterococci. They are surface proteins that show similarities with other adhesins of streptococci (Lowe *et al*., 1995). The exact mechanism of the virulence of EfaAfs/fm has not been identified yet. However, Low *et al.* (2003) showed that the expression of genes encoding this antigen (*efaCBA*) is induced in serum and is regulated by manganese concentration because it builds a manganese (Mn^{+2}) specific ion transport system. Hew *et al.* (2007) confirms this upregulation of these genes in Brain Heart Infusion (BHI) medium, which contains only 0.27 µg/g of free Mn^{+2}.

Enterococcal surface protein

The enterococcal surface protein (Esp), which was first described by Shankar *et al.* (1999), is a high-molecular-weight surface protein (approx. 206 kDa) that seems to appear more often in infection-associated isolates of *E. faecalis* (Shankar 2001). The expression of Esp is important for *E. faecalis* for initial attachment and to form biofilm on abiotic surfaces (Toledo-Arana *et al*., 2001). This finding emphasizes the frequent association of *E. faecalis* isolates harboring the gene *esp* encoding this surface protein with nosocomial urinary tract infections (Shankar *et al.*, 2001). Although Esp contains characteristics of surface protein receptors designated MSCRAMM (Patti *et al.*, 1994) that adhere to components of the host, there is no evidence for the domains binding to host factors in Esp. The Esp

found in *E. faecium* shares 90% homology with Esp of *E. faecalis* and appears to be prevalently present in clinical isolates (Eaton and Gasson, 2002).

Cytolysin

One of the most investigated virulence factors in *Enterococcus* is cytolysin (also referred to as hemolysin), a β-hemolytic toxin that can disrupt membranes of other bacteria and eukaryotic cells (Gilmore *et al*., 1994; Ike *et al.,* 1984; Jett *et al.*, 1992; Miyazaki *et al.*, 1993). Cytolysin is capable of lysing rabbit, horse, and human erythrocytes (Kobayashi, 1940; Gaspar *et al.*, 2009).

The complete cytolysin operon consists of eight genes (*cylM, cylB, cylA, cylI, cylR1, cylR2, cylL$_L$*, *cylL$_S$*), which can be located on plasmids or on the chromosome often within the pathogenicity island (Shankar et al., 2004; Ike and Clewell, 1992). The cytolysin consists of two non-identical subunits CylL$_S$ and CylL$_L$, which are modified post-translationally by the gene product CylLM. The secretion and activation of cytolysin is accomplished through CylB and CylA. The gene product of *cylI* provides cell immunity to the active cytolysin. The cytolysin operon is regulated by a quorum-sensing mechanism involving the products of *cylR$_1$*/*cylR$_2$* that show relatedness to proteins of two-component regulatory systems. It has been shown that the presence of target cells enhances the expression of the cytolysin operon and it was proven that this operon habors a mechanism with the ability to actively probe the environment for possible cytolysin targets (Haas *et al.*, 2002; Coburn *et al.*, 2004).

The active cytolysin has been proven to enhance the disease severity in many animal models, e.g. in a rabbit endophthalmitis model and an endocarditis model (Chow *et al.*, 1993; Jett *et al.*, 1992). Studies have shown that there is an interaction of virulence factors within the pathogenic behavior. Chow *et al.* (1993) have demonstrated that the joint expression of cytolysin and aggregation substance results in an eightfold increased lethality through endocarditis in comparison to the expression of either one of these factors expressed alone. A cytolysin encoded within the pathogenicity island is also associated with the virulence factor Esp (Shankar *et al.*, 2004).

Gelatinase and Serine protease

The enterococcal gelatinase (GelE) is an extracellular metalloendopeptidase, containing a zinc ion in the active site. GelE can hydrolyze gelatine, collagen, fibrinogen, insulin,

hemoglobin, casein, endothelin-1 and even enterococcal sex-pheromone metabolites (Mäkinen *et al.*, 1989; Mäkinen and Mäkinen, 1994). The gelatinase activity shows a broad range of pH optimum, between 6 and 8 (Mäkinen *et al.*, 1989). The first study that demonstrated the virulent effect of enterococcal GelE was done by Gold *et al.* (1975). They detected a proteolytic *E. faecalis* strain (OG1) causing caries in rats. Many animal studies have shown a correlation between the presence of virulence factors and experimental disease (Dupont *et al.*, 1998; Jett *et al.*, 1998; Singh *et al.*, 1998; Schlievert *et al.*, 1998), and epidemiological studies with human isolates from clinical origin do verify the incidence and activity of this virulence trait (Coque *et al.*, 2002; Kanemitsu *et al.*, 2001). GelE contributes to the development of chronic intestinal inflammation in disease susceptible, interleukin-10-deficient mice. It has been proven that enterococcal GelE in addition with other pro-inflammatory cytokines supposedly damages the epithelial barrier function (Steck *et al.,* 2011). The gene encoding GelE occurs mostly combined in an operon-type structure together with a gene for a serine protease, *sprE* (Qin *et al.*, 2000; Singh *et al.*, 2005). In animal models both have been suggested to play a major role in the infection process (Sifri *et al.*, 2002). The expression of GelE and SprE is controlled by a quorum sensing mechanism-involving *fsrB*. The enterococcal SprE was found to aid adhesion of the bacterium to dentin, supposedly by exposing binding sites for the adhesins or by modifying the adhesins (Hubble *et al.*, 2003).

Whereas Vergis *et al.* (2002) conclude that the putative virulence factors in *E. faecalis* are not associated with an increased fourteen-day mortality rate among bacteremic persons. Although other factors or properties of *E. faecalis* may be important to its ability to cause human diseases, it is not immediately apparent which factors are associated with disease severity.

1.4 Genome flexibility of *E. faecalis*

The first sequenced strain of *E. faecalis* V583 (Paulsen *et al.*, 2003) was one of the first vancomycin-resistant isolates from a blood culture derived from a chronically infected patient, documented in the late 80s in the United States (Sahm *et al.*, 1989). The genome of strain V583 consists over 25 % of mobile genetic elements (Paulsen *et al.*, 2003), which is nearly outstanding to all sequenced bacterial genomes today. The mechanism underlying the uptake of these many elements is unknown. These mobile elements in strain V583 are including phage regions, over 30 insertion elements, conjugative transposons and a pathogenicity island (PAI) encoding over 100 open reading frames (ORFs) some of which

with known virulence factors such as enterococcal surface protein, cytolysin and aggregation substance (Paulsen *et al.*, 2003). It has been suggested that PAI can move as an integrative conjugative element (ICE) because it shows phage-related integration and excision proteins, and terminal direct repeats. However, Manson *et al.* (2010) showed that for an integration of this specific PAI the donor cell must have a pheromone responsive plasmid and they further showed that plasmid mobilization appears to be an important mechanism for horizontal gene transfer spreading the antibiotic resistance in the genus *Enterococcus*.

Another highly investigated strain of *E. faecalis* OG1RF, a rifampicin and fusidic acid resistant derivative of the human isolate OG1 (Dunny *et al.*, 1978), is lacking a region of around 478 kb within its chromosome and neither plasmid pTEF3 nor the two pheromone responsive, independently replicating plasmids (pTEF1/2) are present in comparison to strain V583 (Bourgogne *et al.*, 2008). Within these absent elements most of the externally acquired DNA and mobile elements found in V583 are missing in OG1RF. Instead, Bourgogne *et al.* (2008) described in OG1RF the presence of two clustered, regularly interspaced short palindromic repeats (CRISPR), CRISPR1-*cas* and CRISPR2 (see Figure 4). The recently characterized CRISPR loci protect bacterial cells from infection with bacteriophages and plasmids underlying a sequence specific defense mechanism (Horvath and Barrangou, 2010; Koonin and Makarova, 2009; Sorek *et al.*, 2008). The CRISPR loci are a family of DNA repeats, which are widely distributed in the genomes of bacteria and archaea. Those repeats have a length of around 24-48 nucleotides and are separated by similarly sized, unique spacers (Grissa *et al.*, 2007). These loci are accompanied by CRISPR-associated (*cas*) genes. These *cas* genes encode proteins that carry domains such as nucleases and helicases (Jansen *et al.*, 2002; Haft *et al.*, 2005; Horvath *et al.*, 2008). The CRISPR loci operate as a diverse defense mechanism that withstands exposure to invading nucleic acids possibly using a RNA-interference-like mechanism (Makarova *et al.*, 2006). The efficacy of the CRISPR defense system relies on the presence and activity of near by located *cas* genes (Horvath and Barrangou, 2010). One of the CRISPR loci in OG1RF, CRISPR1-*cas*, is accompanied by partly conserved *cas* genes (*cas1*, *cas2*, *csn1*, *csn2*) whereas the other CRISPR2 locus is lacking any *cas* genes. *E. faecalis* V583 possesses only the CRISPR2 locus indicating the lack of prevention of the uptake of foreign genetic elements. Another CRISPR locus with associated functional genes (CRISPR3-*cas*) was discovered by Palmer and Gilmore (2010). Significant association between the absence of CRISPR and the presence of antibiotic resistance traits in clinical isolates, *versus*

the presence of CRISPR elements in commensal strains has been demonstrated (Palmer and Gilmore, 2010).

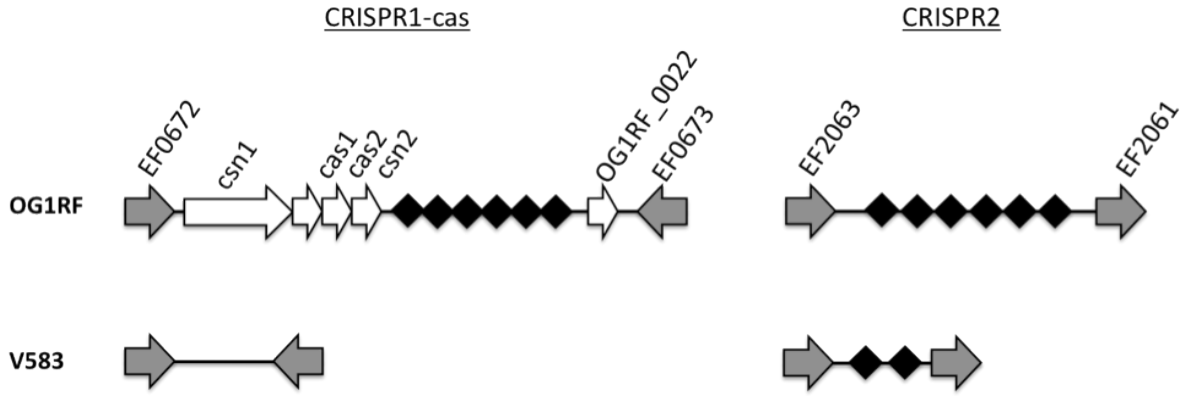


Figure 4: CRISPR loci in *E. faecalis* genomes of strains OG1RF and V583. Grey arrows show genes homologue to *E. faecalis* V583. CRISPR locus-specific genes are shown in white arrows, and CRISPR spacers are represented by black diamonds. CRISPR3-cas is not presented because of its absence in OG1RF and V583. This figure is modified from Palmer and Gilmore (2010).

1.5 Aim of the study

Strains of *E. faecalis* and *E. faecium* are human commensals and even probiotic effects have been claimed for some strains (Domann *et al.*, 2007). In the past 30 years, both *E. faecalis* and *E. faecium* have been recognized as opportunistic pathogens causing a variety of diseases, which to date appeared to be difficult to treat. The intrinsic ability to survive even under harsh conditions may allow the bacterium to persist in hospital environments and to survive host defenses. Many studies have either focused primarily on the genomic variation between commensal and infectious strains, or concentrated on the bacterium's reaction on proteome and transcriptome level facing different stress conditions or mimicking conditions in an infected host. However, currently known virulence factors do not fully explain the behavior of these bacteria namely the possible switch from a harmless commensal into a life-threatening pathogen.

The aim of this work is to characterize the distribution of known virulence factors in *E. faecalis* among different strains and to identify novel fitness and adaptive functions, to establish a better understanding of the ambivalent nature of this species. Therefore, the genetic background of different *E. faecalis* isolates with respect to known virulence factors should be investigated to clarify if a distinct classification of isolates is possible with regards to their origin of isolation and an alleged behavior associated with that. Furthermore, novel fitness factors of *E. faecalis* should be identified, which characterize the adaptation ability of this organism to the host as a first step in a switch from a

commensal to a pathogen. Their knowledge should contribute towards a mechanistic understanding of disease pathology at the interface between food born bacteria and the intestinal epithelium of the host. The ambivalent nature of strains within this species should be enlighted through the analysis of *E. faecalis* strain OG1RF, a human isolate with some known virulence factors. The identification of these fitness and adaptive factors should be achieved along differential analyses of changes in the proteome and transcriptome of *E. faecalis* OG1RF *in vitro* and *in vivo*, respectively, by analysing different growth conditions in both cell cultures and monoassociated wild type mice.

2 Material and Methods

2.1 Materials

2.1.1 Devices

Devices used in this work are listed in Table 2 in alphabetical order.

Table 2: Devices used in this work.

Device	Model	Manufacturer
Agarose gel chamber 25 x 20 cm	Easy Cast electrophoresis system	Owl Separation Systems, Portsmouth, NH, USA
Agarose gel chamber 13.8 x 12 cm	Easy Cast electrophoresis system	Owl Separation Systems, Portsmouth, NH, USA
Autoclaves	2540 ELV	Systec GmbH, Wettenberg, Germany
	Varioklav	H + P Labortechnik, Oberschleißheim, Germany
Incubation	Certomat BS-1	B. Braun Biotech International, Melsungen, Germany
	Hereaus B5042E	Hereaus Instruments, Hanau, Germany
	Hereaus VT 5042 EK/NZ	Hereaus Instruments, Hanau, Germany
	WiseCube®WIS-ML02	Wise Laboratory Instruments
Centrifuges	Sigma 1 K 15	Sigma Labortechnik, Osterrode am Harz, Germany
	Sigma 6-16K	Sigma Labortechnik, Osterrode am Harz, Germany
	J-6	Beckman, Palo alto, CA, USA
	J-2	Beckman, Palo alto, CA, USA
	Hermle Z383 K	Hermle Labortechnik, Wehningen, Germany
	Hermle Z382 K	Hermle Labortechnik, Wehningen, Germany
	Hermle Z216 MK	Hermle Labortechnik, Wehningen, Germany
Deep-freezer	Platinum 500	Angelantoni Industries s.p.a, AS biomedical division, Massa Martana, Italy
Electrophoresis Power supplies	MPP 2 x 3000 Power Supply	MWG Biotech AG, Ebersberg, Germany
	Electrophoresis Power Supply EPS 3000	Pharmacia Biotech, Cambridge, England
	Electrophoresis Power Supply EPS 3301 XL	Pharmacia Biotech, Cambridge, England

Device	Model	Manufacturer
	2197 Supply PPS 200-1D	MWG Biotech AG, Ebersberg, Germany
Incubation hood	Certomat H	B. Braun Biotech International, Melsungen, Germany
Isoelectric focusing	Hoefer IEF 100	Hoefer Inc., Holliston, MA, USA
Laminar flow sterile work bench	HERA safe	Heraeus Instruments, Hanau, Germany
Scanner	Epson Expressiom 1600pro	Seiko Epson Corporation, Tokio, Japan
MALDI	MALDI target plate	Bruker Dalonics, Bremen, Germany
	MALDI-TOF-MS spectrometer; Microflex LT	Bruker Dalonics, Bremen, Germany
Microscope	Axiolab	Carl Zeiss MicroImaging GmbH, Germany
Nanodrop	Nanodrop1000	Peqlab Biotechnologie GmbH, Erlangen, Germany
PCR-Cycler	Primus 96 plus	MWG Biotech, AG, Ebersberg, Germany
	Mastercycler gradient	Eppendorf AG, Hamburg, Germany
pH meter	InLab® Semi-Micro, pH 0-14 (electrode)	Mettler-Toledo, Gießen, Germany
	Knick pH 761 Calimatic (measuring device)	Knick elektronische Geräte, Berlin, Germany
Photometer	NovaspeIIq	Pharmacia Biotech, Cambridge, England
Pipettes	Pipetman	Gilson-Abomed, Langenfeld, Germany
Pure watersystem	Euro 25 and RS 90-4/UF	SG Wasseraufbereitung GmbH, Barsbüttel, Germany
Sample Preparation System	Fast-Prep®-24	MP Biomedical, Irvine CA, USA
Shaking	Certomat R	B. Braun Biotech International, Melsungen, Germany
	Vortex 2 Genie	Scientific Industries Inc., Bohemia, NY, USA
Stirring	RCT-Basic	Mettler-Toledo, Gießen, Germany
Thermo block	Techne DRI-Block DB3	Thermo-Dux Gesellschaft für Laborgerätebau mbH, Wertheim, Germany
Ultra sonification	SONOPLUS/SH70G	Bandelin electronic, Berlin, Germany
UV table	Herolab UVT 28M	Herlab GmbH Laborgeräte, Wiesloch, Germany
Vertical Gel Electrophoresis	SE900 Vertical Slab Gel Electrophoresis Unit	Hoefer Inc., Holliston, MA, USA

Device	Model	Manufacturer
Water bath	Lauda BD	LAUDA Dr. D. Wobser GmbH & Co., Lauda-Königshofen, Germany

2.1.2 Chemicals

Chemicals used in this work are listed in Table 3 in alphabetical order.

Table 3: Chemicals used in this work.

Chemicals and media	Purity	Manufacturer
α-cyano-4-hydroxycinnamic acid	≥98 %	Bruker Daltonics, Bremen, Germany
Acetic acid	99 - 100 % (glacial)	Merck, Darmstadt, Germany
Acetonitirle	for HPLC	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Acrylamide/bis solution	(19:1); 30 % (w/v)	SERVA, Heidelberg, Germany
Agar	european agar	Difco, BD Sciences, Heidelberg, Germany
Agarose	for electrophoresis	Biozym Scientific GmbH, Oldendorf, Germany
Ammonium persulfate (APS)	electrophoresis grade	SERVA, Heidelberg, Germany
Antibiotic/antimycotic solution	molecular biology grade	Invitrogen Life Technologie GmbH, Darmstadt, Germany
β-mercaptoethanol	99%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Boric acid	≥99.5 %	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Brain heart infusion (BHI) broth	for microbiology	SIGMA-Aldrich, Steinheim, Germany
3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS)	research grade	SERVA, Heidelberg, Germany
Chloroform:isoamyl alcohol (24:1)	research grade	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Columbia blood agar plate, Merckoplate®	for microbiology	Merck, Darmstadt, Germany
Diethylpyrocarbonate (DEPC)	research grade	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Dimethyl sulfoxide (DMSO)	≥99.5 % p. a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Dimidium bromide	≥98 %,for electrophoresis	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Disodium hydrogen phosphate ($Na_2HPO_4 \bullet H_2O$)	p. a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Chemicals and media	Purity	Manufacturer
Dipotassium phosphate (K_2HPO_4)	p. a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
1,4 Dithio-D,L-Threitol (DTT)	high purity	GERBU Biotechnik, GmbH, Gaiberg, Germany
6 x DNA loading dye	-	Fermentas GmbH, St. Leon-Rot, Germany
Ethanol, denatured	99 % with 1 % methylethylketone	Chemikalien und Laborbedarf Nierle, Freising, Germany
Ethanol, absolute	≥99,8 %	VWR, Prolabo, Foutenay-sous-Bois, France
Ethylendiaminetetraacetic acid (EDTA)	for molecular biology	SIGMA-Aldrich, Steinheim, Germany
Fetal bovine serum (FBS)	-	Invitrogen Life Technologie GmbH, Darmstadt, Germany
Formaldehyde 37 wt % in H_2O	-	SIGMA-Aldrich, Steinheim, Germany
Formaldehyde 10 wt % buffered with phosphate (formalin)	-	Fisher Scientific, Fairlawn, NJ, USA
Formic acid	98-100 %	Merck, Darmstadt, Germany
Gelatine	-	Merck, Darmstadt, Germany
Glycerol	99.5 %, high purity	GERBU Biotechnik, GmbH, Gaiberg, Germany
Glycine	p. a.	Merck, Darmstadt, Germany
Glycogen	molecular biology grade	Merck, Darmstadt, Germany
HCl 37 %	p.a.	Merck, Darmstadt, Germany
Horse blood, defibrinated	-	Acila Dr. Weidner GmbH, Weiterstadt, Germany
4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	for molecular biology	GERBU Biotechnik, GmbH, Gaiberg, Germany
Iodoacetamide	≥99 %, for HPLC	SIGMA-Aldrich, Steinheim, Germany
Insulin-transferrin-selenium A GIBCO	-	Invitrogen Life Technologie GmbH, Darmstadt, Germany
Interferon-gamma (IFN-γ)	-	Invitrogen Life Technologie GmbH, Darmstadt, Germany
Isoamyl alcohol	>98.5 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Isopropanol	p.a.	Scharlau Chemi S. A., Sentmenat, Spain
Lysozyme	-	SERVA, Heidelberg, Germany
Methanol	HPLC-grade	Mallinckrodt Baker B. V., Deventer, Netherlands
Magnesium sulfate (MgSO4 * 7 H2O)	p.a.	Merck, Darmstadt, Germany

Chemicals and media	Purity	Manufacturer
Mutanolysin	-	SIGMA-Aldrich, Steinheim, Germany
p-amino benzoic acid	≥99,8 %	SIGMA-Aldrich, Steinheim, Germany
Panthothenic acid	p.a.	SIGMA-Aldrich, Steinheim, Germany
Peptone from casein	for microbiology	Merck, Darmstadt, Germany
Phosphate buffered salnie (PBS)		Invitrogen Life Technologie GmbH, Darmstadt, Germany
Phosphoric acid	-	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Potassium chloride (KCl)	p.a.	Merck, Darmstadt, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	p.a.	Merck, Darmstadt, Germany
Primer	-	MWG-BiotechAG, Ebersberg, Germany
RLT buffer	-	Qiagen, Hilden, Germany
RPMI Media 1640 + L-Glutamine	-	Invitrogen Life Technologie GmbH, Darmstadt, Germany
Roti®-Blue 5 x concentrated	-	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
ServalytTM carrier ampholyte pH 3-10	analytical grade	SERVA, Heidelberg, Germany
Silver nitrate	>99.9 %, p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium acetate	p.a.	Merck, Darmstadt, Germany
Sodium carbonate (Na_2CO_3)	>99.5 %, p.a., anhydrous	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium chloride (NaCl)	-	Merck, Darmstadt, Germany
Sodium dodecyl sulfate (SDS)	research grade	SERVA, Heidelberg, Germany
Sodium hydroxide (NaOH)	-	Merck, Darmstadt, Germany
Sulfuric acid (HCl)	p.a.	Merck, Darmstadt, Germany
Tertiary butanol	>99.5 %	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Tertamethylethylenediamine (TEMED)	p.a.	Merck, Darmstadt, Germany
Thiourea	>99 %	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Thiosulfate	>99 %	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Tris base	ultra pure	ICN Biomedicals, Inc., Ohio, USA
Tris-HCl	p.a.	Merck, Darmstadt, Germany

Chemicals and media	Purity	Manufacturer
Trypsin-EDTA	-	Invitrogen Life Technologie GmbH, Darmstadt, Germany
Trifluoroacetic acid	≥99.0 %for HPLC /GC	SIGMA-Aldrich, Steinheim, Germany
Tumor necrosis factor-alpha (TNF-α)	-	Invitrogen Life Technologie GmbH, Darmstadt, Germany
Tween 80	-	Mallinkrodt Baker B. v., Deventer, NL
Urea	>99.5 %	Carl Roth GmbH + Co. KG, Karlsruhe
Yeast extract	for microbiology	Merck, Darmstadt, Germany

2.1.3 Expendable materials

Expendables used in this work are listed in Table 4 in alphabetical order.

Table 4: Expendables used in this work.

Material	Type	Manufacturer
Anaerocult®	C mini	Merck, Darmstadt, Germany
Cell culture flasks	25 cm^2, 75 cm^2, sterile, filter cap	Greiner bio-one, Kremsmünster, Austria
Electrode wicks	standard size	SERVA, Heidelberg, Germany
Immobilized pH gradient (IPG) strips	24 cm, pH 4-7	GE Healthcare, Freiburg, Germany
Reaction tubes	200 µl, 1.5 ml, 2 ml	Eppendorf, Hamburg, Germany
Sample cups for isoelectric focusing (IEF)	6 cups each strip	SERVA, Heidelberg, Germany
Sterile ml tubes	15 ml, 50 ml	Sarstedt, Nümbrecht, Germany
Sterile filter	Filtropur S 0.2 (0.2 µm)	Sarstedt, Nümbrecht, Germany

2.1.4 Kits

Kits used in this work are listed in Table 5 in alphabetical order.

Table 5: Kits used in this work.

Kit	Type	Manufacturer
E.Z.N.A. Bacterial DNA Kit	DNA isolation	Omega Bio-Tek Inc., Norcross, GA, USA
Ribominus® transcriptome isolation kit	Depletion of ribosomal RNA from bacterial total RNA	Invitrogen, Carlsbad, California, USA
RNeasy Mini Kit	RNA isolation	Qiagen AG, Hilden, Germany
Taq Core Kit	DNA polymerase	MP Biomedicals Solon, Ohio, USA

Kit	Type	Manufacturer
TURBO DNA-free™ Kit	DNA degradation from RNA samples	Applied Biosystems Inc., Foster City, California, USA

2.1.5 DNA and protein marker

DNA marker GeneRuler™ 100 bp Plus DNA ladder and GeneRuler™ 3 k bp plus DNA ladder used for size comparison were purchased from Fermentas GmbH (St. Leon-Rot, Germany). Protein marker used for size estimation was purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany) unstained SDS PAGE protein marker 6.5 - 200 kDa.

2.1.6 Bacterial strains

All enterococcal strains used in this work for PCR screening were obtained from the TMW culture collection and are listed in numerical order in Table 6 and 7.

Sixteen recently sequenced strains of *E. faecalis* (Palmer *et al.*, 2010) listed in Table 8 were screened *in silico* for the presence of virulence genes and CRISPR-*cas csn1* loci by the platform provided by Broad Institute that made also the genome sequences publicly available (http://www.broadinstitute.org/).

Table 6: *E. faecalis* strains used in this study. Strains marked with * are isolated from patients of various ages that suffered divers diseases, Klinikum rechts der Isar, Roger Vogelmann, Technische Universität München.

Species	TMW strain no.	Designation	Origin
E. faecalis	2.63	food isolate	beer
E. faecalis	2.136	environmental isolate	isogenic with DSMZ 20060
E. faecalis	2.354	environmental isolate	duck feces
E. faecalis	2.520	environmental isolate	iguana feces
E. faecalis	2.622	environmental isolate	isogenic with OG1RF (human oral isolate), Barbara Murray, University of Texas, USA
E. faecalis	2.629	food isolate	goat cheese
E. faecalis	2.630	food isolate	sheep's milk cheese
E. faecalis	2.645	clinical isolate	isogenic with strain 10, Gehard Reuter, Freie Universität Berlin, Germany
E. faecalis	2.647	food isolate	isogenic with strain A/F2, Gerhard Reuter, Freie Universität Berlin, Germany
E. faecalis	2.648	food isolate	isogenic with strain 34.5, Gerhard Reuter, Freie Universität Berlin, Germany
E. faecalis	2.777	environmental isolate	Symbioflor®1, SymbioPharm, Herborn, Germany
E. faecalis	2.798	environmental isolate	infant feces

Species	TMW strain no.	Designation	Origin
E. faecalis	2.815	environmental isolate	fermented belladonna
E. faecalis	2.852	clinical isolate	isogenic with strain V583, Ingolf Nes, Norwegian University of Life Sciences, Norway
E. faecalis	2.900	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.901	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.902	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.903	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.904	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.905	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.906	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.907	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.908	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.909	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.910	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.911	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.912	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.913	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.914	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.915	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.916	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.917	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.918	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.919	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.921	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.922	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.923	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.924	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.925	clinical isolate	Klinikum rechts der Isar, Technische Universität München*

Species	TMW strain no.	Designation	Origin
E. faecalis	2.926	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.927	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.928	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.929	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.930	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.931	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.932	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.933	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.934	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.935	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.936	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.937	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
E. faecalis	2.947	clinical isolate	Klinikum rechts der Isar, Technische Universität München*

Table 7: *E. faecium* strains used in this study. Strains marked with * are isolated from patients of various ages that suffered divers diseases, Klinikum rechts der Isar, Roger Vogelmann, Technische Universität München.

Species	TMW strain no.	Designation	Origin
E. faecium	2.938	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
	2.939	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
	2.940	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
	2.941	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
	2.942	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
	2.943	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
	2.944	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
	2.945	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
	2.946	clinical isolate	Klinikum rechts der Isar, Technische Universität München*
	1.1045	environmental isolate	duck feces

Table 8: Recently sequenced strains of *E. faecalis* by Palmer *et al.* (2010). In this study these sixteen strains were screened *in silico* for the presence of virulence genes and CRISPR-*cas csn1* loci by platform provided from Broad Institute (http://www.broadinstitute.org/).

Species	Isolate	Source	Reference
E. faecalis	AR01/DG	dog mastitis	Manson *et al.*, 2003
	ATCC 4200	blood	ATCC; Schilling and Birkhaug 1927
	CH188	liver	Rice *et al.*, 1991
	D6	pig	McBride *et al.*, 2007
	DS5 (ATCC 14508)	unknown	ATCC; Clewell *et al.*, 1974
	E1Sol	commensal	Gardner *et al.*, 1969
	Fly1	drosophila (wild-captured)	McBride *et al.*, 2007
	HIP11701	clinical	Weigel *et al.*, 2003
	JH1	clinical	Jacob and Hobbs, 1974
	Merz96	blood	Harrington *et al.*, 2004
	T1	unkown	Maekawa *et al.*, 1992
	T2	urine	Maekawa *et al.*, 1992
	T3	urine	Maekawa *et al.*, 1992
	T8	urine	Maekawa *et al.*, 1992
	T11	urine	Maekawa *et al.*, 1992
	X98 (ATCC 27276)	infant feces	ATCC; Brock *et al.*, 1963

2.1.7 Primers used in this study

Oligonucleotides for screening and sequencing purposes are listed in Table 9 and were synthesized by Eurofins MWG-Operon (Ebersberg, Germany). Primers developed in this study were designed on bases of public available sequences: GenBank nucleotide sequence database under following accession numbers: AE016830.1 and AF454824.1.

Table 9: Primer sequences used in this study. References (Ref.): a) this study; b) Eaton and Gasson (2001); c) Gaspar *et al.* (2009); d) Palmer and Gilmore (2010); e) Ehrmann *et al.* (2003).

Primer	Sequence (5` to 3`)	Gene	PCR-Product length (bp)	Ref.
ace for	CCGAATTGAGCAAAAGTTC	*ace*	746	a
ace rev	AGTGTAACGGACGATAA			
TE3	AAGAAAAAGAAGTAGACCAAC	*as*	923	b
as rev	ACCTACAGCGTCCCAATCAC			a
TE49	AACATTCAGCAAACAAAGC	*cob*	1,405	b
TE50	TTGTCATAAAGAGTGGTCAT			
cytolysin for	GGTTGCCATTGAAAAATATCTTCTAGTGGAGTATCCAGG	*cytolysin locus*	8,364	c
cytolysin rev	GTGATTGATTGGCTTATTTCATCATCATCACTTTTGAGC			
TE5	GACAGACCCTCACGAATA	*efaAfs*	705	d
TE6	AGTTCATCATGCTGTAGTA			
TE 37	AACAGATCCGCATGAATA	*efaAfm*	735	d
TE 38	CATTTCATCATCTGATAGTA			
esp for	GAGTTAGCGGGAACAGGTCA	*esp*	617	a
TE36	GCGTCAACACTTGCATTGCCGAA			b

Primer	Sequence (5` to 3`)	Gene	PCR-Product length (bp)	Ref.
fsrB for	GTTTGTCCCATCCATTGTCC	*fsrB*	346	a
fsrB rev	TTTATTGGTATGCGCCACAA			
gelE for	AATTGCTTTACACGGAACGG	*gelE*	547	a
gelE rev	AGCCATGGTTTCTGGTTGTC			
sprE for	CTTGTCTGCAAATGCAGAAG	*sprE*	660	a
sprE rev	CGCCATTGGAATGAACACCA			
C1-*cas csn1* for	CAGAAGACTATCAGTTGGTG	CRISPR1-*cas csn1*	783	d
C1-*cas csn1* rev	CCTTCTAAATCTTCTTCATAG			
C1-*cas* for	GCGATGTTAGCTGATACAAC	CRISPR1-*cas*	315	d
C1-*cas* rev	CGAATATGCCTGTGGTGAAA			
C2 for	CTGGCTCGCTGTTACAGCT	CRISPR2	variable	d
C2 rev	GCCAATGTTACAATATCAAACA			
C3-*cas csn1* for	GCTGAATCTGTGAAGTTACTC	CRISPR3-*cas csn1*	258	d
C3-*cas csn1* rev	CTGTTTTGTTCACCGTTGGAT			
C3 for	GATCACTAGGTTCAGTTATTTC	CRISPR3-*cas*	224	d
C3 rev	CATCGATTCATTATTCCTCCAA			
616V	AGAGTTTGATYMTGGCTCAG	16S rRNA	815	e
609R	ACTACYNGGGTATCTAAKCC			
M13V	GTTTTCCCAGTCACGAC	unspecific	variable	e

2.2 Methods

2.2.1 Microbiological methods and experiments

2.2.1.1 Media and growth conditions

All enterococci were cultivated in Brain Heart Infusion (BHI) medium under aerobic conditions at 37°C. For long-term storage strains were held in glycerol stocks.

2.2.1.2 *E. faecalis* growing under aerobic and anaerobic conditions

For anaerobic and aerobic growth experiments, *E. faecalis* strain OG1RF was grown at 37°C in brain heart infusion broth (BHI) at 200 rpm aerobically or strictly anaerobic in an anaerobic incubator (Heraeus Instruments, Hanau, Germany). For proteome analysis, *E. faecalis* were harvested at mid exponential growth phase (OD_{590} 0.5-0.6).

2.2.1.3 Detection of gelatinase activity

Determination of gelatinase-producing strains was carried out on lysogeny broth (LB) agar (10 g peptone, 5 g yeast extract, 5 g NaCl, 15 g agar per liter) containing 3 % (w/v) gelatine. After incubation at 37 °C for 24 h, plates were stored at 4 °C for 5 h according to Eaton and Gasson (2001).

2.2.1.4 Detection of cytolysin activity

The phenotypic detection of the enterococcal strains for hemolytic activity was performed on Columbia blood agar plates containing 5 % (v/v) sheep blood and additionally on LB agar supplemented with 5 % (v/v) defibrinated horse blood. According to Gaspar *et al.* (2009), blood agar plates were incubated under aerobic and anaerobic conditions at 37 °C (Anaerocult ®A, Merck, Germany). The production of cytolysin (β-hemolysis) appeared in a clear zone around grown enterococcal colonies due to hemolyzed erythrocytes within the agar.

2.2.1.5 Interaction of *E. faecalis* with mouse colonic epithelial cells *in vitro*

The Ptk6 null mouse colonic epithelial cell line (further on referred as Ptk6 cells) was grown according to standard cell culture techniques (Whitehead *et al.*, 2008) in a humidified 5 % CO_2 atmosphere at 33°C to almost 100 % confluence in 75 cm^2 cell culture flasks (Cellstar® Filter cap flasks, Greiner Bio-One GmbH, Frickenhausen, Germany). The used cell culture medium was RPMI medium (with L-glutamine) supplemented with 5 % (v/v) fetal bovine serum (FBS), 1 % (v/v) insulin-transferrin-selenium A, 1 % (v/v) antibiotic/antimycotic solution (a/a) and 2 ng/ml interferon-γ (IFN-γ). The medium was changed every 2-3 days of on-going cultivation. After confluent growth of the cells, they were washed twice with 10 ml of phosphate-buffered saline (PBS). Then 2 ml of trypsin-EDTA solution was applied allowing the detachment and passage of cells. After an incubation time of trypsin-EDTA solution for 4 minutes at 37°C, cells were harvested and diluted with 10 ml culture medium and then centrifuged at 300 x g for 5 min. The supernatant was discarded and the remained cell pellet was then resuspended in fresh culture medium for seeding.

For cell culture experiments *E. faecalis* OG1RF (TMW 2.622) in an early to mid exponential growth phase (OD_{590} 0.3-0.4) was exposed to confluent grown Ptk6 cells (further indicated as CC) for 3 hours. The Ptk6 cells derived from passage 20-45 were grown at 37°C to differentiate. Two days prior to the experiment, cell culture media was used without antibiotic/antimycotic (a/a) solution and IFN-γ.

As a reference *E. faecalis* OG1RF was simultaneously exposed to cell culture media (further indicated as CM) in the same way as CC but without the presence of Ptk6 cells. The sampling of *E. faecalis* for proteome analysis was carried out after 3 hours of exposure to Ptk6 cells (CC) as well as sole cell culture media (CM). The pH value and colony

forming units were checked before and after the exposure of *E. faecalis*. The experiment was carried out in triplicates and a multiplicity of "infection" with a ratio of 400 was applied. For both conditions CC and CM samples were taken out of one batch of *E. faecalis* overnight culture. After the experiment was carried out, the samples were directly processed for protein extraction in preparation of the two-dimensional gel electrophoresis (see 2.2.4.3).

2.2.1.6 Monoassociation of *E. faecalis* in mice

The mouse experiment was carried out in the lab of Prof. R. Balfour Sartor (University of North Carolina, Chapel Hill, NC, USA). The mice were held under strictly germ free conditions in the National Gnotobiotic Rodent Resource Center at the University of North Carolina, Chapel Hill. Nine germfree wild type mice 129 SvEv TAC (derived by Dr. Edward Balish, University of Wisconsin, Madison, WI, USA) were monoassociated with *E. faecalis* strain OG1RF (TMW 2.622) at an age of 11-12 weeks. Mice were fed with standard autoclavable diet Prolab® RMH 3500 in which calories are provided by 26.20 % protein, 15.46 % fat and 58.34 % carbohydrates. The protein part consists of nineteen proteinogenic, essential free amino acids, the fat part consists mainly of long chain, unsaturated fatty acids and the carbohydrate part comprises mainly of starch (see Appendix for LabDiet® PMI Nutrition International, Purina Mills, Inc Richmond, Indiana, USA). The inoculation of mice with *E. faecalis* was done by gavage feeding and rectal swabbing. Mice were killed one week after initial bacterial colonization. The presence of a pure culture of *E. faecalis* in mice was verified by plating ten-fold dilution series of colon content on BHI agar plates. The intestinal contents were collected and deep-frozen directly after killing and dissection. The fecal samples were preserved at -80 °C until RNA isolation for next generation SOLiD^{TM} sequencing analysis (see 2.2.3.1).

For histology analysis and scoring respectively tissue sections of small intestine, the cecal tip, and colon were fixed in 10 % (v/v) buffered formalin. After fixation the tissue samples were embedded in paraffin and cut in sections of 5 µm size. Afterwards the tissue samples were leached from paraffin and stained with hematoxylin and eosin (H&E staining) according to Berg *et al.* (1996).

The stained tissue samples were classified in histology scores in the range of 0 (no damage) to 12 (severe damage) in a blinded fashion by assessing the degree of lamina propria mononuclear cell filtration, crypt hyperplasia, goblet cell depletion and

architectural distortion as described by Katakura *et al.* (2005). The scoring of mouse tissue samples used in this study has been done by Dr. S. Kisling (Lehrstuhl für Biofunktionalität der Lebensmittel, Technische Universität München).

2.2.2 Molecular Biological Methods

2.2.2.1 DNA isolation from enterococci

Prior to DNA isolation all *Enterococcus* strains were grown in BHI broth at 37°C. DNA isolation was done with the E.Z.N.A.® Bacterial DNA kit (Omega Biotek Inc., Norcross, Georgia, USA) according to the manufacturer's instructions. The result of DNA isolation was monitored by analytical agarose gel electrophoresis and the amount of DNA was determined by NanoDrop1000 (Peqlab GmbH, Erlangen, Germany) analysis.

2.2.2.2 General DNA amplification

The isolated DNA was amplified by polymerase chain reaction (PCR). The PCR reactions were performed on thermo cycler by Eppendorf (Mastercycler® Gradient, Eppendorf AG, Hamburg, Germany) in a total of 50 µl, using 80 pmol of each primer, 1.5 mM $MgCl_2$, 10 mM each of dNTPs, 2U of Taq DNA, 1x Buffer mix (Qbiogene, USA) and sample DNA. Amplification conditions were as follows: an initial denaturation step of 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 1 min, primer annealing for 1 min (at an appropriate temperature, calculated by melting temperature of primer minus 3 °C) and extension at 72 °C for a duration adjusted according to PCR-product length (Taq polymerase replicates about 1 kb strand per min), followed by a final elongation at 72 °C for 5 min.

2.2.2.3 Agarose gel electrophoresis

The generated PCR products were electrophoretically separated on 1.5 % (w/v) agarose gels (in total 100 ml) prepared with 0.5 x TBE buffer that was stored as 10 x TBE buffer (108 g/l Tris, 26.2 g/l boric acid, 9.0 g/l EDTA, pH 8.9). The PCR samples were mixed with loading dye in ratio 6:1 and applied onto prepared agarose gel. Samples of a general PCR were applied on gels of about 12 x 13.8 cm size with small cavities (of 10 µl capacity) and electrophoresis was performed at 110 V for 1 hour. Electrophoretic analysis of RAPD-PCR patterns was carried out in 1.3 % (w/v) 0.5 x TBE gel (in total 220 ml) at 170 V for 3 hours in an electrophoresis chamber of 20 x 25 cm size with large cavities of 15 µl capacity. By the application of ready-to-use DNA size standards (see 2.1.5) as a

molecular weight marker the generated PCR product size was determined.
After gels have been stained with dimidium bromide the banding profiles were visualized under UV light (wavelength 320 nm) and digitalized by gel documentation system from INTAS-science imaging instruments GmbH.

2.2.2.4 Detection of virulence genes and CRISPR associated (*cas*) genes by general DNA amplification

A total of 62 *Enterococcus* isolates from clinical, food and environmental origins were used in this study (see Table 6 and Table 7 for strains and origins of isolation). Out of these, 52 *E. faecalis* and 10 *E. faecium* isolates were tested. All strains used in this study were verified as belonging to the species *E. faecalis* or *E. faecium* by sequencing the 815 bp amplification product of the 16S rRNA gene generated by primers 616V and 609R (primer sequence see Table 9). Sequencing of this PCR product was performed by GATC Biotech GmbH (Konstanz, Germany). Additionally, this PCR target was also used as a positive control for each strain analyzed by PCR.
Specific primers for the following virulence associated and CRISPR associated (*cas*) genes were used: *ace, as*, *cob*, cytolysin operon, *efaAfs, efaAfm*, *esp*, *fsrB*, *gelE*, *sprE,* CRISPR1-*cas csn1*, CRISPR2 loci, CRISPR3-*cas csn1*. The primer sequences and their amplification products are listed in Table 9.
The PCR screening for the complete cytolysin locus with an amplification product of 8,364 bp (Gaspar *et al.*, 2009) was performed using Phusion® High-Fidelity DNA Polymerase (Finnzymes Oy, Vantaa, Finland) according to the recommendations of the manufacturer. All primers were tested before being used with *E. faecalis* strains OG1RF and V583. All screening PCRs were performed in an Eppendorf Gradient Cycler (Eppendorf, Hamburg, Germany). Negative or indistinct results were conducted at least twice to achieve a definite result.

2.2.2.5 In silico screening for virulence associated genes and CRISPR associated (cas) genes

Sixteen *E. faecalis* strains of recently sequenced enterococcal genomes (Palmer *et al.*, 2010) were screened *in silico* for the presence of virulence genes and CRISPR-*cas csn1* loci by platform provided from Broad Institute that made also the genome sequences publicly available (http://www.broadinstitute.org/). For a detailed list of *E. faecalis* strains

analyzed *in silico* see Table 8.

2.2.2.6 Analysis of genetic fingerprinting by RAPD-PCR

RAPD (random amplification of polymorphic DNA) analyses were performed using single primer M13V (see Table 9) as described by Ehrmann *et al.* (2003). The PCR reaction mix contained 5 µl of 10 x PCR buffer without $MgCl_2$, 7 µl of 25 mM $MgCl_2$, 2 µl dNTPs (each 10 mM), 0.5 µl primer M13V (100 pmol/ µl), 0.3 µl Taq polymerase (5U/ µl) and 0.5 µl of ~100 ng extracted DNA. The PCR cycling program started with 3 cycles of 94 °C for 3 min, 40 °C for 5 min, 72 °C for 5 min, which was followed by 32 cycles of: 94 °C for 1 min, 60 °C for 2 min, and 72 °C for 3 min. The PCR products were electrophoretically separated as described above (2.2.2.3). The GeneRuler™ 3 k bp plus DNA ladder (Fermentas, Canada) was used as a molecular weight marker. Generated RAPD-PCR fingerprints were analyzed by BioNumerics^{TM} software (version 3.0; Applied Maths BVBA, Belgium). The analysis of the similarities of the generated RAPD-PCR fingerprints was calculated on the basis of the Pearson product-moment correlation coefficient. A cluster analysis was derived from the matrix of similarities by the Unweighted Pair Group Method using Arithmetic Mean (UPGMA) cluster algorithm.

2.2.3 Methods of transcriptome analysis

2.2.3.1 Extraction of total bacterial RNA out of mouse fecal samples

In the following procedure all buffers or pure water were treated with diethylpyrocarbonate (DEPC) to inactivate RNases and prevent RNA degradation. Prior RNA extraction of *in vivo*, mouse fecal samples were prepared according to a slightly modified method by Kang *et al.* (2009). Mouse fecal samples were weighed in aliquots (each 0.1 g) and mixed with equal volume of phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4 \cdot H_2O$, 1.76 mM K_2HPO_4; pH 7.4). After high-speed centrifugation at 10,000 x g for 1 min the supernatant was discarded and the pellet was resuspended in equal volume of dissociation solution (DSS: 0.1 % (v/v) Tween 80, 1 % (v/v) methanol, 1 % tertiary butanol adjusted to pH 2 with HCl; Whitehouse *et al.*, 1994). The samples were centrifuged at low-speed for 3 min at 200 x g. Afterwards, the supernatant was collected and centrifuged at 10,000 x g for 1 min. The supernatant was discarded, the pellet was resuspended in 200 µl TE buffer and than 600 µl RLT buffer (including 10 µl/ml of β-mercaptoehtanol) was added. This suspension was mixed with pre-cooled silica beads to

start cell disruption by using FastPrep®-24 at 5.5 m/sec speed for 45 sec (MP Biomedicals, Cleveland, Ohio, USA). The suspension was applied on columns of the RNeasy mini Kit (Qiagen, Hilden, Germany) and further total RNA isolation was done according to the manufacturer's instructions. Further procedure of total RNA treatment for next generation SOLiD™ sequencing analysis see section 2.2.3.3.

2.2.3.2 Extraction of total RNA form pure cultures

E. faecalis liquid culture samples (7.5 ml) for isolation of total RNA were pelleted with ice-cold methanol (2.5 ml) by centrifugation at 10,000 x g for 15 min at 4 °C and washed with 2 ml cooled TE buffer. After another centrifugation at 10,000 x g for 5 min at 4 °C the supernatant was discarded and the remaining cell pellet was resuspended in 200 µl TE buffer. The cell suspension was immediately mixed with 600 µl RLT buffer supplemented with β-mercaptoethanol, 10 µl/ml RLT buffer (Qiagen, Hilden, Germany), and applied on pre-cooled silica beads (Lysing Matrix B; MP Biomedicals, Cleveland, Ohio, USA). The cell disruption was done using FastPrep®-24 (MP Biomedicals, Cleveland, Ohio, USA) for 45 sec with 5.5 m/sec speed. After the beads had settled the supernatant was transferred in a new 1.5 ml tube and mixed with 160 µl of ice-cold 100 % (v/v) ethanol. This suspension was applied on columns of the RNeasy mini Kit (Qiagen, Hilden, Germany) and further total RNA isolation was done according to the manufacturer's instructions. The obtained RNA is further referred to as reference sample in transcriptome analysis.

2.2.3.3 RNA treatment for next generation SOLiD™ sequencing analysis

Before total RNA was separated from co-extracted rRNA and DNA residues, the integrity of the total isolated RNA was assessed by gel electrophoresis in a 1.5 % (w/v) agarose gel that was prepared with 0.5 x TBE buffer. The degradation of RNA under running on the agarose gel was avoided by cleaning electrophoresis chamber with 1 % (w/v) SDS solution before starting the electrophoresis.

Indices of protein and volatile compound contamination were calculated by the absorption ratios A_{260}/A_{280} and A_{260}/A_{230} measured by NanoDrop1000 spectrophotometer (Peqlab GmbH, Erlangen, Germany). The ratio of the absorbance at 260 nm and 280 nm was used to assess the purity of RNA. A ratio of about 2 was accepted as pure. The ratio of the absorbance at 260 nm and 230 nm may indicate the presence of co-purified contaminants and samples only with a value in the range between 1.8 and 2.2 were accepted for further

analysis. RNA samples extracted from mouse fecal content from nine mice (five male and four female mice) were originated from all intestinal segments: the small intestine, caecum and colon. These samples were pooled to finally one *in vivo* total RNA sample only if the quality requirements were met.

The total RNA sample was depleted of rRNA using Ribominus™ Transcriptome Isolation Kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. Afterward the treated RNA was precipitated with ethanol in which the RNA sample was well mixed with 1 µl glycogen (20 µg/µl), 2.5 x sample volumes of 100 % ethanol and $1/10^{th}$ sample volume of 3 M sodium acetate. After the precipitation of RNA by incubation at -80 °C for 2 hours, the sample was treated with RNase free DNase to avoid DNA contamination in further analysis. This has been done by the use of TURBO DNA-free™ Kit (Applied Biosystems Inc., Foster City, California, USA) according to manual's instructions. As mentioned above, a second precipitation has been done but in this step without using glycogen. Finally, the amount of residual messenger RNA (mRNA) for transcriptome analysis was measured again by NanoDrop1000 spectrophotometer.

For next generation SOLiD™ sequencing analysis (sequencing by oligonucleotide ligation and detection, developed by Life Technologies, Carlsbad, California, USA) the samples, RNA from mouse experiment and RNA from pure culture, were sent to CeGat GmbH (Tübingen, Germany).

2.2.3.4 Next generation SOLiD™ sequencing data analysis and processing

SOLiD sequencing was perfomed at CeGat GmbH (Tübingen, Germany). Generation of cDNA, library preparations, fragment library protocols, and all SOLiD-run parameters followed standard Applied Biosystem protocols. The generated data files of the sequencing were formatted in qual and csfata files. The data was converted and mapped using Galaxy software (http://usegalaxy.org/) according to the following procedure given by personal communication with Richard Landstorfer (Prof. Scherer, Chair for Microbial Ecology, Technische Universität München). The data were mapped with Bowtie for SOLiD (version 1.1.2) on the available genome sequence from *E. faecalis* OG1RF that was obtained from the National Center for Biotechnology Information (NCBI; http://ncbi.nlm.nih.gov/; accession number CP002621). After the mapping process the generated sequence alignment/map (sam) format was filtered and converted into the binary alignment/map (bam) data format. This file was arranged by picard (via SortSam and BuildBam Index).

This was visually appraised by Artemis software (version 13.2.0). The bioinformatic analysis determined counts (number of reads covering a gene) and RPKM values (reads per kilobase per million mapped reads; Mortazavi *et al.*, 2008), and has been done by Svenja Simon (University of Konstanz, Department of Computer and Information Science, Germany). The counts were normalized using a previously described method (Mortazavi *et al.*, 2008) in which reads are adapted for the sequencing depth and for the length of a given gene. For determination of counts and RPKM values, the previously generated bam files were imported into statistic software R (verison 2.14.1) (Grömping, 2010) using Rsamtools (http://bioconductor.org/). For onward processing, the Bioconductor packages GenomicRanges and IRanges was used (Gentleman *et al.*, 2004 and http://www.bioconductor.org/). Gene locations were determined by GenBank entry of *E. faecalis* OG1RF under accession number CP002621 in NCBI database (http://ncbi.nlm.nih.gov/). Ptt files and locations of 5S, 16S and 23S rRNA genes were determined by rnt file from information in CP002621. The method countOverlaps was used to determine the reads overlapping a 5S, 16S or 23S rRNA gene. These reads are discarded from further analysis, since the presence of rRNA at naturally high proportions hinders the detection of potential low transcribed genes through normalization. The tool of countOverlaps is additionally used to determine the number of reads overlapping a gene on the same strand (counts). These counts were normalized by gene length in kilobases and the number of mapped reads in millions. For the value million mapped reads we use the number of reads mapped to the genome minus the reads overlapping a 5S, 16S or 23S rRNA gene (see above). The transcription level can then be quantified as RPKM values and further calculated through values of the logarithm base 2 into logarithm fold changes (logFC). To define a gene as significantly differentially expressed its p-value was calculated and a significance level of $p \leq 0.05$ was set as a threshold.

2.2.4 Methods of protein analyses

2.2.4.1 Analysis of whole cell mass spectral profile by MALDI-TOF-MS

All enterococcal isolates were grown for 20 hours on Columbia sheep blood agar plates at 37°C. Colonies were picked and resuspended in 300 µl of mutanolysin solution (20 u/ml) and incubated at 37°C for 30 min. 900 µl of ethanol (100 %) were added. The mixture was centrifuged at maximum speed for 2 min. After removal of the supernatant, the pellet was centrifuged again. Residual ethanol was completely removed by pipetting and the pellet

was allowed to dry at room temperature. Subsequently, 30 µl of 70 % (v/v) formic acid were added and mixed with the pellet by vortexing. Next 30 µl of acetonitrile were added and mixed thoroughly. The solution was centrifuged at maximum speed for 2 min again and 1.5 µl of the supernatant were spotted on the MALDI target plate (Bruker Daltonics, Bremen, Germany) in two replicates. Immediately after drying, 1.5 µl of the matrix solution was added to each spot and allowed to air dry. The matrix used was a saturated solution of α-cyano-4-hydroxycinnamic acid dissolved in 50 % (v/v) acetonitrile with 0.025 % (v/v) trifluoroacetic acid. Brukers Bacterial Test Standard (Bruker Daltonik GmbH, Bremen, Germany) was used as mass calibration standard. Samples were then processed in the MALDI-TOF-MS spectrometer (Microflex LT; Bruker Daltonics) with flex control software (Bruker Daltonics, Bremen, Germany). Each spectrum was obtained by averaging 240 laser shots acquired in the automatic mode at the minimum laser power necessary for ionization of the samples. The spectra were analyzed in an *m/z* range of 2 to 20 kDa. Dr. M. Pavlovic carried out the generation of MALDI-TOF-MS spectra of all enterococcal isolates used in this study at Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit (Oberschleißheim, Germany).

2.2.4.2 MALDI-TOF-MS data analysis

Data analysis was performed using LIMPIC for reduction of background noise and the baseline drift (Mantini *et al.*, 2007). Multiple spectra (6 to 8) of each strain were processed on the basis of the *peak detection rate* (PDR) of 0.6, expressed by the ratio between the number of spectra containing the considered peak and the total number of analyzed spectra (Mantini *et al*., 2007). The obtained spectra were imported into BioNumerics™ and the cluster analysis was performed as described for the RAPD analysis (see passage 2.2.2.6).

2.2.4.3 Protein extraction method for two dimensional (2D) gel electrophoresis

The protein extraction was done by a slightly modified method according to Behr *et al.* (2007). Bacteria cells were washed with Tris EDTA buffer (TE buffer containing 10mM Trisbase, 1mM EDTA, pH 8.0) and resuspended for cell lysis with TE buffer containing lysozyme (50 mg/ml) for 30 min at 37 °C. After centrifugation at 5000 x g for 5 min the supernatant was discarded and the cell pellet was resuspended in 400 µl SDS buffer (0.9 % (w/v) SDS, 100 mM Tris base, pH 8.6) and disrupted by sonication (HD-70/Bandelin, 2 cycles of 30 sec; power 90 %, cycle 30 % and intermediate cooling on ice). Then 500 µl

Chaps lysis buffer (6 M urea, 2 M thiourea, 4 % (w/v) Chaps, 1 % (w/v) DTT, 1.5 % (v/v) Servalyt^{TM} 3-10) was added and solubilised proteins by shaking for 20 min at 4 °C. The residual cell debris was separated by centrifugation for 30 min at 18,000 x g and 4 °C. Aliquots of supernatants were stored at -80 °C (Behr *et al*., 2007).

2.2.4.4 Determination of protein concentration

The protein concentration was determined by using the Bio-Rad Protein Assay. This assay is based on the method of Bradford (Bradford, 1976) and measures spectroscopic the concentration of proteins. Before Bio-Rad dye reagent was mixed with protein standards and protein samples for measuring, the dye stock solution was diluted with four parts of pure water. The prepared series of protein standards of bovine albumin fraction V (BSA) in a concentration range between 200 and 1400 µg/ml were mixed with the prepared dye reagent in a ratio of 1:50. After 5 minutes of incubation the protein standards and samples were measured at 595 nm wavelength. The concentration of protein samples was calculated based on the generated standard curve from measured BSA standards.

2.2.4.5 Two dimensional (2D) gel electrophoresis

Two dimensional gel electrophoresis begins with the first dimension, the isoelectric focusing (IEF) of protein samples on immobilized pH gradient (IPG) dry strips (Görg *et al.*, 2000). The IEF was performed with Hoefer IEF 100 isoelectric focusing unit (Hoefer Inc., Holliston, MA, USA) on 24 cm IPG strips with pH range of 4-7 at 20 °C. Drying of IPG strips during IEF was prevented through a cover with silicon oil. The protein samples were applied on the gel strips by anodic cup loading after the IPG dry strips were rehydrated overnight to their gel thickness (0.5 mm) in a reswelling solution containing 6 M urea, 2 M thiourea, 0.5 % (w/v) Chaps, 0.2 % (w/v) DTT, 0.2 % (v/v) Servalyt^{TM} 3-10 (Görk *et al.*, 2000). The IEF was carried out according to the following protocol: 1000 V (4 h, gradient), 12 000 V (6 h, gradient), 2.0 W (64 000 V h, constant), and 1000 V (2 h, constant).

Prior to the second dimension, the vertical gel electrophoresis, IPG strips were equilibrated by the use of two equilibration solutions (pH 8.8) containing 50 mM Trisbase, 5 M Urea, 2.5 % (w/v) SDS, 30 % (v/v) glycerol, and 2 % (w/v) DTT in the first and 4 % (w/v) iodacetamide instead of DTT in the second step (Görg *et al.*, 1987). After the equilibration of the IPG strips, they were applied to vertical SDS polyacrylamide gels with a total

acrylamide concentration of T 11 %, and cross-linker concentration 3 %. An overnight polymerization of SDS polyacrylamide gels (PAGE) was initiated by adding 3.5 ml 10 % (v/v) ammonium persulfate solution and 27.5 µl tetramethylethylenediamine (TEMED) into 500 ml of gel reagent. The SDS-polyacrylamide gel electrophoresis (PAGE) was run according to the following settings: 60 mA for 1 h, followed by 80 mA for 4 h and finally 165 mA overnight at 15 °C. The running buffer contained 24 mM Tris, 200 mM glycine and 0.1 % (w/v) SDS.

The proteins were visualized through silver or colloidal coomassie staining. The silver staining has been done by a modified procedure according to Blum *et al.* (1987). For the fixation the gels were incubated in a solution containing 40 % (v/v) ethanol and 10 % (v/v) acetic acid. Two following washing steps were carried out in a solution of 30 % (v/v) ethanol, followed by an incubation for 1 min in 0.2 % (w/v) thiosulfate containing reagent. After three washing steps with distilled water the gels were incubated for 20 min in sliver nitrate reagent containing 2 % (w/v) silver nitrate and 0.2 % (v/v) of formaldehyde (37 %). After another three washing steps with distilled water, a developer (30 % (w/v) sodium carbonate, 0.2 % (v/v) of formaldehyde (37 %), and 0.03 mM sodium thiosulfate) was applied to the point were visual protein spots appeared.

The colloidal coomassie staining was done by a solution consisting of 1 x Roti®-Blue, 0.2 % (v/v) methanol and 0.01 % (v/v) phosphoric acid. For destaining and hence clarifying the background of the gels, they were washed in distilled water. After staining, gels were scanned (Epson Expression 1600pro, Seiko Epson Corporation, Tokyo, Japan) and the generated Tiff files were analyzed by Progenesis SameSpots^{TM} software (version 4.1; Nonlinear Dynamic Ltd., UK).

2.2.4.6 Protein identification

Relevant proteins were excised from the coomassie-stained gel (Roti-Blue, see section above) and sent to Zentrallabor für Proteinanalytik (ZfP of Ludwig-Maximilians-Universität München, Germany) for LC-ESI MS/MS analysis. The identification of proteins from the genome sequenced *E. faecalis* OG1RF, was genrally done according to Behr *et al.* (2007). All available protein sequences from *E. faecalis,* were obtained from the National Center for Biotechnology Information (NCBI; http://ncbi.nlm.nih.gov/). For protein identification, the NCBI database was searched by WU-BLAST2 program (Gish, 1996). The "Mascot generic format (MGF) formatted" (Perkins *et al.*, 1999) files, derived

from LC-ESI MS/MS analysis, were processed to peptide sequences with PepNovo (Frank *et al.*, 2005; Frank and Pevzner 2005, Frank *et al.*, 2007; Frank, 2009). Therefore, MGF files were split in parts and processed in parallel by an open sharedroot computer cluster (ATIX; http://opensharedroot.org/) using a message passing interface (MPI) for job control (Open MPI: 11th European PVM/MPI Users`Group Meeting, Hungary, Sept. 2004). BASH scripts and programs written in C generated and submitted catenations of peptide sequences to MS-BLAST for protein identification (Shevchenko *et al.*, 2001). Personal communication with Dr. Jürgen Behr.

3 Results

3.1 Distributions of virulence and CRISPR-*cas* genes

In total, 62 *Enterococcus* isolates (see Table 6 and 7) were screened for virulence and CRISPR-*cas* genes. The virulence genes were detected in *E. faecalis* isolates only, with the exception of *esp,* the *Enterococcus* surface protein, which was present in 27 out of 52 *E. faecalis* isolates and also in nine *E. faecium* strains. Ace, which encodes an adhesin protein in *E. faecalis* was found in 51 isolates and was the most frequent virulence gene found in the PCR tested strains. The gene encoding for endocarditis antigen EfaAfs was found in 34 *E. faecalis* strains whereas the equivalent of *E. faecium* (*efaAfm*) was present in all 10 *E. faecium* strain used in this study. The pheromone encoding gene cob was only present in 22 *E. faecalis* isolates and was one of the less frequent genes detected. The cytolysin operon was detected in 19 isolates, which all showed the phenotypic expression (β-hemolysis) on horse blood agar plates, only with one exception (TMW 2.928). The gelatinase gene (*gelE*) was present in 38 *E. faecalis* strains. The distribution of CRISPR-associated (*cas*) genes was tested by the presence of following genes: CRISPR1-*cas csn1*, CRISPR3-*cas csn1* according to (Palmer and Gilmore, 2010). In parallel the absence of CRISPR1-*cas* and CRISPR3-*cas* loci was determined by the detection of the junction EF0672-0673 and EF1760-1759 (Palmer and Gilmore, 2010). The CRISPR2 locus was detected by primers targeting the conserved position. We detected a PCR-product with variable length in 47 *E. faecalis* strains. However, the efficacy of the CRISPR defense system relies on the presence and activity of near by located *cas* genes (Horvath and Barrangou, 2010). These crucial genes are missing in the CRISPR2 locus and thus we neglected to list it in Table 10. Eighteen *E. faecalis* strains were *cas* positive (CRISPR1-*cas* and CRISPR3-*cas*) and negative for one of the junctions indicating the CRISPR loci appear in the locations between EF0672 and EF0673 (CRISPR1-*cas*) (Bourgogne *et al.*, 2008; Palmer and Gilmore, 2010) or EF1760 and EF1759 (CRISPR3-*cas*) as compared with the genome of V583 (Palmer and Gilmore, 2010). It was found that strains containing less virulence genes tended to have more frequently detected *cas* genes (see Table 10). However, a statistically significant correlation between the presence of CRISPR-*cas* loci and the absence of a virulence gene could only be determined for the cytolysin operon, *cob* encoding the pheromone, and *as* encoding the aggregation substance (p-value < 0.01; analyzed by the Fisher exact test). According to Palmer and Gilmore (2010) one CRISPR-

cas locus was identified in three *E. faecium* genomes. However, this locus could not be detected in our ten *E. faecium* strains.

Table 10: Summary of PCR screening results of virulence and CRISPR-associated genes. Strains are listed according to the number of detected virulence genes, from most present to less present virulence genes. Source of strains is marked as followed a) clinical isolate, b) environmental isolate, c) food isolate and d) unknown. Strains marked with [*)] were tested *in silico* based on sequences published by Palmer and Gilmore (2010) with Broad Institute.

strain	virulence genes									CRISPR-*cas* and corresponding flanking regions			
	fsrB	*gelE*	*sprE*	*cytolysin operon*	*ace*	*efaAfs*	*as*	*esp*	*cob*	CRISPR 1-*cas*	CRISPR 1-*cas* flank	CRISPR 3-*cas*	CRISPR 3-*cas* flank
X98[b *)]	+	+	+	+	+	+	+	+	+	+	-	-	+
JH1[a *)]	+	+	+	+	+	+	+	+	+	-	+	-	+
2.929[a]	+	+	+	+	+	+	+	-	+	-	+	-	+
2.928[a]	+	+	+	+	+	+	+	-	+	-	+	-	+
2.927[a]	+	+	+	+	+	+	+	-	+	-	+	-	+
2.914[a]	+	+	+	+	+	+	+	-	+	-	+	-	+
2.915[a]	-	+	+	+	+	+	+	+	+	-	+	-	+
2.902[a]	-	+	+	+	+	+	+	+	+	-	+	-	+
T1[d *)]	+	+	+	+	+	+	+	-	+	-	+	-	+
Merz96[a *)]	+	+	+	+	+	+	+	-	+	-	+	-	+
HIP11701[a *)]	-	+	+	+	+	+	+	+	+	-	+	-	+
2.930[a]	+	+	+	+	+	+	+	-	-	-	+	-	+
2.908[a]	+	+	+	+	+	+	+	-	-	-	+	-	+
2.907[a]	+	+	+	+	+	+	-	-	+	-	+	-	+
2.926[a]	+	+	+	+	+	-	+	-	+	-	+	-	+
2.901[a]	+	+	+	-	+	+	+	-	+	-	+	-	+
2.630[c]	+	+	+	-	+	+	+	+	-	+	-	-	+
2.648[c]	+	+	+	-	+	+	+	+	-	-	+	-	+
2.924[a]	-	-	+	+	+	+	+	+	+	-	+	-	+
2.905[a]	-	-	+	+	+	+	+	+	+	-	+	-	+
2.922[a]	-	+	+	+	+	+	+	+	-	-	+	-	+
CH188[a *)]	+	+	+	-	+	+	-	+	+	-	+	-	+
T2[a *)]	-	+	+	+	+	+	-	+	+	-	+	-	+
T8[a *)]	-	+	+	+	+	+	-	+	+	-	+	-	+
T11[a *)]	+	+	+	-	+	+	+	-	+	-	+	+	-
D6[b *)]	-	-	-	+	+	+	+	+	+	+	-	-	+
AR01/DG[b *)]	+	+	+	-	+	+	+	-	+	-	+	-	+
2.947[a]	+	+	+	+	+	-	+	-	-	-	+	-	+
2.354[b]	+	+	+	-	+	-	+	-	+	-	+	-	+
2.852[a]	+	+	+	-	+	-	+	-	+	-	+	-	+
2.815[b]	+	+	+	-	+	-	+	-	+	-	+	-	+
2.629[c]	+	+	+	-	+	-	+	+	-	+	-	-	+
2.921[a]	+	+	+	-	+	+	-	+	-	+	-	-	+
2.936[a]	+	+	+	-	+	+	+	-	-	-	+	-	+
2.798[b]	+	+	+	-	+	-	-	+	+	+	-	-	+
2.911[a]	-	-	+	+	+	+	+	+	-	-	+	-	+
2.932[a]	-	+	+	+	+	-	+	+	-	-	+	-	+
2.63[c]	-	+	+	+	+	-	+	-	+	-	+	-	+
2.900[a]	-	+	+	-	+	+	-	+	+	-	+	-	+
2.910[a]	-	+	+	-	+	+	-	+	+	-	+	-	+
2.925[a]	+	-	+	-	+	+	-	+	+	-	+	-	+
2.906[a]	+	-	+	-	+	+	-	+	+	+	-	-	+
2.937[a]	+	-	+	-	+	+	+	+	-	+	-	-	+
2.136[b]	+	-	+	-	+	-	+	+	+	+	-	-	+
T3[a *)]	+	+	+	-	+	+	-	-	+	-	+	-	+
E1Sol[b *)]	+	+	+	-	+	+	-	-	+	+	-	-	+
Fly1[b *)]	+	+	+	-	+	+	-	-	+	-	+	+	-
2.912[a]	+	+	+	-	+	+	-	-	-	+	-	-	+
2.916[a]	+	+	+	-	+	+	-	-	-	-	+	+	-
2.919[a]	+	+	+	-	+	+	-	-	-	+	-	-	+
2.923[a]	+	+	+	-	+	+	-	-	-	+	-	-	+
2.933[a]	+	+	+	-	+	-	-	+	-	+	-	-	+
2.931[a]	-	+	+	-	+	+	-	+	-	+	-	-	+
2.917[a]	-	+	+	-	+	+	-	+	-	+	-	-	+
2.918[a]	-	+	+	-	+	+	-	+	-	+	-	-	+
2.913[a]	-	+	+	-	+	+	-	+	-	-	+	-	+
2.934[a]	-	-	+	+	+	-	+	+	-	-	+	-	+
2.904[a]	-	-	+	+	+	+	-	+	-	-	+	-	+
2.903[a]	-	-	+	-	+	+	+	-	+	-	+	-	+
ATCC4200[a *)]	-	+	+	-	-	+	-	+	+	+	-	-	+
DS5(ATCC14508)[d *)]	-	-	-	+	+	+	-	+	+	+	-	-	+
2.622[b]	+	+	+	-	+	-	-	-	-	+	-	-	+
2.520[b]	+	+	+	-	+	-	-	-	-	+	-	-	+
2.647[c]	-	+	+	-	+	-	+	-	-	+	-	-	+
2.935[a]	+	-	+	-	+	-	-	-	-	-	+	-	+
2.645[a]	-	-	+	-	+	-	+	+	-	-	+	-	+
2.909[a]	-	-	-	-	+	+	-	+	-	-	+	-	+
2.777[b]	-	-	-	-	-	-	+	-	-	-	+	-	+

Additionally, recently sequenced strains of *E. faecalis* by Palmer *et al.* (2010) were screended *in silico* for the presence of virulence genes and CRISPR-*cas csn1* loci and are listed in Table 8. These strains fit the tendency of possessing frequent virulence genes and less CRISPR-*cas* loci. In spite of this tendency there are still strains that did not match this surveillance (X98, 2.630, T11, D6, 2.629, 2.921).

3.2 Phenotypic screening of gelatinase and cytolysin activity

The detection of β-hemolytic activity was done on Columbia blood agar plates containing 5 % (v/v) of sheep blood and on LB agar plates containing 5 % (v/v) of horse blood to test the varying sensitivity of erythrocytes from different species against enterococcal cytolysin. Additionally, the impact of oxygen availability was compared with the β-hemolytic activity and accordingly its expression. A β-hemolytic phenotype was identified by a clear zone around grown colonies. A distinct expression of a β-hemolysis was detected using horse blood agar plates under anaerobic conditions. From the 52 *E. faecalis* strains eighteen showed a cytolysin phenotype (see Table 5).

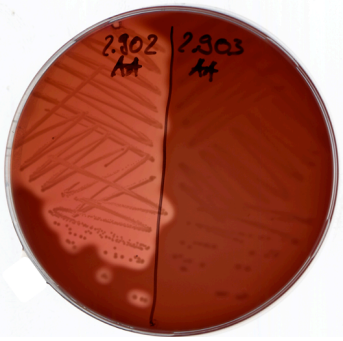


Figure 5: Phenotypic expression of β-hemolytic activity on LB agar plates containing horse blood. Strain TMW 2.902 showed a positive phenotype through a clear zone in the agar plate around grown colonies and strain TMW 2.903 showed no β-hemolytic activity.

Figure 6: Phenotypic analysis of gelatinase activity on lysogeny broth agar plates containing gelatine. Strain TMW 2.630 showed gelatinase activity through opacity in the agar plate around grown colonies

The gelatinase activity of Enterococci was tested on LB agar plates containing gelatine (3 % (w/v)). A release of gelatinase by enterococci was verified by a definite opacity appearing within the agar around grown colonies (see Figures 6). A total of 22 strains showed a gelatinase phenotype that was only detected in strains possessing *fsrB* and *gelE*. Two strains did not show a gelatinase phenotype although they possess *fsrB* and *gelE* indicating that these genes may be defective in their sequence (TMW 2.923, TMW 2.933).

Neither β-hemolytic activity nor gelatinase activity was detected in the ten *E. faecium* strains and therefore they are not listed in Table 11.

Table 11: Results of phenotypic detection of gelatinase (yellow) and cytolysin (red) activity. Strains are listed according to numbers in TMW culture collection.

Species	**TMW strain no.**	Columbia blood agar containing 5 % (v/v) sheep blood		LB agar containing 5 % (v/v) horse blood		LB agar conatining 3 % (v/v) gelatine
		aerobic	anaerobic	aerobic	anaerobic	Gelatinase
E. faecalis	2.63					
	2.136					
	2.354					
	2.520					
	2.622					
	2.629					
	2.630					
	2.645					
	2.647					
	2.648					
	2.777					
	2.798					
	2.815					
	2.852					
	2.900					
	2.901					
	2.902					
	2.903					
	2.904					
	2.905					
	2.906					
	2.907					
	2.908					
	2.909					
	2.910					
	2.911					
	2.912					
	2.913					
	2.914					
	2.915					
	2.916					
	2.917					
	2.918					
	2.919					
	2.921					
	2.922					
	2.923					
	2.924					
	2.925					
	2.926					
	2.927					
	2.928					
	2.929					
	2.930					
	2.931					
	2.932					
	2.933					
	2.934					
	2.935					
	2.936					
	2.937					
	2.947					

3.3 Genetic analysis of *Enterococcus* isolates by RAPD-PCR

For all 52 *E. faecalis* and ten *E. faecium* strains that were present in TMW culture collection at that time RAPD-PCR was generated. The spectrum analyzed by BioNumerics™ software, which uses Unweighted Pair Group Method with Arithmetic Mean (UPGMA) for comparative analysis derived five major groups of enterococci (see

Figure 7: cluster I-V). At a cut-off level of 65 % species-specific differentiation of *E. faecalis* and *E. faecium* was achieved, cluster I-IV containing species *E. faecalis* and cluster V *E. faecium*. While cluster III contained strains of various sources of isolation, the RAPD-PCR fingerprint using the M13V primer (cut-off level 70 %) generated two clusters (I, II), which almost exclusively contained clinical isolates, with only one exception in cluster I (TMW 2.647). Furthermore, one environmental isolate (TMW 2.798 - designated as cluster IV) showed a RAPD-PCR fingerprint, which did not match any of the generated groups (see RAPD patterns in the Appendix). Cluster V contained all *E. faecium* strains.

3.4 Proteome analysis of *Enterococcus* isolates by MALDI-TOF-MS

The generated MALDI-TOF-MS spectra were analyzed by BioNumerics^{TM} software, which uses UPGMA for comparative analysis. For this analysis only peaks with a detection rate (PDR) of 0.6 were included. This method clearly differentiated at strain level, and a general clustering in five main groups was only feasible under a cut-off level of 10 %. Within these groups (I-IV) the isolates showed neither a relation of their phenotype of gelatinase or β-hemolysis nor any relation to the origin of isolation. Even on species-specific basis one exception (TMW 2.942) appeared as an independent group (see Figure 8: III).

3.5 Proteome analysis –Two dimensional (2D) gel electrophoresis

Protein samples of *E. faecalis* strain OG1RF (TMW 2.622) analyzed by 2D electrophoresis were derived from aerobic/anaerobic and cell culture experiments. Each experiment is presented separately in the following chapters 3.5.1 to 3.5.2. In the first dimension the whole-cell protein fraction of *E. faecalis* was separated using IPG strips in the pH range of 4 to 7 with a separation distance of 24 cm. The followed SDS PAGE separation, second dimension, had a separation range from 10-250 kDa using 11 % (v/v) polyacrylamide gels. Silver stained gels were analyzed for differentially expressed proteins with Progenesis SameSpots^{TM} software (version 4.1; Nonlinear Dynamic Ltd., UK). Identified protein spots were excised on preparative gels stained with coomassie and were send to ZfP (Zentrallabor für Proteinanalytik, Ludwig-Maximilians-Universität München) for LC-ESI MS/MS analysis. The generated data were processed to peptide sequences with PepNovo and further analyzed as described in detail in section 2.2.3.9.

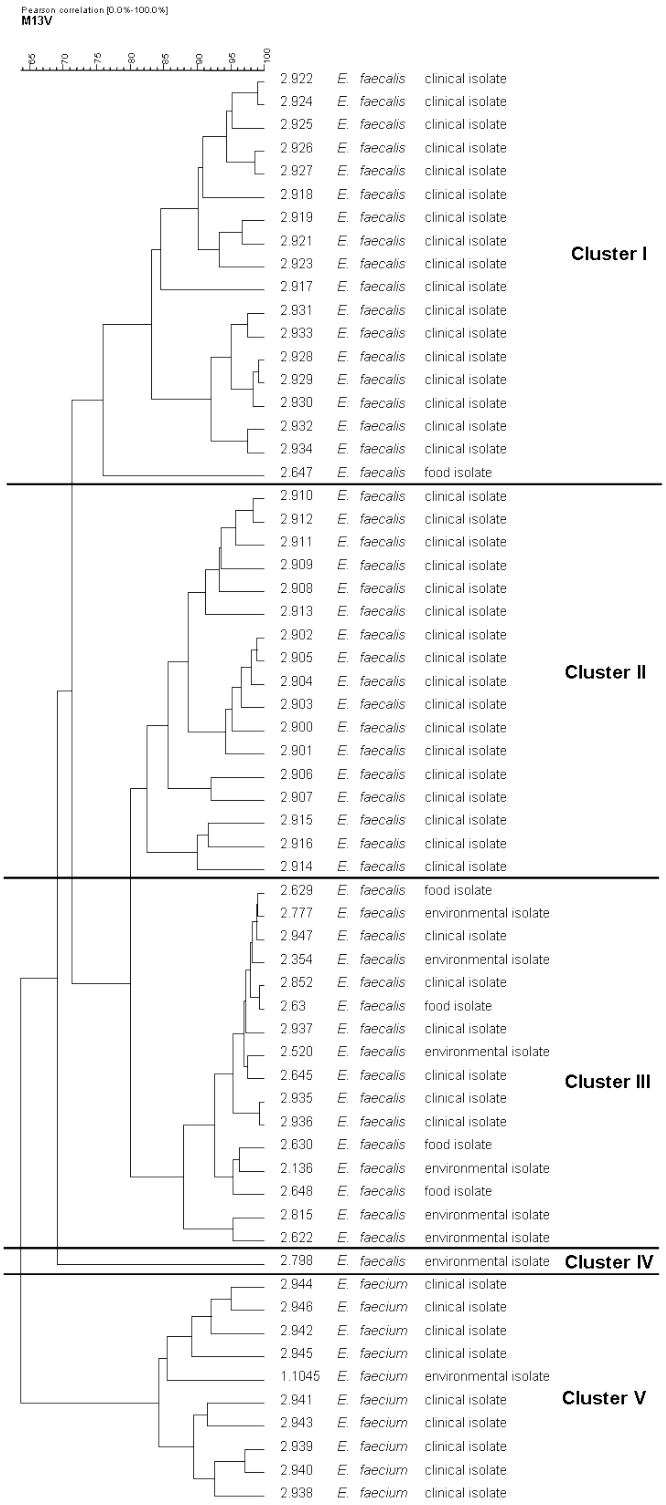


Figure 7: Cluster analysis of genetic fingerprints of 52 *E. faecalis* isolates and 10 isolates of *E. faecium* generated by the use of RAPD-PCR. At a cut-off level of 65 % a species-specific clustering is shown (cluster I-IV versus cluster V). Similarity coefficients were calculated using the method of Pearson, and the clustering was calculated using UPGMA approach.

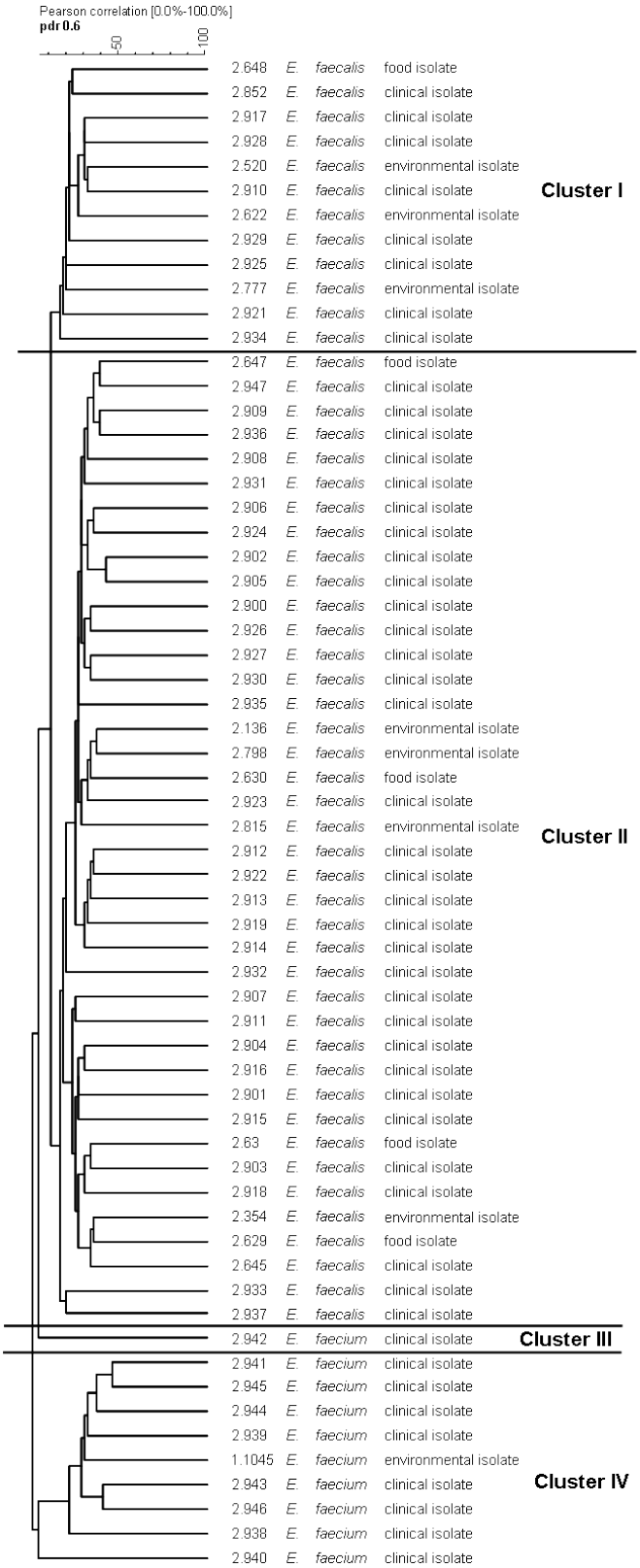


Figure 8: Cluster analysis of mass spectra from 52 *E. faecalis* and 10 *E. faecium* isolates. For the analysis only peaks with a detection rate (PDR) of 0.6 were included. Similarity coefficients were calculated using the method of Pearson, and the clustering was calculated using UPGMA approach. Four clusters were generated (I-IV).

3.5.1 Proteome analysis of *E. faecalis* grown under aerobic and anaerobic conditions

Strain *E. faecalis* OG1RF was grown either under aerobic conditions through agitation (200rpm), or otherwise through anaerobic conditions. Samples were taken from both approaches at mid exponential growth phase (OD_{590} 0.5-0.6), see Figure 9.

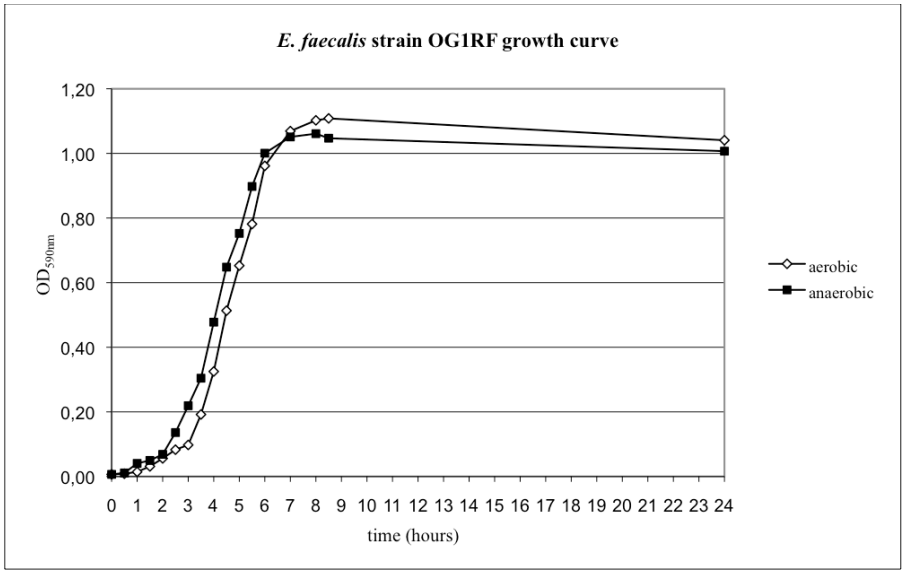


Figure 9: Growth-curves of *E. faecalis* OG1RF (TMW 2.622) in BHI medium under aerobic (open square) and anaerobic (black square) growth conditions. Each time point represents the mean value of three biological replicates. Optical density (OD) was measured at a wavelength of 590 nm at certain time points.

Each growth condition was assessed in triplicate. In total, eight differentially expressed proteins could be identified. Five of the eight proteins were induced upon aeration and were absent under anaerobic growth conditions, whereas the other three proteins were expressed under anaerobic conditions but not under aerobic growth conditions (see Figure 10 and Figure 11). For details see Table 14 and Table 15, the identified proteins with their corresponding change in protein expression (n-fold) and their significance level (p-value) were calculated by Progenesis SameSpots^{TM} software (version 4.1). The identified proteins and their function are presented in Tables 12 and 13.

Two of the identified proteins involved in the aminoacyl-tRNA biosynthesis: asparaginyl-tRNA ligase (AsnS) and phenylalanyl-tRNA synthetase subunit alpha (PheS). AsnS catalyzes the attachment of L-asparagine to its cognate transfer RNA (tRNA) under the consumption of energy through the degradation of ATP. The alpha subunit of phenylalanyl-tRNA synthase is part of the enzyme catalyzing the same reaction as AsnS, but instead of L-asparagine L-phenylalanine is attached to its specific tRNA.

The aerobically induced SufC protein is an ABC-type ATPase that is involved in the biosynthesis of iron-sulfur clusters through an active enterococcal SufCDSUB complex (Riboldi *et al.*, 2009). In contrast to other ABC ATPases that are part of ABC transporters and located in the cell membrane, the SufC does not contain transmembrane domains and is present in the cytosol (Nachin *et al.*, 2003).

Table 12: Proteins identified from 2D-electrophoresis that were expressed of *E. faecalis* strain OG1RF after growing under aerobic conditions. Accession numbers designated gi refers to NCBI database entries. Proteins are listed according to their molecular weight (Mw).

Protein	Gene ID	Mw (kDa)	Accession no.	Function
PBP4	OG1RF_11907	74	gi | 327535760	penicillin-binding protein 4
AsnS	OG1RF_11805	51	gi | 327535658	asparagine-tRNA ligase
PheS	OG1RF_10893	39	gi | 327534746	phenylalanyl-tRNA synthetase subunit alpha
SufC	OG1RF_11829	30	gi | 327535682	ABC superfamily ATP binding cassette transporter, ABC protein
Gls24	OG1RF_10071	28	gi | 327533924	stress response regulator Gls24 protein

Also aerobically expressed was one protein that is part of the enterococcal cell membrane: penicillin-binding protein 4 (PBP4). PBP4 is and is responsible for the resistance of *E. faecalis* to β-lactam antibiotics (Duez *et al.*, 2001). Another identified protein expressed under aerobic growth conditions was the stress response regulator Gls24 protein. This is an important protein for the stress response of various challenges, e.g. glucose starvation (Giard *et al.*, 2000)

Table 13: Proteins identified from 2D-electrophoresis that were expressed of *E. faecalis* strain OG1RF after growing under anaerobic conditions. Accession numbers designated gi refers to NCBI database entries. Proteins are listed according to their molecular weight (Mw).

Protein	Gene ID	Mw (kDa)	Accession no.	Function
ArcB	OG1RF_10100	39,00	gi |327533953	ornithine carbamoyltransferase
ArcC	OG1RF_10101	32,00	gi |327533954	carbamate kinase
SdaA	OG1RF_10094	30,00	gi |327533947	L-serine dehydratase, iron-sulfur-dependent, α subunit

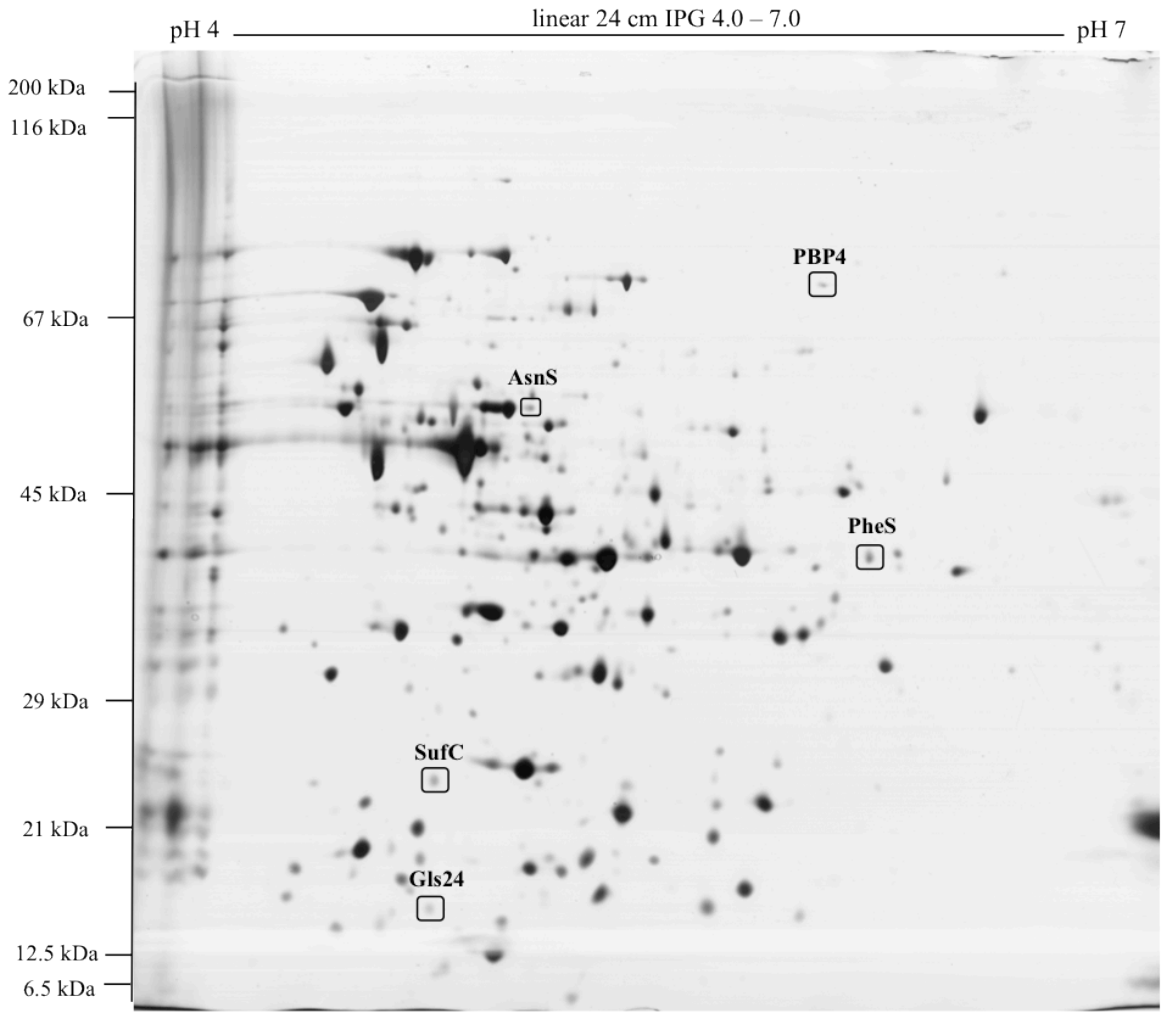
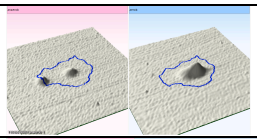
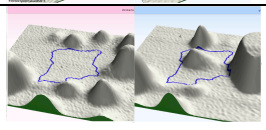
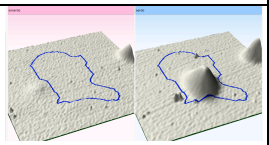
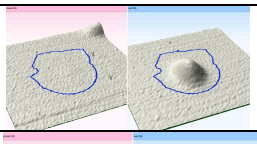
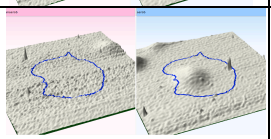


Figure 10: Two-dimensional electrophoretic analysis of silver stained total protein from *E. faecalis* strain OG1RF (TMW 2.622) grown under aerobic conditions. Marked proteins (PBP4, AsnS, PheS, SufC and Gls24) were only expressed under aerobic growth of *E. faecalis* OG1RF.

Table 14 Induced proteins in *E. faecalis* OG1RF grown under aerobic conditions. The picture of protein spot, p-value and fold difference were generated and calculated by Progenesis SameSpots™ software (version 4.1; Nonlinear Dynamic Ltd., UK). Protein spot on the left (highlighted in light-red) is originated from anaerobic growth and protein spot on the right (highlighted in blue) is originated from aerobic growth.

Protein	Protein spot in different conditions	p-value	Fold difference
PBP4		0.019	2.5
AsnS		0.025	5.0

Protein	Protein spot in different conditions	p-value	Fold difference
PheS		0.011	7.0
SufC		0.027	4.5
Gls24		0.022	4.3

In comparison to aerobically induced proteins a total of three proteins were differentially expressed in OG1RF under anaerobic conditions (see Figure 11 and Tables 13 and 15). Two of these three proteins are part of the arginine catabolism, which is called arginine deiminase (ADI) pathway: the ornithine carbamoyltransferase (ArcB) and carbamate kinase (ArcC).

Another anaerobically induced protein that was identified is the alpha subunit of L-serine dehydratase (SdaA), which catalyzes the degradation of L-serine to pyruvate and ammonium.

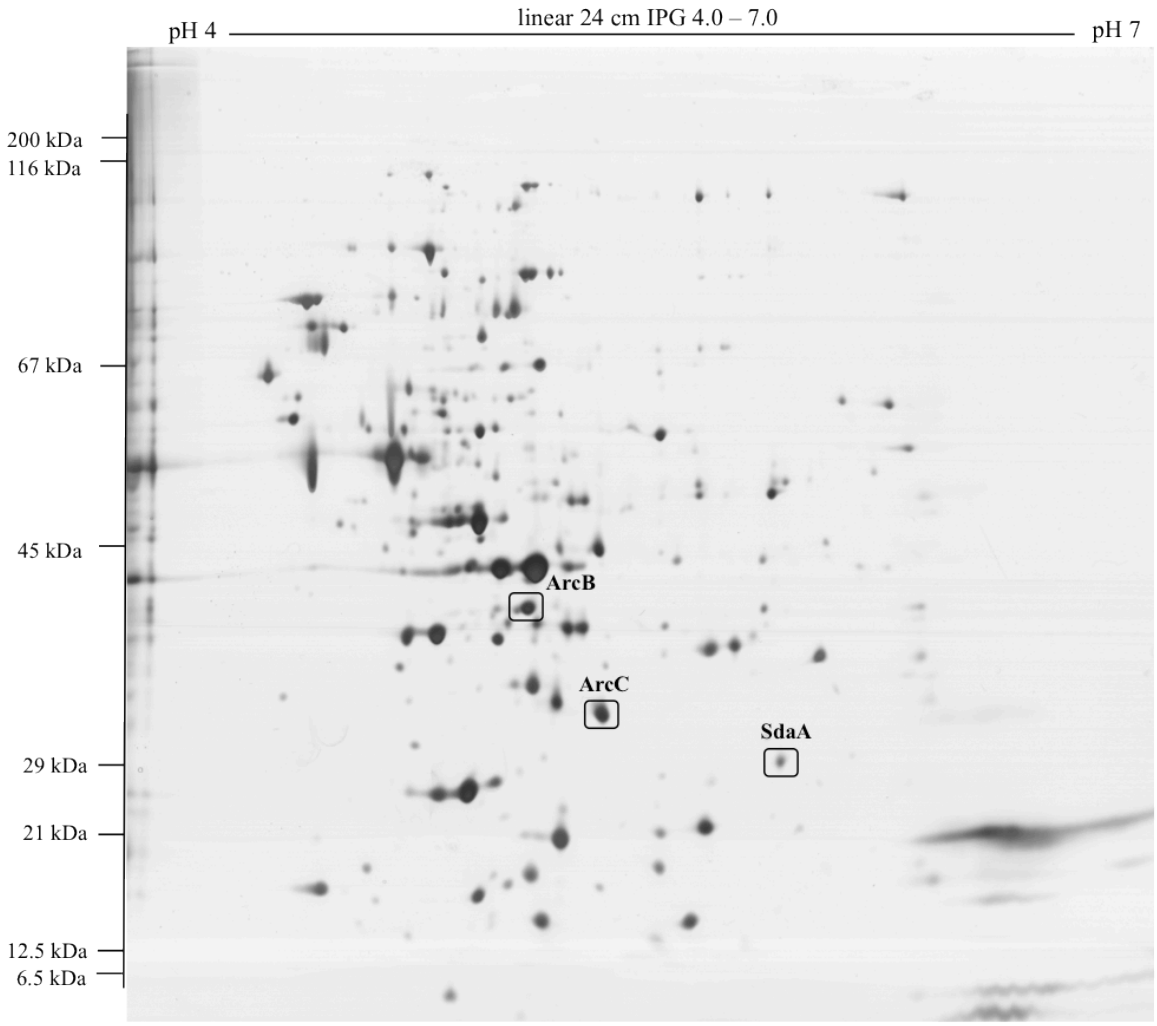
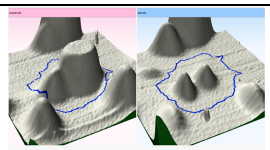
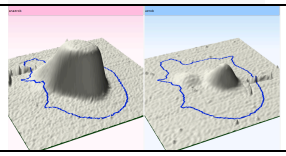
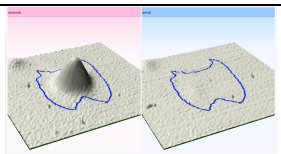


Figure 11: Two-dimensional electrophoretic analysis of silver stained total protein from *E. faecalis* strain OG1RF (TMW 2.622) grown under anaerobic conditions. Marked proteins (ArcB, ArcC and SdaA) were only expressed under anaerobic growth of *E. faecalis* OG1RF.

Table 15: Induced proteins in *E. faecalis* OG1RF grown under anaerobic conditions. The picture of protein spot, p-value and fold difference were generated and calculated by Progenesis SameSpots™ software (version 4.1; Nonlinear Dynamic Ltd., UK). Protein spot on the left (highlighted in light-red) is originated from anaerobic growth and protein spot on the right (highlighted in blue) is originated from aerobic growth

Protein	Protein spot in different conditions	p-value	Fold difference
ArcB		0.034	2.4
ArcC		0.028	3.2

Protein	Protein spot in different conditions	p-value	Fold difference
SdaA		0.00004	10.4

3.5.2 Proteome analysis of *E. faecalis* in contact with mouse colonic epithelial cells *in vitro*

For the experiment, *E. faecalis* OG1RF (TMW 2.622) was exposed to Ptk6 null mouse colonic epithelial cell line (Ptk6 cells). *E. faecalis* cells were in the early to mid exponential growth phase (OD_{590} 0.3-0.4) when applied to the cell culture at a multiplicity of infection (MOI) of 400 (further indicated as CC). As a reference *E. faecalis* OG1RF was simultaneously exposed to cell culture media only (further indicated as CM). The sampling of *E. faecalis* for proteome analysis was done after three hours of exposure to Ptk6 cells as well as to sole cell culture media (CM). The experiment was done in triplicates and for both conditions CC and CM samples were taken out of one batch of *E. faecalis* overnight culture. The pH value and CFU/ml were checked before and after the experiment to ensure that a difference in protein expression is not a cause of different growth phases. The change of both parameters was similar in conditions with (CC) and without cell culture (CM) (Table 16).

Table 16: Determination of cell counts of *E. faecalis* OG1RF and pH value in cell culture media including confluent Ptk6 cells (CC) and cell culture media without Ptk6 cells (CM) at the start and endpoint of the experiment.

	pH value in supernatant		Cell counts of *E. faecalis* (CFU/ml)	
E. faecalis exposed to cell culture (CC) or sole to cell culture medium (CM)	CC	CM	CC	CM
Start of experiment	7.3 ±0.2		2.4×10^8	
End of experiment (after 3 hours)	6.7 ±0.3	6.6 ±0.3	3.8×10^8	3.9×10^8

Four protein spots could be identified as expressed in the presence of Ptk6 cells, see Table 17 and 19.

Table 17: Proteins identified from 2D-electrophoresis that were expressed of *E. faecalis* strain OG1RF after 3 hours of exposure to mouse epithelia cells (Ptk 6) *in vitro*. Accession numbers designated gi refers to NCBI database entries. Proteins are listed according to their molecular weight (Mw).

Protein	Gene ID	Mw (kDa)	Accession no.	Function
AsnS	OG1RF_11805	51	gi | 327535658	asparagine-tRNA ligase
RmlA	OG1RF_11734	32	gi | 327535587	Glucose-1-phosphate - thymidylyltransferase
OmpR	OG1RF_10965	27	gi | 327534818	DNA-binding response regulator
BudA	OG1RF_10986	25	gi | 327534839	α-acetolactate decarboxylase

E. faecalis OG1RF expresses asparagine-tRNA ligase (AsnS), which is involved in the biosynthesis of aminoacyl-tRNA. AsnS catalyzes the attachment of L-asparagine to its compatible cognate tRNA.

Glucose-1-phosphate-thymidylyltransferase (RmlA) was also expressed in the presence of mouse colonic epithelial cells. This enzyme is part of the extracellular enterococcal cell wall polysaccharide biosynthetic process and catalyzes the junction of a nucleotide with a sugar. Specifically, thymidine diphosphate glucose and pyrophosphate is generated out of D-glucose 1-phosphate and thymidine triphosphate (dTTP).

Another induced protein in OG1RF was the DNA-binding response regulator of the OmpR family, which is part of a two-component system. This regulator shows high protein sequence similarities (75 %) to response regulators YycF in *Bacillus* (*B.*) *subtilis* and *Staph. aureus* as well as to VicR in *Streptococcus* (*S.*) *pneumoniae*. A previously identified consensus recognition sequence of YycF in *B. subtilis* was published 5`-TGT(A/T)A(A/T/C)-$N_5$-TGT(A/T)A(A/T/C)-3` by Dubrac and Msadek (2003). An *in silico* search was performed for this motif in the complete genome of *E. faecalis* OG1RF (CP002321.1) with nucleic acid pattern search function (FUZZNUC) of EMBOSS database (http://proteomics.leeds.ac.uk/emboss/fuzznuc.html). A list of 30 hits could be found in the genome on either strand of strain OG1RF. According to characteristics of DNA-binding response regulators, matches located within open reading frames were eliminated. Five potential binding sites within a 400 bp region upstream from a translation initiation codon (ATG or GTG) were identified. Among these, only three genes encode proteins with a putative or known function, OG1RF_10090 encoding a putative lipoprotein, OG1RF_10218 encoding a thioesterase and OG1RF_12493 encoding the beta

subunit of a DNA-directed RNA-polymerase (see Table 18).

Table 18: Alignment of nucleotide sequences of putative OmpR-regulated promoter regions in sequenced strain OG1RF. Accession numbers designated CP002621.1 (genome OG1RF), AE, and OG1RF refers to NCBI database entries.

Range of consensus sequence in genome CP002621.1	**Strand**	**Sequence**	**Putative regulated gene and its product**
102,098-102,114	+	TGTTACATTAATGTTAC---N_{27}---ATG	OG1RF_10090, putative lipoprotein (AEA92777.1)
228,935-228,951	+	TGTTAAGTCAATGTTAC---N_{323}---GTG	OG1RF_10218, thioesterase (AEA92905.1)
956,700-956,716	+	TGTTATAATTTTGTAAA---N_{45}---GTG	OG1RF_10914, hypothetical protein (AEA93601.1)
558,233-558,249	-	TGTAACTAGATTGTAAA---N_{150}---ATG	OG1RF_10532, hypothetical protein (AEA93219.1)
2,635,030-2,635,046	-	TGTAACGTAAATGTAAT---N_{40}---GTG	OG1RF_12493, DNA-directed RNA polymerase subunit beta (AEA95180.1)

The third protein identified in OG1RF that was induced by the exposition to mouse epithelial cells was α-acetolactate decarboxylase (BudA). This enzyme plays a key role in the regulation of the α-acetolactate pool in the process of pyruvate catabolism and biosynthesis of branched-chain amino acids.

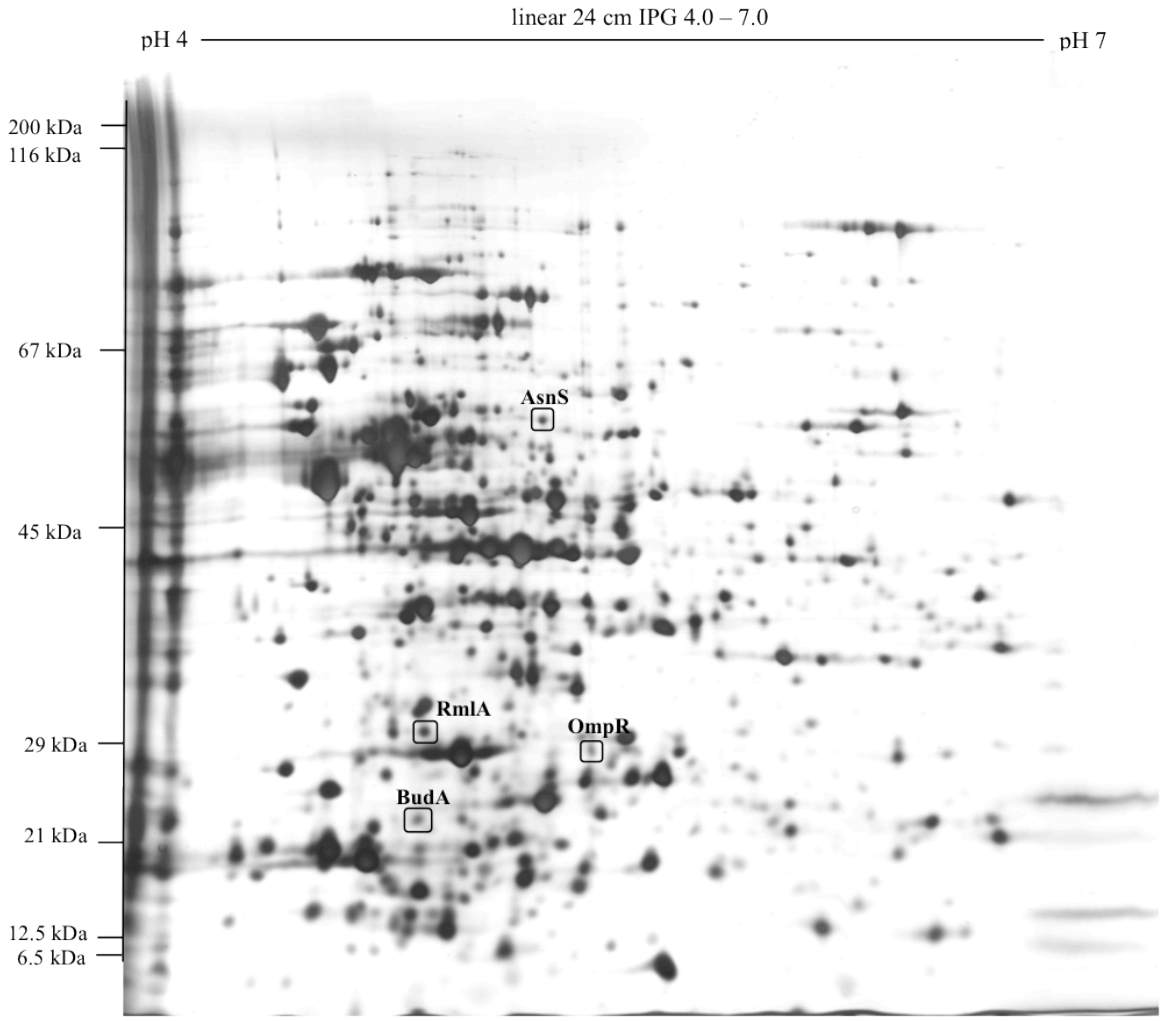
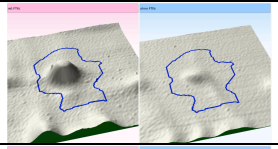
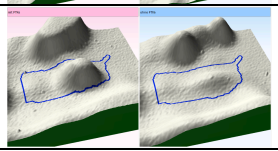
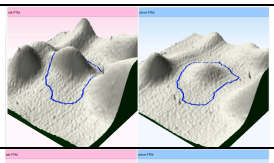
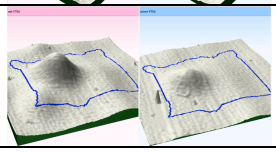


Figure 12: Two-dimensional electrophoretic analysis of silver stained total protein from *E. faecalis* OG1RF (TMW 2.622) exposed to mouse colonic epithelial cells for three hours. Four marked proteins (AsnS, RmlA, OmpR and BudA) were only expressed in OG1RF during its exposition to mouse epithelial cells.

Table 19: Induced proteins in *E. faecalis* OG1RF through contact with mouse colonic epithelial cells *in vitro*. The picture of protein spot, p-value and fold difference were generated and calculated by Progenesis SameSpots™ software (version 4.1; Nonlinear Dynamic Ltd., UK). Protein spot on the left (highlighted in light-red) is originated from OG1RF in contact with mouse epithelial cells and protein spot on the right (highlighted in blue) is originated from OG1RF without mouse epithelial cells

Protein	Protein spot in different conditions	p-value	Fold difference
AsnS		0.007	2.9
RmlA		0.023	2.5

Protein	Protein spot in different conditions	p-value	Fold difference
OmpR		0.008	1.7
BudA		0.043	2.6

3.6 Transcriptome analysis of *E. faecalis* monoassociated in wild type mice *in vivo* by next generation SOLiD™ sequencing analysis

For *in vivo* transcriptome analysis of *E. faecalis* strain OG1RF (TMW 2.622), nine germfree wild type mice (129 SvEv TAC) were monoassociated with *E. faecalis* at 11-12 weeks of age. The intestinal content containing strain OG1RF was collected through dissection after one week of bacterial colonization. The total RNA of *E. faecalis* was extracted out of the fecal content of five male and four female mice, and was pooled to one RNA sample for further transcriptome analysis.

The verification of a pure culture of *E. faecalis* in the tested mice and the number of colony forming units (CFU) was confirmed by plating ten-fold dilution series of mice colon content on BHI agar plates. A CFU of 2.9 x 10^9 per 1 gram of mice colon content could be detected.

For transcriptome data analysis a reference RNA sample of *E. faecalis* OG1RF has been extracted of a bacteria culture grown in BHI media under anaerobic conditions at 37°C. *E. faecalis* cells were harvested at mid exponential growth phase. The intestinal tissue

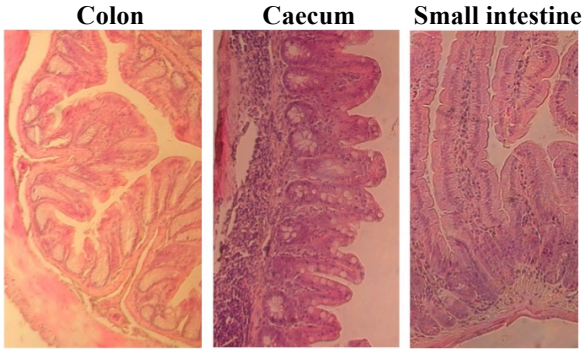


Figure 13: Hematoxylin and eosin staining of intestinal tissue sections from wild type mice, which were monoassociated for one week with *E. faecalis* OG1RF. No impact of OG1RF on intestinal tissue has been observed.

samples of monoassociated wild type mice used in this study were classified by a histological score of 0, which indicates no tissue damage (see Figure 13). The amount and quality of both total bacterial RNA samples, the *in vivo* sample and reference sample, were determined through NanoDrop1000 spectrophotometer measuring and through gel electrophoresis (see Table 20 and Figure 14).

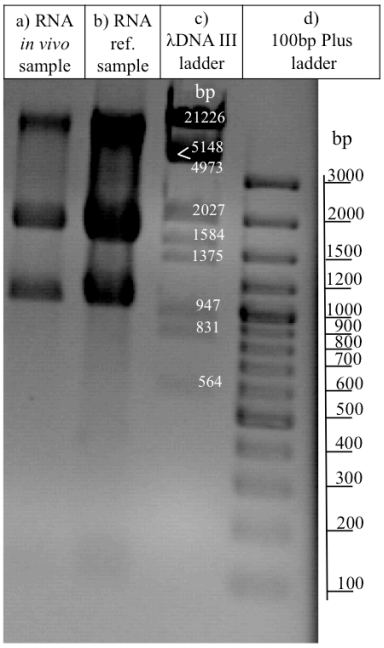


Figure 14: Quality and integrity of total RNA: a) Total RNA from OG1RF extracted from *in vivo* experiment, b) Total RNA from OG1RF grown under reference (ref.) conditions in BHI media, c) λDNA/EcoRI+HindIII ladder, d) GeneRuler™ 100 pb Plus ladder. In lane a) and b) bands of 23S rRNA and 16S rRNA are indicating the efficient quality of total RNA.

Furthermore, the total RNA samples were removed from co-extracted rRNA and DNA residues by the use of Ribominus™ Transcriptome Isolation Kit and TURBO DNA-free™ Kit. For next generation SOLiD™ sequencing analysis (sequencing by oligonucleotide ligation and detection, developed by Life Technologies, Carlsbad, California, USA) samples were sent to CeGat GmbH (Tübingen, Germany).

Table 20: RNA amount measured with spectrophotometer. Total RNA amount (ng/µl) from OG1RF of *in vivo* and reference (ref.) sample before rRNA depletion and DNase treatment. The RNA amount (ng/µl) from OG1RF of *in vivo* and ref. sample after rRNA depletion and DNase treatment.

Sample		ng/µl	Ratio of Absorption 260nm/280nm	Ratio of Absorption 260nm/230nm
before rRNA and DNA depletion	total RNA *in vivo*	40	1.99	2.09
	total RNA ref.	438	1.87	2.10
after rRNA and DNA depletion	RNA *in vivo*	10	1.92	2.26
	RNA ref.	65	1.80	2.22

3.6.1 Results of transcriptome analysis of *E. faecalis* monoassociated in wild type mice *in vivo*

The generated data files of the sequencing were in qual and csfata format. These data were converted and mapped using Galaxy software (http://usegalaxy.org/) as described in 2. Material and Methods section 2.2.3.4. The data were mapped with Bowtie for SOLiD (version 1.1.2) on the available genome sequence from *E. faecalis* OG1RF. The generated sequence alignment/map (sam) format was filtered and converted into a file in binary alignment/map (bam) format. This file was arranged via SortSam and BuildBam Index tools. The data were further processed at University of Konstanz by Svenja Simon, Department of Computer and Information Science, Germany. The generated sequence reads were quantified to transcription levels in reads per kilobase per million mapped reads (RPKM), which reflects the molar concentration of a transcript in the sample by normalizing RNA length and the total read number in the measurement (Mortazavi, 2008). A valid RPKM value was set as a minimum of 10 on the basis of three genes that are part of the primary metabolism and possessed a similar RPKM value in both conditions (RPKM values of *ung* encoding uracil-DNA glycosylase: 14 /40; *pyrC* encoding dihydroorotase: 73/36; *aroE* encoding shikimate dehydrogenase: 51/91; *rluD2* encoding 23S rRNA pseudouridylate synthase: 92/96).

Finally, we defined a gene to be significantly differentially expressed if its calculated significance level (p-value) was ≤ 0.05, and the absolute value of the logarithm (base 2) fold change (logFC) was greater than 6.55 for an up-regulated gene and smaller than -6.95 for a down-regulated gene. These logFC thresholds were arranged according to the calculated ratios of RPKM values of the *in vivo* experiment sample and the reference sample in which the reference was set as the denominator. If a RPKM value appeared to be zero no ratio could be calculated and its rate of difference was handled separately. These data were presented as absolute difference and are marked with n.a. in Table 21 and 22.

Out of 2579 genes that are present in strain OG1RF were 1316 genes identified as significantly differentially expressed. The group of highly up-regulated genes that meet all criteria given above consists of 97 genes (see Table 21). The group of genes that were strongly down-regulated consists of 62 genes (see Table 22).

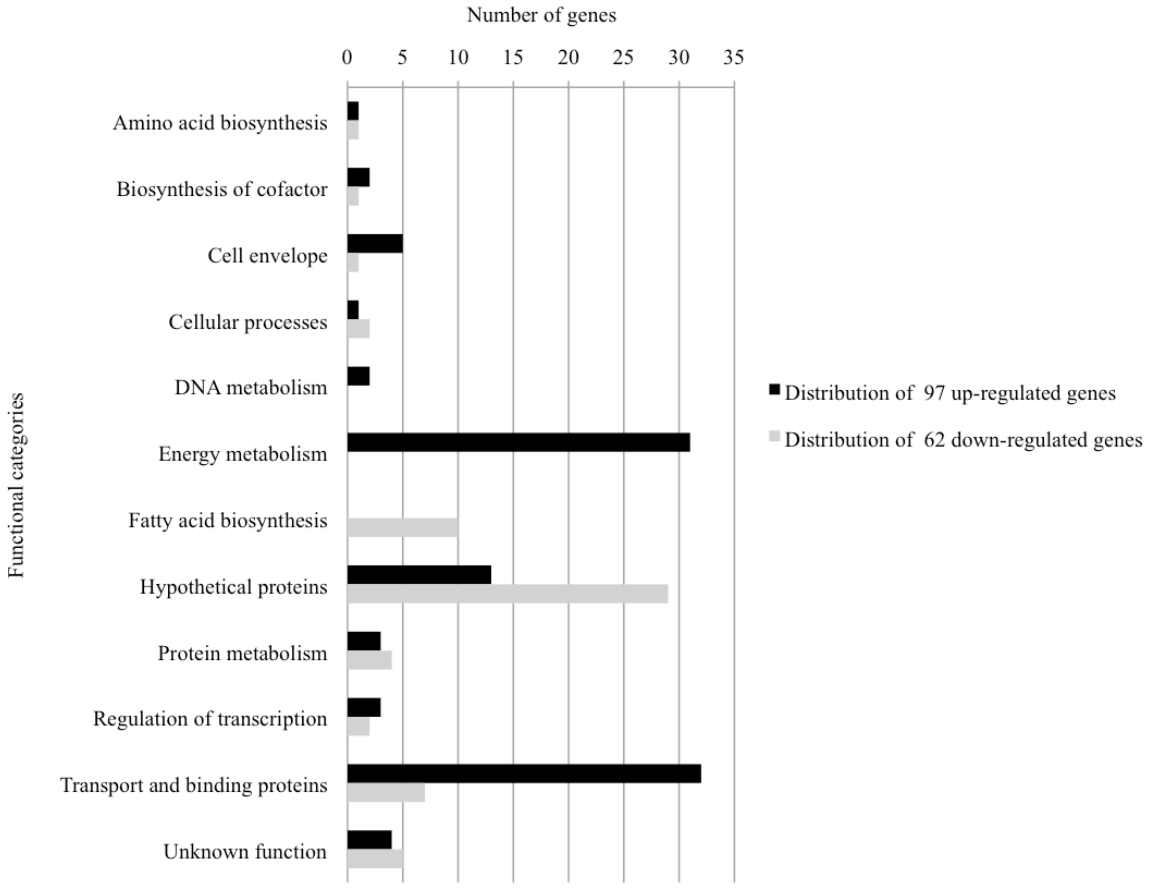


Figure 15: Numbers and functional categories of 159 genes most differentially expressed genes in *E. faecalis* strain OG1RF monoassociated in wild type mice. Genes are grouped according to their functional class. Only $\log_2$ fold-changes of over 6.55 or below -6.95 as well as a p-value ≤ 0.05 were considered as significantly up- or down-regulated and further criteria were achieved. For more information see text.

The group of 97 highly up-regulated genes in *E. faecalis* strain OG1RF, exposed to the intestinal tract of mice, consists of a major proportion of 32 genes that are encoding transporters involved in the uptake of energy substrates as well as their associated signal transduction pathway through corresponding kinases (e.g. PTS family porter component IIA). In detail, 21 genes encode components of different phosphoenolpyruvate-dependent sugar phosphotransferase systems (PTS). Mainly the PTS uptake system specific for mannose, fructose and sorbose were up-regulated (nine genes). Also, PTS family porters that are specific for lactose, cellobiose, mannitol, ascorbate or galatitol were within the 21 genes encoding PTS systems. Besides the significantly transcribed PTS systems, seven genes of ABC transporters are transcribed equivalently. Genes for a chitin-binding protein, a major facilitator family transporter, a molybdopterin-binding domain protein and a cytosine/purine permease are included this group of up-regulated membrane associated

proteins.

Another group within the 97 highly up-regulated genes consists of 31 genes encoding enzymes that are responsible for the energy metabolism (see Figure 15). Out of these three gene clusters of operon type structures could be detected: part of the *iol* operon (*iolE*, OG1RF_11782; *iolG*, OG1RF_11782; *iolG2*, OG1RF_11782), the *glp* operon (*glpF2*,

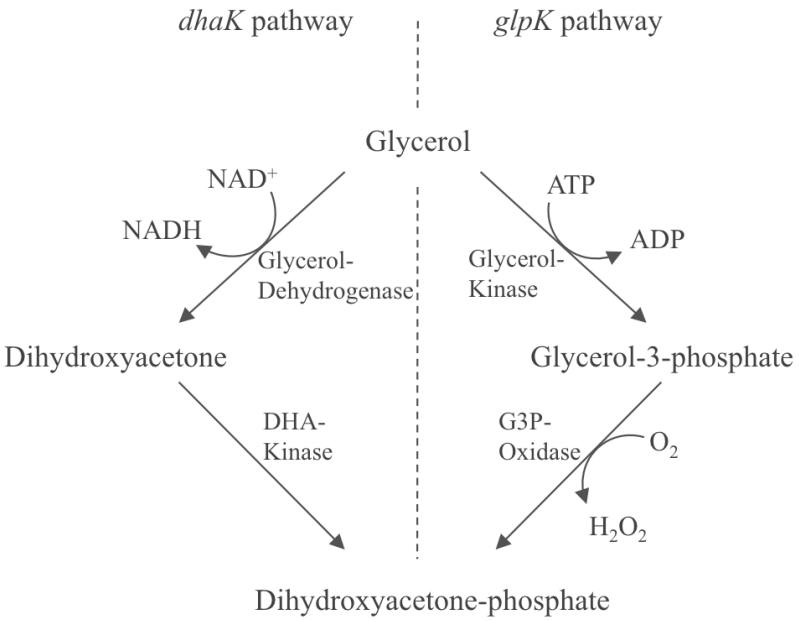


Figure 16: Model for aerobic glycerol catabolism through *glpK* and *dhaK* pathways. In the *glpK* pathway, glycerol is first phosphorylated to glycerol-3-phosphate by glycerol kinase (encoded by *glpK*) and then oxidized to dihydroxyaceton-phosphate by glycerol-3-phosphate-oxidase (encoded by *glpO*). In the *dhaK* pathway, glycerol is first oxidized to dihydroxyacetone by glycerol dehydrogenase (encoded by *gldA*) and then phosphorylated to dihydroxyacetone-phosphate by dihydroxyactone kinase (encoded by *dhaK*). This figure is modified from Bizzini *et al.* (2010).

OG1RF_11590; *glpO*, OG1RF_11591 and *glpK,* OG1RF_11592), and the *bkd* operon (*bkdCBAD*, OG1RF_11370-73; *buk*, OG1RF_11374; *ptb,* OG1RF_11375). In *E. faecalis* OG1RF the *iol* gene cluster consists of eight genes from which three were highly up-regulated. The remaining genes of this operon were also up-regulated, but with logFC between 3.0 and 6.2 not in the group of the highest up-regulated genes. The *iol* operon encodes enzymes necessary for the degradation of myo-inositol into glyceraldehyde-3-phosphate.

The highest up-regulated genes with a logFC over 10, are encoding the *glp* operon that is responsible for the glycerol dissimilation. In *E. faecalis* OG1RF glycerol can be

metabolized in two pathways: *glpK* and *dhaK* pathways, see Figure 16 (Bizzini *et al.*, 2010). Both were up-regulated but only *glpK* was in the group of highest up-regulated genes.

The *bkd* operon encodes enzymes that constitute a catabolic pathway for branched-chain α-keto acids (Ward *et al.*, 1999). Two carbamate kinases (*arcC2*, OG1RF_10274 and *arcC3*, OG1RF_11956), although not adjacently located in the genome of OG1RF, were also significantly expressed. A chitinase (*chiC*, OG1RF_10250) responsible for the degradation or biosynthesis of polysaccharides was transcribed as well as its adjacent and relating chitinase-binding protein (OG1RF_10251). Another up-regulated gene involved in the metabolism of polysaccharides, such as the previously mentioned chitinase, was the 6-phospho-β-glucosidase encoding gene (*selA*, OG1RF_10198). Two genes that are encoding proteins involved in electron transport chain, malate:quinone oxidase and flavodoxin, showed also an up-regulation in OG1RF. Other high up-regulated genes responsible for the organisms' energy requirement were mainly involved in carbohydrate or amino acid degradation, such as 6-phosphogluconate dehydrogenase (*gnd2*, OG1RF_12405) and dihydropyrimidase (*hydA*, OG1RF_11960) respectively.

Genes, which are grouped as part of the cell envelope, are surface or membrane proteins (OG1RF_10450: WxL domain surface protein; OG1RF_11777: putative lipoprotein; OG1RF_12558: cell wall surface anchor family protein) and enzymes that are involved in the biosynthesis or degradation of surface polysaccharides and lipopolysaccharides (OG1RF_10107: family 20 glycosyl hydrolase; *endOF3*, OG1RF_12167: mannosyl-glycoprotein endo-β-N-acetylglucosaminidase).

Other functional categories in which the highly up-regulated genes could be classified are protein metabolism (*selD*, OG1RF_11948 encoding selenide water dikinase; OG1RF_11949 encoding putative cysteine desulfurase; *ygeY*, OG1RF_11958 encoding M20/DapE family protein YgeY), amino acid biosynthesis (*gltA*, OG1RF_11941 encoding glutamate synthase), cellular processes (OG1RF_10325 encoding immunity protein PlnM) and regulation of transcription (OG1RF_11232 encoding DNA-binding/PRD domain protein and OG1RF11309 encoding AraC family transcriptional regulator).

Two of the highly up-regulated genes are involved in DNA metabolism: OG1RF_10314 (*rhiN*) encoding RhiN protein and OG1RF_11306 encoding MuT/NUDIX family protein.

Four genes could not be classified in a category of function due to the fact that their biological role in *E. faecalis* is unclear: OG1RF_11369 encoding a regulatory protein, OG1RF_11950 encoding a YgfJ family molybdenum hydroxylase accessory

protein, OG1RF_11951 selenium-dependent molybdenum hydroxylase 1 and OG1RF_11955 endoribonuclease inhibitor of translation. Two genes classified in the functional category of biosynthesis of cofactors are genes *moaC* (OG1RF_11181) and *moaB* (OG1RF_11184) encoding molybdenum cofactor biosynthesis protein C and B.

The group of high up-regulated genes further includes thirteen genes encdoding hypothetical proteins whose sequence did not match significantly any gene in NCBI database entries after searching with Blast program (htttp://www.ncbi.nih.gov/).

Table 21: Differentially expressed genes ranked by gene identification (ID). All listed genes were significantly up-regulated (p-value ≤ 0.05), the absolute value of the base 2 logarithm indicating the fold change (logFC) in gene expression in *E. faecalis* OG1RF in intestinal tract of wild type mice (*in vivo*). Genes marked with "n.a." (not applicable) no logFC could be calculated because the RPKM value of the reference sample was zero. In this case the absolute expression magnitude with RPKM value > 10 of the gene is given in parentheses.

Gene ID	Gene name	Product	logFC	Functional category
OG1RF_10107	-	family 20 glycosyl hydrolase	6,71	cell envelope
OG1RF_10198	*aldA*	aldehyde dehydrogenase (NAD(+))	6,83	energy metabolism
OG1RF_10234	*celA*	6-phospho-beta-glucosidase	6,85	energy metabolism
OG1RF_10235	*celB*	PTS family lactose-N,N'-diacetylchitobiose-beta-glucoside (lac) porter component IIBC	8,78	transport and binding protein
OG1RF_10250	*chiC*	chitinase C1	n.a. (RPKM absolute 997.58)	energy metabolism
OG1RF_10251	-	chitin-binding protein/carbohydrate-binding protein	n.a. (RPKM absolute 1011.7)	transport and binding protein
OG1RF_10264	*mqo*	malate:quinone oxidoreductase	n.a. (RPKM absolute 211.0)	energy metabolism
OG1RF_10271	-	hypothetical protein	6,64	hypothetical protein
OG1RF_10272	-	hypothetical protein	7,40	hypothetical protein
OG1RF_10273	-	major facilitator family transporter	7,19	transport and binding protein
OG1RF_10274	*arcC2*	carbamate kinase	7,37	energy metabolism
OG1RF_10296	*mtlA2*	PTS family mannitol porter, EIICB component	n.a. (RPKM absolute 155.18)	transport and binding protein
OG1RF_10297	*mltF2*	PTS family fructose/mannitol porter component IIA	n.a. (RPKM absolute 319.60)	transport and binding protein
OG1RF_10298	*mtlD*	mannitol-1-phosphate 5-dehydrogenase	8,51	energy metabolism
OG1RF_10314	*rhiN*	RhiN protein	n.a. (RPKM absolute 15.07)	DNA metabolism
OG1RF_10325	-	immunity protein PlnM	n.a. (RPKM absolute 86.61)	cellular processes
OG1RF_10340	-	PTS family mannose/fructose/sorbose porter component IIC	n.a. (RPKM absolute 16.24)	transport and binding protein
OG1RF_10449	-	hypothetical protein	9,36	hypothetical protein

Gene ID	Gene name	Product	logFC	Functional category
OG1RF_10450	-	WxL domain surface protein	n.a. (RPKM absolute 662.42)	cell envelope
OG1RF_10451	-	hypothetical protein	n.a. (RPKM absolute 366.93)	hypothetical protein
OG1RF_10560	-	PRD domain protein	n.a. (RPKM absolute 56.16)	regulation of transcription
OG1RF_10562	-	hypothetical protein	n.a. (RPKM absolute 40.63)	hypothetical protein
OG1RF_10665	-	ABC superfamily ATP binding cassette transporter, ABC protein	7,25	transport and binding protein
OG1RF_10691	-	hypothetical protein	n.a. (RPKM absolute 18.96)	hypothetical protein
OG1RF_10746	-	PTS family lactose/cellobiose porter component IIC	n.a. (RPKM absolute 96.71)	transport and binding protein
OG1RF_10747	-	hypothetical protein	n.a. (RPKM absolute 109.29)	hypothetical protein
OG1RF_10748	-	hypothetical protein	n.a. (RPKM absolute 108.26)	hypothetical protein
OG1RF_10749	-	hypothetical protein	n.a. (RPKM absolute 123.53)	hypothetical protein
OG1RF_10848	*galM*	galactose mutarotase	n.a. (RPKM absolute 100.00)	energy metabolism
OG1RF_10907	*ulaD*	hexulose-6-phosphate synthase	n.a. (RPKM absolute 33.58)	energy metabolism
OG1RF_10935	*ansB*	N4-(beta-N-acetylglucosaminyl)-L-asparaginase	8,08	energy metabolism
OG1RF_10936	*celA4*	PTS family porter, enzyme I	n.a. (RPKM absolute 118.69)	transport and binding protein
OG1RF_10937	*celB2*	PTS family oligomeric beta-glucoside porter component IIC	8,73	transport and binding protein
OG1RF_10938	-	hypothetical protein	6,91	hypothetical protein
OG1RF_11004	-	ABC superfamily ATP binding cassette transporter, membrane protein	n.a. (RPKM absolute 21.38)	transport and binding protein
OG1RF_11181	*moaC*	molybdenum cofactor biosynthesis protein C	n.a. (RPKM absolute 38.40)	biosynthesis of cofactor
OG1RF_11184	*moaB*	molybdenum cofactor biosynthesis protein B	n.a. (RPKM absolute 60.07)	biosynthesis of cofactor
OG1RF_11185	-	molybdopterin-binding domain protein	n.a. (RPKM absolute 72.38)	transport and binding protein
OG1RF_11186	*modA*	molybdenum ABC superfamily ATP binding cassette transporter, binding protein	6,55	transport and binding protein
OG1RF_11232	-	DNA-binding/PRD domain protein	n.a. (RPKM absolute 10.77)	regulation of transcription
OG1RF_11235	-	PTS family porter component IIA	n.a. (RPKM absolute 16.12)	transport and binding protein
OG1RF_11306	-	MutT/NUDIX family protein	n.a. (RPKM absolute 33.35)	DNA metabolism
OG1RF_11309	-	AraC family transcriptional regulator	n.a. (RPKM absolute 53.14)	regulation of transcription
OG1RF_11369	-	regulatory protein	n.a. (RPKM absolute 212.84)	unknown function
OG1RF_11370	*bkdC*	branched-chain alpha-keto acid	n.a. (RPKM absolute 543.85)	energy metabolism
OG1RF_11371	*bkdB*	3-methyl-2-oxobutanoate dehydrogenase	n.a. (RPKM absolute 391.69)	energy metabolism
OG1RF_11372	*bkdA*	3-methyl-2-oxobutanoate dehydrogenase	n.a. (RPKM absolute 424.38)	energy metabolism

Gene ID	Gene name	Product	logFC	Functional category
OG1RF_11373	*lpd2*	dihydrolipoyl dehydrogenase	9,73	energy metabolism
OG1RF_11374	*buk*	butyrate kinase	n.a. (RPKM absolute 166.76)	energy metabolism
OG1RF_11375	*ptb*	branched-chain phosphotransacylase	n.a. (RPKM absolute 174.71)	energy metabolism
OG1RF_11511	-	PTS family mannose/fructose/sorbose porter component IID	7,51	transport and binding protein
OG1RF_11513	*levE*	PTS family mannose/fructose/sorbose porter, IIB component	6,94	transport and binding protein
OG1RF_11514	*bgaL2*	beta-galactosidase	6,98	energy metabolism
OG1RF_11556	*lacA*	galactose-6-phosphate isomerase LacA subunit	n.a. (RPKM absolute 11.38)	energy metabolism
OG1RF_11557	-	PTS family fructose/mannitol porter component IIA	n.a. (RPKM absolute 27.84)	transport and binding protein
OG1RF_11558	-	PTS system, IIB component	n.a. (RPKM absolute 14.36)	transport and binding protein
OG1RF_11559	-	PTS family galactitol (gat) porter component IIC	6,94	transport and binding protein
OG1RF_11590	*glpF2*	MIP family glycerol uptake facilitator protein GlpF	11,63	energy metabolism
OG1RF_11591	*glpO*	glycerol-3-phosphate oxidase	11,44	energy metabolism
OG1RF_11592	*glpK*	glycerol kinase	10,75	energy metabolism
OG1RF_11611	-	phosphosugar isomerase	7,38	energy metabolism
OG1RF_11612	-	phosphosugar-binding protein	10,16	energy metabolism
OG1RF_11613	-	PTS family mannose/fructose/sorbose porter component IID	n.a. (RPKM absolute 444.41)	transport and binding protein
OG1RF_11614	-	PTS family mannose/fructose/sorbose porter component IIC	n.a. (RPKM absolute 277.49)	transport and binding protein
OG1RF_11615	*ulaB*	PTS family ascorbate porter, IIB component	8,85	transport and binding protein
OG1RF_11616	-	PTS family mannose/fructose/sorbose porter component IIA	n.a. (RPKM absolute 327.56)	transport and binding protein
OG1RF_11761	-	ABC superfamily ATP binding cassette transporter, binding protein	7,88	transport and binding protein
OG1RF_11762	-	carbohydrate ABC superfamily ATP binding cassette transporter, membrane protein	7,27	transport and binding protein
OG1RF_11763	-	ABC superfamily ATP binding cassette transporter, membrane protein	9,25	transport and binding protein
OG1RF_11767	-	ABC superfamily ATP binding cassette transporter, membrane protein	8,26	transport and binding protein
OG1RF_11771	-	brp/Blh family beta-carotene 15,15'-monooxygenase	n.a. (RPKM absolute 12.95)	energy metabolism
OG1RF_11776	-	hypothetical protein	8,33	hypothetical protein
OG1RF_11777	-	putative lipoprotein	n.a. (RPKM absolute 37.48)	cell envelope
OG1RF_11782	*iolE*	myo-inositol catabolism protein IolE	7,56	energy metabolism
OG1RF_11783	*iolG*	inositol 2-dehydrogenase	n.a. (RPKM absolute 257.42)	energy metabolism
OG1RF_11784	*iolG2*	inositol 2-dehydrogenase	n.a. (RPKM absolute 200.76)	energy metabolism
OG1RF_11941	*gltA*	glutamate synthase	7,67	amino acid biosynthesis

Gene ID	Gene name	Product	logFC	Functional category
OG1RF_11943	-	flavodoxin	6,63	energy metabolism
OG1RF_11948	*selD*	selenide, water dikinase	n.a. (RPKM absolute 134.00)	protein metabolism
OG1RF_11949	-	putative cysteine desulfurase	6,95	protein metabolism
OG1RF_11950	*ygfJ*	YgfJ family molybdenum hydroxylase accessory protein	9,24	unknown function
OG1RF_11951	-	selenium-dependent molybdenum hydroxylase 1	7,09	unknown function
OG1RF_11955	-	endoribonuclease inhibitor of translation	8,54	unknown function
OG1RF_11956	*arcC3*	carbamate kinase	8,60	energy metabolism
OG1RF_11958	*ygeY*	M20/DapE family protein YgeY	7,39	protein metabolism
OG1RF_11959	*dpaL*	diaminopropionate ammonia-lyase	6,62	energy metabolism
OG1RF_11960	*hydA*	dihydropyrimidinase	n.a. (RPKM absolute 83.93)	energy metabolism
OG1RF_12166	-	hypothetical protein	n.a. (RPKM absolute 12.46)	hypothetical protein
OG1RF_12167	*endOF3*	Mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase	9,22	cell envelope
OG1RF_12262	-	PTS system fructose IIA component	n.a. (RPKM absolute 10.83)	transport and binding protein
OG1RF_12280	-	cytosine/purine permease	n.a. (RPKM absolute 11.14)	transport and binding protein
OG1RF_12288	-	hypothetical protein	7,85	hypothetical protein
OG1RF_12399	*sorA*	PTS family mannose/fructose/sorbose porter component IIA	6,76	transport and binding protein
OG1RF_12400	*sorB2*	PTS family mannose/fructose/sorbose porter, IIB component	8,65	transport and binding protein
OG1RF_12402	*sorC*	PTS family mannose/fructose/sorbose porter component IIC	7,63	transport and binding protein
OG1RF_12405	*gnd2*	6-phosphogluconate dehydrogenase	6,73	energy metabolism
OG1RF_12558	-	cell wall surface anchor family protein	8,42	cell envelope

The group of 62 significantly down-regulated genes in *E. faecalis* strain OG1RF, exposed to the intestinal tract of mice, contains of 29 genes encoding hypothetical proteins (see Figure 15). Additional sequence analysis of those 29 genes with Blast program on NCBI database did not reveal significantly similar genes (htttp://www.ncbi.nih.gov/).

The second largest group of down-regulated genes includes an operon of ten genes that are responsible for fatty acid biosynthesis (*fab*): OG1RF_12178 to OG1RF_12187 encoding acetyl-CoA carboxylase carboxyl transferase subunit α (*accA*), -β (*accD*), acetyl-CoA carboxylase subunit A (*accC*), hydroxymyristoyl-[acyl-carrier-protein] dehydratase (*fabZ2*), acetyl-CoA carboxylase biotin carboxyl carrier subunit (*accB*), β-ketoacyl-acyl-carrier-protein synthase II (*fabF2*), 3-oxoacyl-[acyl-carrier-protein] reductase (*fabG3*),

malonyl-CoA-[acyl-carrier-protein] transacylase (*fabD*), enoyl-[acyl-carrier-protein] reductase II (*fabK*) and acyl carrier protein (*acpP*). These genes with their function are illustrated in Figure 17.

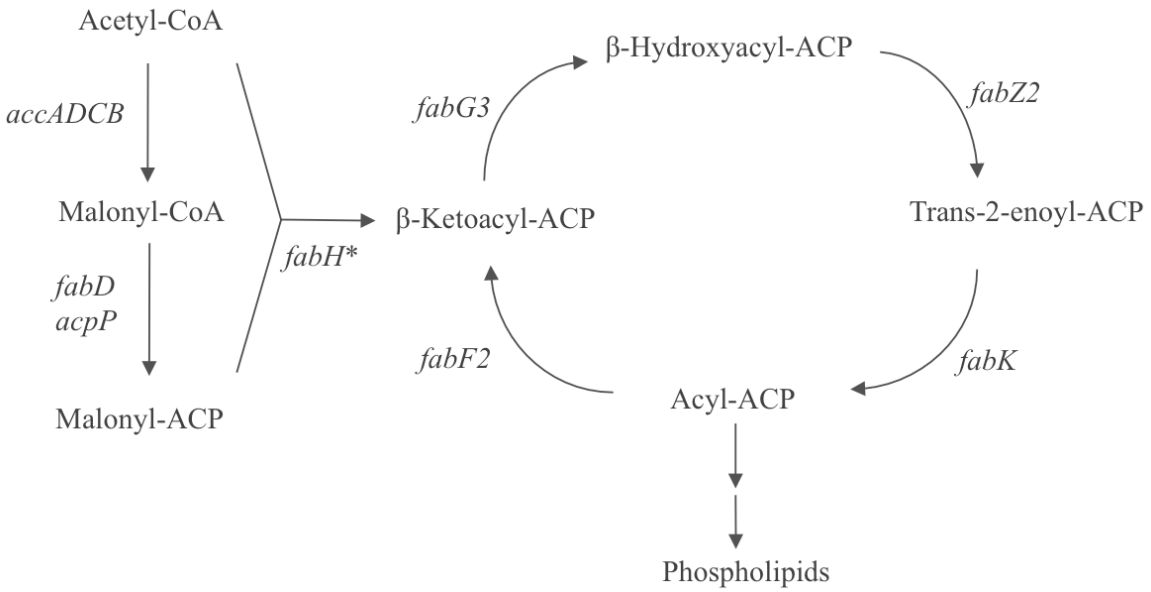


Figure 17: Overview of fatty acid biosynthesis and the detected down-regulated genes in *E. faecalis* OG1RF. Genes encoding enzymes catalyzing the corresponding reaction are presented in italics. Gene marked with * had a $\log_2$ fold-change of -5.33 and is not listed within the lowest down-regulated genes. All other genes showed in this figure had $\log_2$ fold-changes below -6.95. CoA means Coenzyme A and ACP means Acyl carrier protein. This figure is modified from Heath and Rock (2004).

Seven genes encoding transport and binding proteins were also prominently down-regulated: two ABC superfamily transporters (OG1RF_11541 and OG1RF_12268), a quaternary ammonium compound-resistance protein SugE (*sugE1*, OG1RF_10248), a NRAMP family Mn^{2+}/Fe^{2+} transporter (OG1RF_10838), an amino acid permease (OG1RF_11654), a ThiW protein that is a predicted thiazole transporter (*thiW*, OG1RF_12138) and a sulfate anion transporter, that has a carboxy-terminal cytoplasmic segment organized around a sulfate transporter and anti-sigma factor antagonist (STAS) domain (OG1RF_12284).

Within the group of yet unknown function consisting of five significantly down-regulated genes encoding proteins of two GNAT family acetyltransferases (OG1RF_11307 and 12348). These *Gcn5*-related N-acetyltransferases (GNAT) catalyze the transfer of an acetyl group originated from acetyl coenzyme A (CoA) to an acceptor of a primary amine. This reaction is implicated in various functions and could not be specifically classified. A putative secreted protein (OG1RF_10696), a competence protein (OG1RF_11881), and

an extracellular protein (OG1RF_12253) were also classified in the group of genes encoding proteins with unknown function.

Two genes, OG1RF_11687 (*rpmB*) and OG1RF_11687 (*rpsN2*), encoding ribosomal proteins and thus they are responsible for protein biosynthesis. The gelatinase and serine protease encoded by *gelE* (OG1RF_11526) and *sprE* (OG1RF_11525) are within the group of down-regulated genes belonging to the organisms' protein metabolism. In general, these enzymes catalyze the degradation of proteins, but they have been demonstrated to be virulence factors in *E. faecalis*.

Two genes involved in the regulation of transcription encode a DNA-binding protein (*hupB*, OG1RF_10081) and an arsenical resistance operon repressor (*arsR*, OG1RF_12237). The down-regulation of this arsenical resistance operon repressor in OG1RF in mice indicates an activated arsenic resistance.

A gene (*metC*, OG1RF_10233) responsible for the biosynthesis of amino acid, the cystathionine γ-synthase, is down-regulated. The generation or hydrolysis of cofactor thiamine is down-regulated (*tenA*, OG1RF_12127) as well as a gene encoding a putative lipoprotein that is possibly involved in the cell envelope (OG1RF_10989).

Table 22: Differentially expressed genes ranked by gene identification (ID). All listed genes were significantly down-regulated (p-value ≤ 0.05), the absolute value of the base 2 logarithm indicating the fold change (logFC) in gene expression in *E. faecalis* OG1RF in intestinal tract of wild type mice (*in vivo*). For genes marked with "n.a." (not applicable) no logFC could be calculated because RPKM value of the experiment sample was zero. In this case the absolute expression magnitude with RPKM value > 10 of the gene in reference sample is given in parentheses.

Gene ID	Gene name	Product	logFC	Functional category
OG1RF_10080	-	hypothetical protein	n.a. (RPKM absolute 279.70)	hypothetical protein
OG1RF_10081	*hupB*	DNA-binding protein	n.a. (RPKM absolute 12.81)	regulation of transcription
OG1RF_10231	-	hypothetical protein	-6,08	hypothetical protein
OG1RF_10233	*metC*	cystathionine gamma-synthase	-6,42	amino acid biosynthesis
OG1RF_10248	*sugE1*	quaternary ammonium compound-resistance protein SugE	n.a. (RPKM absolute 35.53)	transport and binding
OG1RF_10285	-	hypothetical protein	-6,12	hypothetical protein
OG1RF_10403	-	hypothetical protein	n.a. (RPKM absolute 54.20)	hypothetical protein
OG1RF_10441	-	hypothetical protein	n.a. (RPKM absolute 125.14)	hypothetical protein
OG1RF_10535	-	hypothetical protein	n.a. (RPKM absolute 17.08)	hypothetical protein
OG1RF_10536	-	hypothetical protein	n.a. (RPKM absolute 12.98)	hypothetical protein

Gene ID	Gene name	Product	logFC	Functional category
OG1RF_10596	-	hypothetical protein	n.a. (RPKM absolute 244.02)	hypothetical protein
OG1RF_10658	-	hypothetical protein	n.a. (RPKM absolute 25.62)	hypothetical protein
OG1RF_10664	-	hypothetical protein	n.a. (RPKM absolute 15.55)	hypothetical protein
OG1RF_10693	-	hypothetical protein	n.a. (RPKM absolute 11.65)	hypothetical protein
OG1RF_10696	-	putative secreted protein	n.a. (RPKM absolute 16.83)	unknown function
OG1RF_10697	-	hypothetical protein	n.a. (RPKM absolute 18.30)	hypothetical protein
OG1RF_10773	-	hypothetical protein	n.a. (RPKM absolute 18.68)	hypothetical protein
OG1RF_10838	-	NRAMP family Mn2+/Fe2+ transporter	-8,27	transport and binding
OG1RF_10839	-	universal stress protein	-7,40	cellular processes
OG1RF_10875	-	hypothetical protein	-10,47	hypothetical protein
OG1RF_10876	-	hypothetical protein	n.a. (RPKM absolute 1847.24)	hypothetical protein
OG1RF_10883	-	hypothetical protein	n.a. (RPKM absolute 57.86)	hypothetical protein
OG1RF_10989	-	putative lipoprotein	n.a. (RPKM absolute 20.88)	cell envelope
OG1RF_11048	-	hypothetical protein	n.a. (RPKM absolute 31.32)	hypothetical protein
OG1RF_11070	-	FtsW/RodA/SpovE family cell division protein	n.a. (RPKM absolute 25.82)	cellular processes
OG1RF_11086	-	hypothetical protein	n.a. (RPKM absolute 128.11)	hypothetical protein
OG1RF_11307	-	GNAT family acetyltransferase	n.a. (RPKM absolute 64.46)	unknown function
OG1RF_11525	*sprE*	SprE protein	-7,19	protein metabolism
OG1RF_11526	*gelE*	gelatinase	-7,15	protein metabolism
OG1RF_11541	*cbiO*	ABC superfamily ATP binding cassette transporter, ABC protein	n.a. (RPKM absolute 56.52)	transport and binding
OG1RF_11654	-	amino acid permease	n.a. (RPKM absolute 125.60)	transport and binding
OG1RF_11685	-	hypothetical protein	n.a. (RPKM absolute 24.13)	hypothetical protein
OG1RF_11687	*rpmB*	50S ribosomal protein L28	n.a. (RPKM absolute 14.90)	protein biosynthesis
OG1RF_11708	-	hypothetical protein	n.a. (RPKM absolute 372.12)	hypothetical protein
OG1RF_11728	-	hypothetical protein	-6,27	hypothetical protein
OG1RF_11756	-	hypothetical protein	n.a. (RPKM absolute 13.81)	hypothetical protein
OG1RF_11817	-	hypothetical protein	n.a. (RPKM absolute 11.45)	hypothetical protein
OG1RF_11881	-	competence protein	n.a. (RPKM absolute 16.65)	unknown function
OG1RF_11897	-	hypothetical protein	n.a. (RPKM absolute 42.70)	hypothetical protein
OG1RF_11978	-	hypothetical protein	n.a. (RPKM absolute 68.32)	hypothetical protein
OG1RF_12127	*tenA*	thiaminase	n.a. (RPKM absolute 27.24)	biosynthesis of cofactor

Gene ID	Gene name	Product	logFC	Functional category
OG1RF_12138	*thiW*	ThiW protein	n.a. (RPKM absolute 40.53)	transport and binding
OG1RF_12178	*accA*	acetyl-coA carboxylase carboxyl transferase subunit alpha	-6,95	fatty acid metabolism
OG1RF_12179	*accD*	acetyl-coA carboxylase carboxyl transferase subunit beta	-7,61	fatty acid metabolism
OG1RF_12180	*accC*	acetyl-CoA carboxylase subunit A	-8,34	fatty acid metabolism
OG1RF_12181	*fabZ2*	(3R)-hydroxymyristoyl-[acyl-carrier-protein] dehydratase	-7,01	fatty acid metabolism
OG1RF_12182	*accB*	acetyl-CoA carboxylase biotin carboxyl carrier subunit	-7,47	fatty acid metabolism
OG1RF_12183	*fabF2*	beta-ketoacyl-acyl-carrier-protein synthase II	-6,10	fatty acid metabolism
OG1RF_12184	*fabG3*	3-oxoacyl-[acyl-carrier-protein] reductase	-7,78	fatty acid metabolism
OG1RF_12185	*fabD*	malonyl-CoA-[acyl-carrier-protein] transacylase	-6,33	fatty acid metabolism
OG1RF_12186	*fabK*	enoyl-(acyl-carrier-protein) reductase II	-7,77	fatty acid metabolism
OG1RF_12187	*acpP*	acyl carrier protein	-6,63	fatty acid metabolism
OG1RF_12219	-	hypothetical protein	n.a. (RPKM absolute 117.43)	hypothetical protein
OG1RF_12237	*arsR*	arsenical resistance operon repressor	n.a. (RPKM absolute 35.53)	regulation of transcription
OG1RF_12253	-	extracellular protein	n.a. (RPKM absolute 12.81)	unknown function
OG1RF_12268	-	ABC superfamily ATP binding cassette transporter, ABC protein	n.a. (RPKM absolute 21.07)	transport and binding
OG1RF_12284	-	sulfate transporter/STAS domain protein	-6,73	transport and binding
OG1RF_12348	-	GNAT family acetyltransferase	-6,13	unknown function
OG1RF_12412	-	hypothetical protein	n.a. (RPKM absolute 54.90)	hypothetical protein
OG1RF_12430	-	hypothetical protein	n.a. (RPKM absolute 33.55)	hypothetical protein
OG1RF_12468	*rpsN2*	30S ribosomal protein S14	n.a. (RPKM absolute 12.81)	protein metabolism
OG1RF_12501	-	hypothetical protein	n.a. (RPKM absolute 74.73)	hypothetical protein

4 Discussion

During this work the distribution of known virulence factors was determined in different *E. faecalis* strains. The absence of any correlation of their distribution to the origin of isolation of the strains suggests that all strains are intrinsically intestinal strains, regardless of their specific source of isolation in which they may be considered as "contaminants". Interestingly, a negative correlation was found of the number of virulence traits with the presence of CRISPR elements/*cas* genes, which indicates that such traits may be more easily acquired in strains with less or no such systems. This also underlines that the currently known virulence factors cannot explain the switch from a commensal to a pathogen, whenever strains with a high number of such factors and concomitant lack of CRISPR elements/*cas* may have a higher probability for that.

In the proteomic *in vitro* study and namely in the transcriptomic *in vivo* study in monoassociated mice novel fitness factors of *E. faecalis* OG1RF describing the adaptation ability to the host as a first step in a switch from a commensal to a pathogen could be identified. These relate to clear changes in the expression of genes involved in the cellular surface and its biosynthesis such as glucose-1-phosphate-thymidylyltransferase (RmlA), phosphoenolpyruvate-dependent sugar phosphotransferase systems (PTS), penicillin-binding protein (PBP4) as well as genes encoding various catabolic pathways. The bacterium also undergoes vast adaptation with respect to its metabolism when it comes to the change of the environment, such as aerobic/anaerobic growth conditions, the contact with mouse colonic epithelial cells *in vitro* or conditions in the intestinal tract of mice.

4.1 Distributions of virulence genes and CRISPR-*cas* genes

In this work, *E. faecalis* and *E. faecium* strains of diverse origin were typed by the distribution of virulence factors and presence of CRISPR elements/*cas* genes in search of a relation of origin and virulence association of strains. The tabulation of the 62 *Enterococcus* isolates showed a multiple number of detected virulence genes, mostly within *E. faecalis* strains, whereas the *E. faecium* isolates were devoid of these virulence genes except for *esp* and *efaAfm*. This finding corroborates the results of Eaton and Gasson (2001) and Coque *et al.* (2002) in which *esp* appears to be more frequent in *E. faecium* than in *E. faecalis*. Generally, studies have identified numerous virulence factors such as gelatinase, collagen-binding protein, enterococcal endocarditis antigen A or cytolysin, although there is no clear association of a single gene or origin of isolation with

pathogenicity (Eaton and Gasson, 2001; Hew *et al.*, 2007; Martin *et al.*, 2005). The incidence of virulence traits is not only limited to isolates from clinical origin, but also a common trait in *Enterococcus* commensal and food isolates. This indefinite occurrence of virulence traits within different strains indicates that all isolates are from intestinal origin. In particular, their persistent nature enables *Enterococcus* its wide spread in diverse environments. We suggest that any *E. faecalis* isolate should just be considered as intrinsically "fecal", irrespective of the specific source of isolation for a single strain as this appears to be rather irrelevant in judging its virulence.

The CRISPR system is a diverse defense mechanism towards invading nucleic acids. CRISPR motives are typically located near CRISPR-associated (*cas*) genes (Koonin and Makarova, 2009; Sorek *et al.*, 2008). No *cas* genes could be detected in the tested *E. faecium* isolates. *E. faecium* is generally referred to as the less virulent species compared to *E. faecalis*, and all strains of this species typically contain much less of the established *Enterococcus* virulence factors. Preferably, this should be attributed to a different lifestyle of this species or other mechanisms limiting virulence factor presence or acquisition may be involved.

Recently, a significant correlation of the absence of CRISPR-*cas* loci and the presence of antibiotic-resistance genes was reported for *E. faecalis* (Palmer and Gilmore, 2010). We could not find such a general inverse correlation between *cas* and genes encoding virulence traits, which is probably attributed to the fact that there is no selective force for the virulence traits as it can be expected from the application of antibiotics towards selection of antibiotic resistance. However, we found that the distribution of *cas* genes correlates inversely with the presence of the cytolysin operon, *cob* and *as*. The cytolysin operon, *cob* and *esp* are reported to reside on the same pathogenicity island, which was found in large, pheromone-responsive plasmids or in the chromosome (Shankar *et al.*, 2001; Shankar *et al.*, 2004). However, we could not find a respective correlation for *esp*. The *cob* and *as* genes promote pheromone production and aggregation promotion, respectively, and thus enhance the probability of genetic exchange. This negative correlation shows that counteraction of a CRISPR-*cas* defense system versus enhanced genetic flexibility in the presence of *cob* and *as* is minimized in these strains, and suggests that CRISPR-*cas* has a role in acquisition prevention of (parts of) the respective pathogenicity island. The absence of virulence factors and also CRISPR-*cas* probably results from the fact that some strains did not at all have the opportunity to pick up these virulence factors from others. While the molecular background is yet to be confirmed by demonstration of active

CRISPR-*cas* systems, this view is further supported by the finding that *cob* and *as* negative strains generally have less virulence traits.

However, the sole possession of virluence factors does not explain the different behavior of *E. faecalis* strains and therefore we focused phenotypical analysis as well as on transcription and translation responses (see following sections).

4.2 Phenotypic screening of gelatinase and cytolysin activity

The most investigated and clearly proven virulence traits (not only in enterococci) are gelatinase and cytolysin (also referred to as hemolysin), which were phenotypically tested on corresponding agar plates in this work. The main part of positive tested phenotypes is comprised from clinical isolates, which is of course an outcome of the focus on isolates from clinical environment in this study but is equivalent to other studies (Mannu *et al.*, 2003; Semedo *et al.*, 2003). Interestingly, no β-hemolysis caused by cytolysin could be detected on Columbia blood agar plates under aerobic conditions, whereas under anaerobic conditions eight strains did express this phenotype. However, the use of horse blood revealed remarkably more cytolysin positive strains than the use of Columbia blood agar plates containing sheep blood. The β-hemolytic activity on horse blood agar plates was even more intense by the application of anaerobic incubation conditions. These results are in agreement with the data of Semedo *et al.* (2003), which verified a different sensitivity of erythrocytes among different species. A similar sensitivity of horse and human erythrocytes against cytolysin produced by enterococci has been determined (Semendo *et al.*, 2003).

The incidence of this virulence trait could only be detected in *E. faecalis* (eighteen strains), which conforms to other studies that showed a higher incidence of cytolysin in this *Entercoccus* species (Coque et al, 1995; Franz *et al.*, 2001). Despite the fact that 17 out of the 18 positive strains were from clinical origin, a correlation could not be found in the clustering of RAPD or MALDI-TOF-MS.

The prevalence of gelatinase activity among all isolates was assessed, and 22 strains possessed this phenotype. Out of the six isolates originating from food three showed a positive gelatinase phenotype, which was unexpected because of a low number of food-associated strains in comparison to the number of the tested isolates from clinical origin. Mostly, gelatinase-positive food strains were originated from cheese, and possibly this is due to the protein-rich environment to utilize cheese protein as a source of amino acids (Franz *et al.*, 2001).

4.3 Comparison of clustering approaches of *Enterococcus* isolates by MALDI-TOF-MS analysis and RAPD-PCR analysis

The application of MALDI-TOF-MS analysis was used to identify bacteria on genus, species (Pribil *et al.*, 2005) and even strain level (Pignogne *et al.*, 2006). The identification and characterization of *Enterococcus* isolates in accordance with their origin using the MALDI-TOF-MS has been subject of recent studies (Giebel *et al.*, 2008; Geijersstam *et al.*, 2007). In contrast to those studies our clustering of the isolates according to their MALDI-TOF-MS profiles did not differentiate according to their origin of isolation. Actually, based on our findings, we would not necessarily expect such a correlation, as the source of isolation may not always be decisive for the traits a strain exhibits. MALDI-TOF-MS profiles and RAPD fingerprints enabled distinction at species level, accounting for the validity of the method, and RAPD discriminated even close to strain level. The occurrence of virulence genes or the *cas* genes did not show a distinct distribution, neither within the groups of MALDI-TOF-MS clustering, nor within the groups of RAPD-PCR clustering. While distribution of strains with various origins was random in MALDI-TOF-MS profiles, two RAPD clusters nearly exclusively contained strains of clinical origin. This may indicate some role of a diseased host in selecting for specific types or indeed some genotypes causing clinical infections with an increased probability, while the biodiversity of *E. faecalis* is broader, also containing commensal strains. In this context it is interesting to note that these isolates clustered together in RAPD analysis were isolated from patients suffering from different diseases (e.g. renal insufficiency, newborns with infection, patients with pneumonia, endocarditis, bacteremia, sepsis, and patients with different kinds of cancer).

4.4 Proteome analysis of *E. faecalis* grown under aerobic and anaerobic conditions

The comparative proteome analysis of strain OG1RF under aerobic and anaerobic growth conditions delivered insights into the strain's metabolic response.

Strain OG1RF growing under aerobic conditions highly expressed two representatives of the protein biosynthesis: asparaginyl-tRNA ligase (AsnS) and subunit alpha of phenylalanyl-tRNA synthetase (PheS). Despite the fact that these two enzymes belong to two different classes of synthetases, both enzymes are relevant in the translation process catalyzing the specific bond of tRNAs with their corresponding amino acids pointing to an enhanced protein biosynthesis of OG1RF in mid exponential growth phase under aerobic conditions. Both amino acids involved in this enzymatic process, apsaragine and

phenylalanine, belong to non-essential amino acids in OG1RF (Murray *et al.*, 1993).

One further protein that was identified to be expressed aerobically is associated with the enterococcal cell membrane: penicillin-binding protein 4 (PBP4). The PBP4 is responsible for the resistance of *E. faecalis* to β-lactam antibiotics (Duez *et al.*, 2001). In general PBP proteins are involved in the synthesis of peptidoglycans and are essential for cell wall structure with its function of selective permeability and protection.

The induced SufC protein is part of the enterococcal SufCDSUB complex that is involved in the biosynthesis of iron-sulfur clusters. In general, iron-sulfur clusters are responsible for many biological processes, e.g. electron transfer, redox and nonredox catalysis and are important prosthetic groups in all organisms. The Suf system is one of three described machineries of iron sulfur biogenesis, whereas in species of the phylum *Firmicutes* it appears to be exclusive. SufC has an ATPase activity that supports iron acquisition, cluster formation and delivery of the cofactor to target proteins (Riboldi *et al.*, 2009). In other species the Suf system has been linked with cellular stress conditions, such as the presence of reactive oxygen species or iron starvation (Nachin *et al.*, 2003). As *E. faecalis* is generally adapted to anaerobic energy gain and growth, the exposure to oxygen exerts oxidative stress, because *E. faecalis* is only capable of aerobic respiration when heme is availabel for incorporation into proteins involved in electron transport (Bryan-Jones and Whittenbury, 1969). Since heme is absent in BHI medium, *E. faecalis* truly dealed with oxidative stress in our experiment, even because the heme-dependent catalase is inactive for detoxification.

Another identified protein expressed under aerobic growth conditions was the stress response regulator Gls24 protein. This protein has been identified in many studies determining the capability of resistance in *E. faecalis* due to stress response to various challenges such as glucose starvation, bile salt, pH 3.7, pH 11.9, 62 °C or H_2O_2 (Giard *et al.*, 2000; Hartke et al., 1998). Studies have shown that Gls24 even plays an important role in the virulence response in animal infection models (Teng *et al.*, 2005; Nannini *et al.*, 2005). The mechanism underlying the function of Gls24 has not yet been identified. However, its appearance under aerobic growth conditions seems to be an additional indicator of a stressed microorganism caused by oxygen as well as reactive oxygen species. Interestingly, in the *in vivo* experiment, in which *E. faecalis* was monoassociated in wild type mice, the gene encoding the stress response regulator Gls24 was significantly up-regulated (with a logFC of 5.1). Whether this up-regulation is caused by oxygen is unclear

because all genes of the other identified proteins - induced through aerobic growth conditions - are not significantly regulated (PBP4 and SufC) or even down-regulated (AsnS and Phes). In contrast, all genes encoding proteins induced during anaerobic growth conditions (*arcB*, *arcC*, *sdaA*) were significantly up-regulated in OG1RF exposed to the intestinal tract of mice. Summing up, we suggest that the identified proteins induced under aerobic growth indicate a stress condition to *E. faecalis* OG1RF and they have to be seen in the context of an adaption process to the exposure of oxygen.

In comparison to aerobically induced proteins a total of three proteins was identified and differentially expressed in OG1RF under anaerobic conditions. Two of these proteins are part of the arginine catabolism, also called arginine deiminase (ADI) pathway (see Figure 18). After L-arginine is deiminated to L-citrulline the ornithine carbamoyltransferase (ArcB) and carbamate kinase (ArcC) are responsible for its further degradation to carbamate, which finally dissociates to carbon dioxide and ammonia (NH_3). This pathway of anaerobic arginine degradation leads to the assembly of energy in the form of ATP. The ADI pathway was originally demonstrated and characterized in the species *E. faecalis.* Despite the fact that the presence of arginine is strongly inducing the ADI pathway, in addition the availability of oxygen, glucose and fumarate act as a significant repressor on this pathway (Jones and Lipmann, 1960). Due to the absence of oxygen our results are in concordance with these studies and support the hypothesis that a change in energy expenditure causes a decrease of the ATP pool during exponential growth phase and leads to an induction of the ADI pathway activity (Forrest, 1965; Jones and Lipmann, 1960). L-Arginine may represent an additional energy source for growth and it offers protection against acid stress (Marquis *et al*., 1987).

The third protein that was found to be induced anaerobically in strain OG1RF was the alpha subunit of L-serine dehydratase (SdaA). The enzyme SdaA plays a role in the energy metabolism through the direct degradation of L-serine to pyruvate and ammonium (NH_4^+). Although, only the up-regulation of the α-subunit could be detected the induction or presence of the residual parts of SdaA are strongly assumed. The generated pyruvate is a key intersection in the network of metabolic pathways. This fact supports the other anaerobically induced proteins we identified and shows the metabolic adaptability of *E. faecalis*. As mentioned above, all genes encoding proteins induced during anaerobic growth conditions (*arcB*, *arcC*, *sdaA*) were identified in transcriptome analysis as significantly up-regulated in OG1RF when exposed to the intestinal tract of mice.

Although the logFC between 2.5 and 3 is not in the highly up-regulated group of genes, it supports the assumption of anaerobic conditions within the intestinal tract.

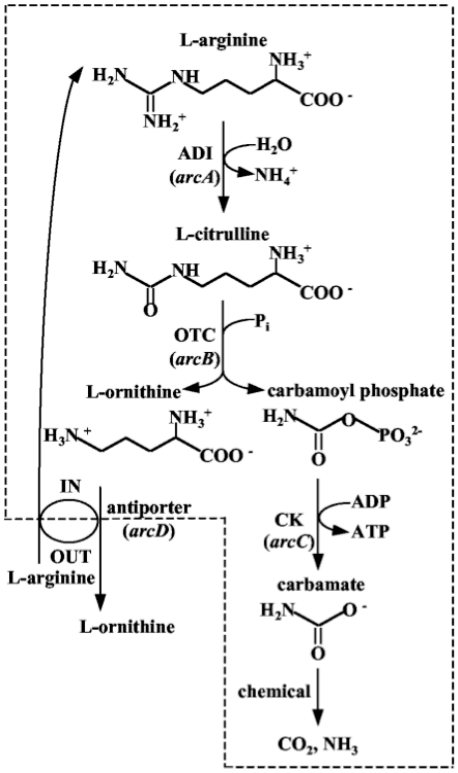


Figure 18: Arginine catabolism in *E. faecalis*. The relevant genes are presented in italics. The cell boundary is marked by broken line. "IN" and "OUT" denotes extracellular and intracellular. Enzymes and their encoding genes of arginine deiminase pathway: arginine deiminase ADI (*arcA*), ornithine carbamoyltransferase OTC (*arcB*) and carbamate kinase CK (*arcC*). Arginine and ornithine antiporter (*arcD*). Figure from Barcelona-Andrés *et al.*, 2002.

4.5 Proteome analysis of *E. faecalis* in contact with mouse colonic epithelial cells *in vitro*

In this experiment we identified four proteins in *E. faecalis* OG1RF whose expression was induced in response to mouse colonic epithelial cells *in vitro*. These proteins were from different functional categories of *E. faecalis*' metabolism. In this experiment *E. faecalis* OG1RF expressed asparagine-tRNA ligase (AsnS), which is responsible for the attachment of L-asparagine to its compatible cognate tRNA indicating an activity of protein biosynthesis in general. Interstingly, this protein is also induced in OG1RF under aerobic growth conditions in BHI medium. However, compared to the *in vivo* experiment of mice monoassociated with OG1RF, a significantly down-regulation was observed in transcriptome analysis.

Another induced protein in *E. faecalis*, when exposed to mouse epithelial cells *in vitro*, was the glucose-1-phosphate-thymidylyltransferase (RmlA). This enzyme is involved in the biosynthesis of enterococcal surface polysaccharides and lipopolysaccharides. The gene encoding this specific transferase (other names were *orfde6*, *epaE* or *EF2194*) is part of the enterococcal polysaccharide antigen (*epa*) gene cluster, which - besides affecting the cell shape - is also involved in adherence and invasion of host tissues (Xu *et al.*, 1998; Hulse *et al.*, 1993). Studies have shown that disruption of the gene encoding glucose-1-phosphate-thymidylyltransferase in *E. faecalis* resulted in a reduced biofilm formation and translocation through eukaryotic cells *in vitro* (Xu *et al.*, 2000; Zeng *et al.*, 2004). This identification clearly shows a response of *E. faecalis* to colonic epithelial cells. Unfortunately, no significant regulation of the encoding gene could be determind in the *in vivo* experiment.

The third induced protein identified in *E. faecalis* OG1RF exposed to mouse epithelial cells was a DNA-binding response regulator of the OmpR family that is part of a two-component system and shows close similarities to response regulators VicR and YycF of other major human pathogens, such as the Gram-positive *S. pneumoniae*, *Staph. aureus* and *B. subtilis*. Therefore we searched *in silico* for a previously published consensus recognition sequence of the YycF regulator by Dubrac and Msadek (2003) in OG1RF. Five genes could be identified as putative regulated genes in which only three possess a known function: putative lipoprotein (OG1RF_10090), thioesterase (OG1RF_10218) and β-subunit of a DNA-directed RNA-polymerase (OG1RF_12493). Unlikely to VicR or YycF in *S. pneumoniae* and *B. subtilis*, respectively, no clearly proven virulence gene seems to be regulated by the identified DNA-binding response regulator in *E. faecalis*. The YycF regulator of *Staph. aureus* binds specifically to genes involved in virulence and cell wall metabolism (Dubrak and Msadek, 2003). However, a differential expression of this DNA-binding response regulator in *E. faecalis* could be observed under stress response to bovine bile (Bøhle *et al.*, 2010). Despite the lack of detailed information about the regulated genes of the DNA-binding response regulator, a shift in its expression indicates a change in gene expression and hence a response to the mouse epithelial cells that could possibly lead to yet uncharacterized virulence traits. A slightly up-regulation (logFC of 1.37) of the gene encoding the DNA-binding response regulator could be detected in OG1RF in mouse intestinal tract. Unfortunately, none of the predicted regulated genes of this regulator identified through *in silico* search (putatuve lipoprotein, thioesterase, hypothetical proteins and β-subunit of a DNA-directed RNA- polymerase) was significantly different in

OG1RF in mice. Anyhow, there must be some regulated genes, which are not identified yet.

The fourth protein identified in OG1RF that was induced by the exposition to mouse epithelial cells was α-acetolactate decarboxylase (BudA). This enzyme plays a key role in the regulation of the α-acetolactate pool in the process of pyruvate catabolism and the biosynthesis of branched-chain amino acids (Curic *et al.*, 1999). The α-acetolactate decarboxylase is regulated due to allosteric activation leucin or valine biosynthesis by activating BudA towards acetoin when branched-chain amino acids are present at a high concentration (Monnet *et al.*, 2003). The activation of BudA is also influenced by a high pyruvate level because of its very low affinity to pyruvate. Also, it is only active when the intracellular pyruvate concentration is high (Dolin and Gunsalus, 1951; Snoep *et al.*, 1992). Through the catalyzed reaction the bacterial cell tries to avoid lactate production and produces neutral 2,3-butanediol instead (see Figure 19).

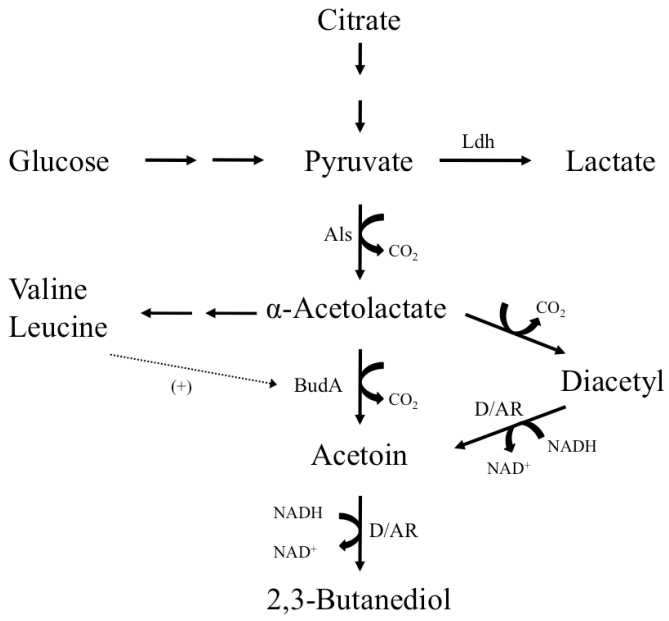


Figure 19: Pathway of diacetyl formation in *E. faecalis*. Ldh, lactacte dehydrogenase, Als, α-acetolactate synthase; BudA, α-acetolactate decarboxylase; D/AR, acetoin and diacetyl reductase. Dotted arrow (+) indicates allosteric acivation. This Figure is modified from Curic *et al.* (1999).

The various functions of these induced proteins in enterococcal metabolism indicate a response to mouse epithelial cells on different levels. Even when only four proteins could be identified in this experiment a definite response of *E. faecalis* strain OG1RF could be demonstrated, which clearly relates to its adaptive response to the presence of the intestinal cells and environment.

4.6 Transcriptome analysis of *E. faecalis* in the intestinal tract of monoassociated mice *in vivo*

In this *in vivo* experiment wild type mice were monoassociated for the duration of one week with *E. faecalis* strain OG1RF to analyze the behavior of *E. faecalis* under conditions within the mice intestinal tract. We used transcriptome data generated from next generation sequencing technology to identify differentially expressed genes in the whole genome to enable a quantitative analysis of gene expression. The genome of strain OG1RF is 2.7 Mb in size and encodes 2,579 genes (Bourgogne *et al.*, 2008). In our transcriptome analysis we identified 1,316 genes in *E. faecalis* OG1RF whose levels are significantly (p-value ≤ 0.05) up- or down-regulated in response to the intestinal tract of living mice. Under these conditions over 50 % of genes in *E. faecalis* OG1RF are differentially expressed. We focused on genes, whose expression levels belong to the highest up-regulated and lowest down-regulated genes. In total, 159 genes belong to these peaks of transcriptional changes.

The major part of highly up-regulated genes consists of transport and binding proteins as well as genes encoding for energy metabolism. Mainly phosphoenolpyruvate (PEP) carbohydrate phosphotransferase systems (PTS) were up-regulated that catalyze the binding, transport and phosphorylation of numerous energy sources.

Studies have shown that *E. faecalis* possesses the ability to adhere to different types of eukaryotic cells and to develop a biofilm. Hufnagel *et al.* (2004) demonstrated in *E. faecalis* a putative sugar-binding transcriptional regulator (referred to as *bop*) as a novel gene operon involved in biofilm formation and showed a significant correlation to prolonged bacteremia in mice. Vebø *et al.*, (2010) also demonstrated an up-regulation of this operon in OG1RF grown in human urine as well as the up-regulation of a cognate maltose PTS system indicating its involvement at biofilm development. However, in our experiment none of these genes were in the group of highly up-regulated genes although the major part of up-regulated genes in OG1RF constists of different PTS. A direct relation to PTS and biofilm formation has been shown in many other Gram-positive bacteria on a regulatory basis (Sutrina *et al.*, 2007). Therefore, besides a highly efficient catalytic function in sugar transport and phosporylation by PTS systems, regulatory functions seem to be an important trait (Deutscher *et al.*, 2006). This regulatory function is linked to the occurrence of carbon catabolite repression (CCR), which explains the hierarchic utilization of carbon sources in bacteria (Contesse *et al.*, 1969). Therefore, as long as preferred sugars are available, transporters and enzymes necessary for metabolizing less favored sugars are

repressed. Studies have shown that the commonly preferred sugars were glucose, fructose, or sorbose. The mechanism underlying the repression could be through the prevention of transcription activation, the repression of transcription or through protein modification. A mechanism of regulation through a PEP-dependent phosphorylation by PTS has been demonstrated by the enhanced activity of the glycerol kinase (GlpK) in *E. faecalis* (Deutscher and Sauerwald, 1986). The presence of rapidly metabolizable PTS sugar inhibits glycerol uptake and metabolism. Inversely, the absence of preferred sugars leads to a decrease in repression. In the Gram-positive *B. subtilis* the expression of *glpFK* operon is mediated by CCR through the expression of gene *ccpA* encoding the catabolite control protein A, which is a transcriptional regulator (Henkin *et al.*, 1991). The *ccpA* is also present in *E. faecalis* and a similar function has been verified (Leboeuf *et al.*, 2000). Whether a regulation through *ccpA* in *E. faecalis* in our experiment has taken place could not be determined because the expression level of *ccpA* (OG1RF_11453) was not significant. However, genes encoding the glycerol catabolic pathway (*glpK* pathway) were the highest up-regulated genes in *E. faecalis* response to the intestinal tract of mice. In addition, the *iol* operon that is controlled by CCR in *B. subitilis* is also part of the high up-regulated genes in *E. faecalis*. This operon is responsible for the myo-inositol catabolism an unusual energy source and enhances the hypothesis of an inactivated repression through the absence of preferred sugars in the environment of *E. faecalis* in the intestinal tract of mice. Additionally, the enzymes of the *iol* operon catalyze myo-inositol into glyceraldehyde-3-phosphate, which is a substrate for the *glpK* pathway and thereby also affect this pathway.

Another high up-regulated gene cluster was the *ptb-buk-bkdCBAD* operon that encodes enzymes responsible for the catabolism of branched-chain α-keto acids (Ward *et al.*, 1999). This gene cluster is regulated by the presence of branched-chain α-keto acids and also by CCR (Ward *et al.*, 2000). The fact that genes encoding a chitinase, responsible for the degradation of polysaccharides, are up-regulated further indicates the absence of favored energy substrates of *E. faecalis* OG1RF and shows that an adaption process is turned on within the intestinal tract of mice. The large group of up-regulated genes responsible for the uptake of energy substrates, such as the ABC transporters and PTS, support the observed change in energy metabolism indicating an eminent energy demand in *E. faecalis* OG1RF in the intestinal tract of mice. A reason for this initiation could be that the *E. faecalis* is in a condition of glucose starvation. A study of Vebø *et al.* (2009) observed a similar up-regulation of genes of the *glpK* operon in *E. faecalis* strain V583 during its

growth in blood, indicating an exhaustion of its glucose reservoir. In our experiment the main part of calories in the mouse feed was provided by carbohydrates (mainly from strach). It seems as though the mouse almost entirely utilized the carbohydrates and left insufficient sugars in the environment of *E. faecalis*.

Some genes differentially expressed are involved in the adaption of the cell membrane composition in *Enterococcus*. This group includes the highly up-regulated genes encoding a WxL domain surface protein, a putative lipoprotein, a cell wall surface anchor family protein, and enzymes that are involved in the biosynthesis or degradation of surface polysaccharides (20 glycosyl hydrolases and mannosyl-glycoprotein endo-β-N-acetylglucosaminidase). Interstingly, the mannosyl-glycoprotein endo-β-N-acetylglucosaminidase cleaves high mannose-type glycans in glycoporteins between N-acetylglucosamine residues of the pentasaccharide core (Koide and Muramatsu, 1974; Roberts *et al.*, 2001). High mannose-type glycans are part of a range of host glycoproteins that are widely distributed within the host's body and are encountered in glycoproteins, such as laminins and thrombospondin (Fujiwara *et al.*, 1988; Furukawa *et al.*, 1989). The modification by endo-β-N-acetylglucosaminidase renders in an altered function of the glycoproteins and the cleaved residues could be used as a source of nutrients. In our experiment, a contact of *E. faecalis* to laminins, which are part of proteins in the basal lamina (a component of the basement membrane), is assumed through natural scaling of epithelial cells as no specific tissue damage was observed. Another reason for the up-regulation of the mannosyl-glycoprotein endo-β-N-acetylglucosaminidase may also be defined through a degradation of mucins. Within the gastrointestinal tract mammalians mucins are cystein rich glycoproteins in which approximate 80 % of the total mass consists of glycans (Johansson *et al.*, 2011). Besides the major part of O-linked oligosaccharides in glycans of mammalians, for which *E. faecalis* has only little ability to degrade, N-linked oligosaccharides are also included in these complex molecules and point to another possible target of nutrient (Corfield *et al.*, 1992).

The distinct down-regulated gene cluster that encodes a complete complex of enzymes responsible for the fatty acid fatty acid biosynthesis (*accABCD*, *fabD*, *acpP*, *fabH*, *fabG3*, *fabZ2*, *fab K*, *fabF2*; see Figure 17) completed the picture of a change in the cell envelope in *E. faecalis* OG1RF. The fact, that the fatty acid biosynthesis is inactive in OG1RF growing in the intestinal tract in mice suggests that an adjusted membrane fluidity is already achieved and a change is unnecessary or OG1RF is in a growth

condition in which all required fatty acids are available, most likely derived from mouse feed, and there is no need for their biosynthesis. Some transcriptome studies have shown that a transcriptome change in those genes is often related to stress responses, such as bile and blood (Bøhle *et al.*, 2010; Vebø *et al.*, 2009).

Interestingly, the two virulence traits in *E. faecalis* OG1RF, serine protease (SprE) and gelatinase (GelE) encoded by *sprE* and *gelE* in an operon type structure together with *frsB*, were significantly down-regulated (see Table 22). These two proteases were grouped in the functional category of protein metabolism because of their enzymatic function to degrade long protein chains into short fragments. The expression of SprE and GelE is regulated by a quorum-sensing system mediated by a pheromone linked to *fsr* genes. A missing signal through quorum-sensing would leave the genes *sprE* and *gelE* unexpressed, indicating that the cell density required for the *fsr* system was not sufficient. However, a cell density of well-colonized *E. faecalis* OG1RF (CFU 2.9 x 10^9 per 1 gram) within the mice intestinal tract should be high enough to activate the quorum-sensing system of GelE and SprE. Another reason for a down-regulation of GelE and SprE could be the absence of substrates for these proteases in the intestinal tract of wild type mice or *E. faecalis* founds sufficient nutrients through the up-regulated PTS.

4.7 Conclusion

The original habitat of *E. faecalis* is the intestinal tract of mammalians whenever it is found as well as in many other niches including various organs of diseased individuals, mother's milk or fermented foods. This indicates the vast adaptive potential of this species. In this work it was demonstrated that this adaptive potential includes specific changes at the cellular surface and its biosynthesis, which may change the hydrophobicity and zeta potential and also specific adherence factors by surface proteins. These proteins may be assigned a specific metabolic function, still their mere presence at the surface forms a target structure, which may contribute to cell interaction. These bacteria also undergo vast adaptation with respect to their metabolism when it comes to a change in their environment. Actually, one can view *E. faecalis* (and other bacteria) as being a "sensor", which can be used to explore the true environmental conditions in the gastrointestinal tract with respect to availability of nutrients and redox conditions compared to the conditions we offer in laboratory media. Indeed, if *E. faecalis* is considered a commensal at first glance, any upregulated gene in the intestinal tract may be better interpreted as a downregulated gene under lab conditions. So what is seen in the transcriptomic data from

E. faecalis in monoassociated mice may certainly have its limits, because the rest of the vast microbiota is absent. Still, it provides deep insight into the "true habitat" of this bacterium. This knowledge can now be used to derive novel evidence-based targets, which can be used to further elucidate the switch from a commensal to a pathogen. Also, it can possibly help to delineate this species into groups of higher and lower risk for applications in a food or medical context versus improved treatment strategies of the so far hard to cure infections.

5 Summary

Enterococcus (*E.*) *faecalis* is considered to be a member of the healthy human intestinal flora. Strains of *E. faecalis* are used as probiotics and are often found as part of the autochthonous flora in a variety of fermented foods, namely cheeses and sausages. However, in recent years this human commensal has been recognized as an opportunistic pathogen in the hospital environment causing diseases such as endocarditis, bacteremia and urinary tract infections. A variety of virulence factors have been described for this species. So far, insight into the commensalism of *E. faecalis* is limited. Therefore, we wanted to identify latent novel fitness and adaptive functions to enable a better understanding of the ambivalent nature of this species. Initially, the genetic background of different *E. faecalis* isolates, with respect to known virulence factors, was investigated to clarify if a classification of isolates is possible with regards to their origin of isolation and if an alleged behavior is associated with that. The absence of any correlation of their distribution to the origin of isolation of the strains suggests that all strains are of intrinsically intestinal origin, regardless of their specific source of isolation. Interestingly, a negative correlation was found between the number of virulence traits and the presence of CRISPR elements/*cas* genes, which indicates that such traits may be more easily acquired in strains with less or without such genetic elements. This underlines that the currently known virulence factors cannot explain the switch from a commensal to a pathogen, although strains with a high number of such factors and concomitant lack of CRISPR elements/*cas* genes may have a higher probability for that.

In this study, the ambivalent nature of strains within this species was highlighted through the analysis of *E. faecalis* strain OG1RF, a human isolate with some known virulence traits. In both the proteomic *in vitro* study and the transcriptomic *in vivo* study in monoassociated mice, novel fitness factors could be identified. These relate to clear changes in the expression of genes involved in the cellular surface and its biosynthesis, for instance phosphoenolpyruvate-dependent sugar phosphotransferase systems (PTS) as well as genes encoding various catabolic pathways some of which may be regulated by PTS. Although the strain OG1RF features some known virulence factors, their expression under the conditions present in the healthy host could not be demonstrated. The bacterium also undergoes vast adaptation with respect to its metabolism when it comes to the change of the environment. In fact, describing the adaptation ability to the host seems to be a key function in the ambivalent nature of this species. The transcriptomic data from *E. faecalis*

in monoassociated mice provides deep insight into the "true habitat" of this bacterium. This knowledge can further be used to derive novel evidence-based targets to elucidate the switch from a commensal to a pathogen and possibly delineate this species into groups of higher and lower risk for applications in a food or a medical context *versus* improved treatment strategies of the so far hard to cure infections.

6 Zusammenfassung

Enterococcus (*E.*) *faecalis* ist Teil einer gesunden Darmflora des Menschen. Manche Stämme von *E. faecalis* finden sogar Anwendung als Probiotika und sind in der autochthonen Flora fermentierter Lebensmittel, wie z.B. Käse- und Wurstprodukten, zu finden. Allerdings hat dieses kommensale Darmbakterium in den letzten Jahren zunehmend an Bedeutung als Erreger von nosokomialen Infektionen gewonnen. *E. faecalis* kann zum Teil schwere Erkrankungen und Entzündungsprozesse, wie z. B. Endokarditis, Bakteriämie und chronische Harnwegsinfektionen verursachen. Viele verschiedene Virulenzfaktoren wurden bereits in der Spezies *Enterococcus* beschrieben. Der Kenntnisstand zu *E. faecalis* als kommensales Bakterium ist dagegen sehr eingeschränkt. Das Ziel dieser Arbeit war es daher latente Eigenschaften zu entdecken die es *E. faecalis* ermöglichen sich an sein Habitat anzupassen und die sein Verhalten innerhalb einer Wirtinteraktion beschreiben. Dazu wurden zunächst die Verbreitung und Häufigkeit von Virulenzgenen innerhalb von Stämmen, die aus klinischer Umgebung, aus Lebensmitteln sowie der Umwelt isoliert wurden, untersucht. Es konnte keine signifikante Korrelation zwischen der Häufigkeit von Virulenzgenen und der klinischen Herkunft sowie der damit vermuteten Pathogenität nachgewiesen werden. Dies deutet darauf hin, dass alle Stämme − unabhängig von ihrem Isolierungsort − ihren eigentlichen Ursprung in der intestinalen Umgebung haben. Das Vorkommen von CRISPR-Elementen und *cas*-Genen verhielt sich invers zu der Häufigkeit von Virulenzfaktoren und zeigt die erschwerte Aufnahme von Virulenzgenen bei Stämmen, die diese genetischen Elemente besitzen. Die bisherigen Virulenzfaktoren können somit nicht die Veränderung eines kommensalen Bakteriums in einem Pathogen erklären, auch wenn vermutlich solche Stämme mit einer hohen Anzahl dieser Faktoren und dem gleichzeitig Fehlen von CRISPR-Elementen und *cas*-Genen eine höhere Wahrscheinlichkeit dafür aufweisen.

Das ambivalente Verhalten von Stämmen innerhalb dieser Spezies wurde anhand des Stamms *E. faecalis* OG1RF untersucht. OG1RF ist ein intensiv erforschter Stamm, der aus der menschlichen Mundhöhle isoliert wurde und einen Teil der bekannten Virulenzfaktoren besitzt. Durch *in vitro* Proteomanalysen sowie durch die Transkriptomanalyse von OG1RF im Mausversuch konnten neue Fitnessfaktoren nachgewiesen werden. Diese erklären sich durch deutliche Veränderungen der Expression von Genen, die in der Zelloberfläche und dessen Synthese beteiligt sind, z B. Phosphotransferasesysteme (PTS) und Gene, die verschiedene katabole Reaktionen zur

Energiegewinnung kodieren und zum Teil sogar durch PTS reguliert scheinen. Obwohl im Genom des Stamms OG1RF ein Teil der bekannten Virulenzfaktoren kodiert ist, wie z.B. Gelatinase, war die Expression dieser Gene im Intestinaltrakt der gesunden Maus nicht nachweisbar. Das Bakterium zeigt eine enorme Anpassungsfähigkeit hinsichtlich seines Metabolismus sobald es zu einer Veränderung der direkten Umgebung kommt. Diese präzise Anpassungsfähigkeit an den Wirt scheint eine Schlüsselfunktion des ambivalenten Charakters dieser Spezies zu sein. Die Transkriptomdaten von *E. faecalis* in monoassoziierten Mäusen erbrachten wichtige Erkenntnisse aus dem eigentlichen Lebensraum dieses Bakteriums, sowie sein Verhalten in diesem. Dieses Wissen kann genutzt werden, um neue evidenzbasierte Ziele abzuleiten, welche die Veränderung von einem kommensalen zu einem pathogenen Bakterium aufzuklären hilft oder eine mögliche Einteilung der Spezies in Risikogruppen ermöglicht, beispielsweise für die Anwendung in Lebensmitteln oder im medizinischen Kontext zur Verbesserung von Behandlungsstrategien bei schwer einzudämmenden Infektionen.

7 References

Adegoke, G.O., Babalola, A.k. (1988) Characteristics of microorganisms of importance in the fermentation of fufu and ogi − two Nigerian foods. Journal of Applied Microbiology **65**, 449-453.

Alemu, M., Laemkom, P., Doi, K., Ohmomo, S., Yajima, M., Nitsinprasert, S. (2006) Characterization of bacteriocin-producing lactic acid bacteria strain N1-33 isolated from edible fermented bamboo shoot. Kasetsart Journal (Natural Science) **40**, 486-498.

Ananou, S., Maqueda, M., Martínez-Bueno, M., Gálvez, A., Valdivia, E. (2005) Control of *Staphylococcus aureus* in sausages by enterocin AS-48. Meat Science **71**, 549-556.

Ahmed, W., Neller, R., Katouli, M. (2005) Host species-specific metabolic fingerprint database for enterococci and *Escherichia coli* and its application to identify sources of fecal contamination in surface waters. Applied Environmental Microbiology **71**, 4461-4468.

Andrewes, F.W., and Horder, T. J. (1906) A study of the streptococci pathogenic for man. The Lancet **168**, 852-855.

Bager, F., Madsen, M., Christensen, J., Aarestrup., F.M. (1997) Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms. Preventive Veterinary Medicine **31**, 95-112.

Balish E., and Warner T. (2002) *Enterococcus faecalis* induces inflammatory bowel disease in interleukin-10 knockout mice. The American Journal of Pathology **160**, 2253-2257.

Barcelona-Andrés, B., Marina, A., Rubio,V. (2002) Gene structure, organization, expression, and potential regulatory mechanisms of arginine catabolism in *Enterococcus faecalis*. Journal of Bacteriology **184**, 6289-6300.

Basualdo J., Sparo M., Chiodo P., Ciarmela M., Minivielle M., (2007) Oral treatment with a potential probiotic (*Enterococcus faecalis* CECZ 7121) appears to reduce the parasite burden of mice infected with Toxocara canis. Annals of Tropical Medicine and Parasitology **101**, 559-562.

Behr, J., Israel, L., Gänzle, M.G., Vogel, R.F. (2007) Proteomic approach for characterization of hop-inducible proteins in *Lactobacillus brevis*. Applied and Environmental Microbiology **73**, 330-3306.

Berg, D.J., Davidson, N., Kühn, R., ;üller, W., Menon, S., Holland, G., Thompson-Snipes, L., Leach, M.W., Rennick, D. (1996) Enterocolitis and colon cancer in Interleukin-10-deficient mice are asociated with aberrant cytokine production and $CD4^+$ TH1-like responses. Journal of Clinical Investigation **98**, 1010-1020.

Bhardwaj, A., Gupta, H., Kapila, S., Kaur, G., Vij, S., Malik, R.K. (2010) Safety assessment and evaluation of probiotic potential of bacteriocinogenic *Enterococcus faecium* KH 24 strain under *in vitro* and *in vivo* conditions. The Interntional Journal of

Food Microbiology **141**, 156-164.

Bizzini, A., Zhao, C., Budin-Verneuil, A., Sauvageot, N., Giard, J.-C., Auffray, Y., Hartke, A. (2010) Glycerol is metabolized in a complex and strain-dependent manner in *Enterococcus faecalis*. Journal of Bacteriology **192**, 779-785.

Blum, H., Beier, H., Gross, H.J (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8**, 93-99.

Bourgogne A., Garsin D.A., Qin X., Singh K.V., Sillanpaa J., Yerrapragada S., Ding Y., Dugan-Rocha S., Buhay C., Shen H., Chen G., Williams G., Muzny D., Maadani A., Fox K.A., Gioia J., Chen L., Shang Y., Arias C.A., Nallapareddy S.R., Zhao M., Prakash V.P., Chowdhury S., Jiang H., Gibbs R.A., Murray B.E., Highlander S.K., Weinstock G.M. (2008) Large scale variation in *Enterococcus faecalis* illustrated by the genome analysis of strain OG1RF. Genome Biology **9**, R110.

Bøhle, L.A., Frægestad, E.M., Veiseth-Kent, E., Steinmoen, H., Nes, I.F., Eijsink, V.G.H., Mathiesen, G. (2010) Identification of proteins related to the stress response in *Enterococcus faecalis* V583 caused by bovine bile. Proteome Science **8**: 37.

Bradford, M.M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry **72**, 248-254.

Brock, T.D., Peacher, B., Pierson, D. (1963) Survey of the bacteriocines of enterococci. Journal of Bacteriology **86**, 702-707.

Bryan-Jones, D.G., Whittenbury, R. (1969) Haematin-dependent oxidative phosphorylation in *Streptococcus faecalis*. Journal of General Microbiology **58**, 247-260.

Centeno, J.A., Menendez, S., Hermida, M, Rodríguez-Otero (1999) Effects of the addtion of *Enterococcus faecalis* on Cebreiro cheese manufacture. The International Journal of Food Microbiology **48**, 97-111.

Centers for Disease Control and Prevention, U.S. (2004) National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992 through June 2004. American Journal of Infection Control **32**, 470-485.

Chow, J.W., Thal, L.A., Perri, M.B., Vazquez, J.A., Donabedian, S.M., Clewell, D.B., Zervos, M.J. (1993) Plasmid.associated hemolysin and aggregation substance production contribut to virulence in experimental enterococcal endocarditis. Antimicrobial agents and Chemotherapy **37**, 247-2477.

Clewell, D.B., Francia, M.V., Flannagan, S.E., An, F.Y. (2002) Enterococcal plasmid transfer: sex pheromones, transfer origins, relaxases, and the *Staphylococcus aureus* issue. Plasmid **48**, 193-201.

Clewell, D.B., Yagi, Y., Dunny, G.M., Schultz, S.K. (1974) Characterization of three plasmid deoxyribonucleic acid molecules in a strain of *Streptococcus faecalis*: identification of a plasmid determining erythromycin resistance. Journal of Bactriology

117, 283-289.

Coburn, P.S., Pillar, C.M., Jett, B.D., Haas, W., Gilmore, M.S. (2004) *Enterococcus faecalis* senses target cells and in response expresses cytolysin. Science 306, 2270-2272.

Coburn, P.S., Gilmore, M.S. The *Enterococcus faecalis* cytolysin: a novel toxin active against eukaryotic and prokaryotic cells. Cellular Microbiology **5**, 661-669.

Cocconcelli, P.S., Cattivelli, D., Gazzola, S. (2003) Gene transfer of vancomycin and tetracycline resistances among *Enterococcus faecalis* during cheese and sausage fermentation. The International Journal of Food Microbiology **88**, 315-323.

Contesse, G., Crépin, M., Gros, F., Ullmann, A., Monod, J. (1969) On the mechanism of catabolite repression. In J.R. Beckwith and D. Zipser (ed.), The lactose operon. Cold Spring Harbor Laboratory Press, 401-415.

Coppola, S., Blaiotta, G., Moschetti, G. (2001) Molecular evaluation of microbial diversity occuring in different types of Mozzarella cheese. Journal of Applied Microbiology **90**, 414-420.

Coque, T.M., Willems, R., Canton, R., Del Campo, R., Baquero, F. (2002). High occurrence of esp among ampicillin-resistant and vancomycin-susceptible *Enterococcus faecium* clones from hospitalized patiens. Journal of Antimicrobial Chemotherapy **50**,1035-1038.

Coque, T.M., Patterson, J.E., Steckelberg, J.M., Murray, B.E. (1995) Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from feces of hospitalized and community-based persons. Journal of Infectious Diseases **171**, 1223-1229.

Corfield, A.P., Wagner, S.A., Clamp, J.R., Kriaris, M.S., Hoskins, L.C. (1992) Mucin degradation in the human colon: production of sialidase, sialate O-acetylesterase, N-acetylneuraminate lyase, arylesterase, and glycosulfatase activities by strains of fecal bacteria. Infection and Immunity **60**, 3971-3978.

Coudron, P.E., Markowitz, S.M., Wong, E.S. (1992) Isolation of a beta-lactamase-producing, aminoglycoside-resistant strain of *Enterococcus faecium*. Antimicrobial Agents and Chemotherapy **36**, 1125-1126.

Curic, M., Stuer-Lauridsen, B., Renault, P., Nilsson, D. (1999) A general method for selection of alpha-acetolactate decarboxylase-deficient *Lactococcus lactis* mutants to improve diacetyl formation. Applied and Environmental Microbiology **65**, 1202-1206.

Deibel, R.H., Silliker, J.H. (1963) Food-poisoning potential of the enterococci. Journal of Bacteriology **85**, 827-832.

Deutscher, J., Francke, C., Postma, P.W. (2006) How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. Microbiology and Molecular Biology Reviews **70**, 939-1031.

Deutscher, J., Sauerwald, H. (1986) Stimulation of dihydroxyacetone and glycerol kinase

activity in *Streptococcus faecalis* by phosphoenolpyruvate-dependent phosphorylation catalyzed by Enzyme I and HPr of the phosphotransferase system. Journal of Bacteriology **166**, 829-836.

Devriese, L.A., Pot, B. (1995) The genus *Enterococcus.* in Wood, B.J.B. and Holzapfel, W.H.: The genera of lactic acid bacteria. Chapmann & Hal, 328-367.

Devriese, L.A., Van de Kerckhove, A., Kilpper-Bälz, R., Schleifer, K.H. (1987) Characterization and identification of *Enterococcus* species isolated from the intestine of animals. International Journal of Systematic Bacteriology **37**, 257-259.

Dolin, M.I., Gunsalus, I.C. (1951) Pyruvic acid metabolism II. An acetoin forming enzyme system in *Streptococcus faecalis*. Journal of Bacteriology **62**, 199-214.

Doucet-Populaire, F., Trieu-Cuot, P. Andremont, A., Courvalin, P. (1992) Conjugal transfer of plasmid DNA from *Enterococcus faecalis* to *Escherichia coli* in digestive tract of gnotobiotic mice. Antimicrobial Agents and Chemotherapy **36**, 502-504.

Domann, E., Hain, T., Ghai, R., Billion, A., Kuenne, C., Zimmermann, K., Chakraborty, T. (2007) Comparative genomic analysis for the presence of potential enterococcal virulence factors in the probiotic *Enterococcus faecalis* strain Symbioflor 1. International Journal of Medical Microbiology **297**, 533-539.

Dubrac, S., Msadek, T. (2004) Identification of genes controlled by the essential YycG/YycF two-component system of *Staphylococcus aureus*. Journal of Bacteriology **186**, 1175-1181.

Duez, C., Zorzi, W., Sapunaric, F., Amoroso, A., Thamm, I., Coyette, J. (2001) The penicillin resistance of *Enterococcus faecalis* JH2-2r results from an overproduction of the low-affinity penicillin-binding protein PBP4 and does not involve a *psr*-like gene. Microbiology **147**, 2561-2569.

Dunny, G.M., Brown, B.L., Clewell, D.B. (1978) Induced cell aggregation and mating in *Streptococcus faecalis*: evidence for bacterial sex pheromone. Proceedings of the National Academy of Science of the USA **75**, 3479-3483.

Dupont, H., Montravers, P., Mohler, J., Carbon, C. (1998) Disparate findings on the role of virulence factors of *Enterococcus faecalis* in mouse and rat models of peritonitis. Infection and Immunity **66**, 2570-2575.

Eaton T.J., Gasson M.J. (2002) A variant enterococcal surface protein Esp_{fm} in *Enterococcus faecium*; distribution among food, commensal, medical, and environmental isolates. FEMS Microbiology Lerrers **216**, 269-275.

Eaton T.J., Gasson M.J. (2001) Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. Applied Environmental Microbiology **67**, 1628-1635.

Ehrmann M.A., Müller M.R., Vogel R.F. (2003) Molecular analysis of sourdough reveals *Lactobacillus mindensis* sp. nov. International Journal of Systematic and Evolutionary Microbiology **53**,7-13.

Engel, H.W.B., Soedirman, N., Rost, J.A., van Leeuwen, W.J., van Embden, J.D. (1980) Transferability of macrolide, lincomycins, and streptogramin resistances between group A, B, and D streptococci, *Streptococcus pneumoniae*, and *Staphylococcus aureus*. Journal of Bacteriology **142**, 407-413.

FAO-WHO working group report on drafting guidelines for the evaluation of probiotics in food. London, Ontario, Canada, April 30 and May 1, 2002 (http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf)

Firmesse, O., Rabout, S., Bermúdez-Humarán, L.G., Corthier, G., Furet, J.-P. (2007). Consumption of Camembert cheeses stimulates commensal enterococci in healthy human intestinal microbiota. FEMS Microbiology Letters **276**, 189-192.

Forrest, W.W. (1965) Adenosine triphosphate pool during the growth cycle in *Streptococcus faecalis*. Journal of Bactriology **90**, 1013-1016.

Foster, T.J., Höök, M. (1998) Surface protein adhesins of *Staphylococcus aureus*. Trends in Microbiology **6**, 484-488.

Foulquié Moreno, M.R., Callewaert, R., Devreese, B., Van Beeuman, J., de Vuyst, L. (2002) Isolation and biochemical characterisation of enterocins produced by enterococci from different sources. Journal of Applied Microbiology **94**, 214-229.

Frank, A.M. (2009) A Ranking-Based Scoring Function for Peptide-Spectrum Matches. Journal of Proteome Research **8**, 2241-2252.

Frank, A.M., Savitski, M.M., Nielsen, L.M., Zubarev, R.A., Pevzner, P.A. (2007) De Novo Peptide Sequencing and Identification with Precision Mass Spectrometry. Journal of Proteome Research **6**, 114-123.

Frank, A., Pevzner, P. (2005) PepNovo: De Novo Peptide Sequencing via Probabilistic Network Modeling. Analytical Chemistry **77**,964-973.

Frank, A., Tanner, S., Bafna, V., Pevzner, P. (2005) Peptide sequence tags for fast database search in mass-spectrometry. Journal of Proteome Research **4**,1287-95.

Franz, C.M., Muscholl-Silberhorn, A.B., Yousif, N.M., Vancanneyt, M., Swings, J., Holzapfel, W.H. (2001) Incidence of virulence factors and antibiotic resistance among enterococci isoltad from food. Applied Environmental Micorbiology **67**, 4385-4389.

Freitas, A.C., Pais, C. Malcata, F.X. Hogg, T.A. (1996) Microbiological characterization of picante da Beira Baixa cheese. Journal of Food Protection **59**, 155-160.

Fujiwara, S., Shinkai, H., Deutzmann, R., Paulsson, M, Timpl, R. (1988) Structure and distributuin of N-linked oligosaccharide chains on various domains of mouse tumour laminin. Biochemical Journal **252**, 453-461.

Furukawa, K., Roberts, D.D., Endo, T., Kobata, A. (1989) Structural study of the sugar chains of human platelet thrombospondin. Archives of Biochemistry and Biophysics **270**, 302-312.

Gardner, P., Smith, D.H., Beer, H., Moellering, R.C. (1969) Recovery of resistance (r)

factors from a drug-free community. The Lancet **294**, 774-776.

Gaspar F.B., Crespo M.T., Lopes M.F. (2009) Proposal for a reliable enterococcal cytolysin production assay avoiding apparent incongruence between phenotype and genotype. Journal of Medical Microbiology **58**, 1122-1124.

Gastmeier, P., Behnke, M., Schwab, F., Geffers, C. (2010) Das Krankenhaus-Infektions-Surveillance-System (KISS)- aktueller Stand. Das Krankenhaus **7**, 645-648.

Geijersstam, A.R., Culak, R., Molenaar, L., Chattaway, M., Røslie, E., Peciuliene, V., Haapasalo, M., Sha, H.N. (2007) Comparative analysis of virulence determinants and mass spectral profiles of Finnish and Lithuanian endodontic *Enterococcus faecalis* isolates. Oral Microbiology and Immunology **22**, 87-94.

Gelsomino, R., Vancanneyt, Cogan, T.M., Swings, J. (2003) Effect of raw-milk cheese consumption on enterococcal flora of human feces. Applied and Environmental Microbiology **69**, 312-319.

Gelsomino, R., Vancanneyt, M., Cogan, T.M., Condon, S., Swings, J. (2002) Source of enterococci in a farmhouse raw-milk cheese. Applied and Environmental Microbiology **68**, 3560-3565.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y.H., Zhang, J. (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biology **5**, R80.

Giard, J.-C., Laplace, J.-M., Rincé, A., Pichereau, Benachour, A., Leboeuf, C., Flahaut, S., Auffray, Y, Hartke, A. (2001) The stress proteome of *Enterococcus faecalis*. Electrophoresis **22**, 2947-2954.

Giard, J.-C., Rincé, A., Capiaux, H., Auffray, Y, Hartke, A. (2000) Inactivation of the stress-and starvation-inducible *gls24* operon has a pleiotrophic effect on cell morphology, stress sensitivity, and gene expression in *Enterococcus faecalis*. Journal of Bacteriology **16**, 4512-4520.

Giebel, R.A., Fredenberg, W., Sandrin, T.R. (2008) Characterization of environmental isolates of *Enterococcus* spp. by matrix-assisted laser desorption/isonization time-of-flight mass spectrometry. Water Research **42**, 931-940.

Gilmore M.S., Segarra R.A., Booth M.C., Bogie C.P., Hall L.R., Clewell D.B. (1994) Genetic structure of the *Enterococcus faecalis* plasmid pAD1-encoded cytolytic toxin system and its relationship to lantibiotic determinants. Journal of Bacteriololgy **176**, 7335-7344.

Giraffa, G. (2003) Functionality of enterococci in dairy products. The International Journal of Food Microbiology **88**, 215-222.

Görg, A., Obermaier, C., Boguth, G., Harder, A., Scheibe, B., Wildgruber, R., Weiss, W. (2000) The current state of two-dimensional electrophoresis with immobilized pH

gradients. Electrophoresis **21**, 1037-1053.

Görg, A., Postel, W., Weser, J., Günther, S., Strahler, J.R., Hanash, S.M., Somerlot, L. (1987) Elimination of point streaking on silver stained two-dimensional gels by addition of iodacetamide to the equilibration buffer. Electrophoresis **8**, 122-124.

Gold, O.G., Jordan, H.V., van Houte, J. (1975) The prevalence of enterococci in the human mouth and their pathogenicity in animal models. Archives of Oral Biology **20**, 473-477.

Grayson, M.L., Eliopoulos, G.M., Wennersten, C.B., Ruoff, K.L., de Girolami, P.C., Ferraro, M.-J., Moellering Jr., R.C. (1991) Increasing resistance to beta-lactam antibiotics among clinical isolates of *Enterococcus faecium*: a 22-year review at one institution. Antimicrobial Agents and Chemotherapy **35**, 2180-2184.

Grissa, I., Vergnaud, G., Pourcel, C. (2007) CRISPRFinder: a web tool to identify cluster regularly interspaced short palindromic repeats. Nucleic Acids Research **35**, 52-57.

Grömping, U. (2009) R: A language and environment for statistical computing. R Foundation for Statistical Computing 1, 1706.

Guarner, F., Schaafsma, G.J. (1998) Probiotics. The International Journal of Food Microbiology **39**, 237-238.

Haas, W., Shepard, B.D., Gilmore, M.S. (2002) Two-component regulator of *Enterococcus faecalis* cytolysin responds to quorum-sensing autoinduction. Nature **415**, 84-85.

Haft D.H., Selengut J., Mongodin E.F., Nelson K.E. (2005) A guild of 45 CRISPR-associated (Cas) protein families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. PLoS Computational Biology **1**, e60.

Hancock, H.H., Sigurdsson, A. Trope, M., Moiseiwitsch, J. (2001) Bacteria isolated after unsuccessful endodontic treatment in a North American population. Oral Surgery Oral Medicine Oral Pathology Endodontics **91**, 579-586.

Hardie, J.M., Whiley, R.A. (1997) Classification and overview of the genera *Streptococcus* and *Enterococcus*. Journal of Applied Microbiology Symposium Supplement **83**, 1S-11S.

Harrington, S.M., Ross, T.L., Gebo, K.A., Merz, W.G. (2004) Vancomycin resistance, esp, and strain relatedness: a 1-year study of enterococal bacteremia. Journal of Clinical Microbiology **42**, 5895-5898.

Hartke, A., Giard, J.-C., Laplace, J.-M., Auffray, Y. (1998) Survival of *Enterococcus faecalis* in an oligotrophic microsom: changes in morphology, development of general stress resistance, and analysis of proteinsynthesis. Applied and Environmental Microbiology **64**, 4238-4245.

Heath, R.J., Rock, C.O. (2004) Fatty acid biosynthesis as a target for novel antibacterials. Current Opinion in Investigational Drugs **5**, 146-153.

Hegstad, K., Mikalsen, T., Coque, T.M., Werner, G., Sundsfjord, A. (2010) Mobile genetic

elements and their contribution to the emergence of antimicrobial resistant *Enterococcus faecalis* and *Enterococcus faecium*. Clinical Microbiology and Infection **16**, 541-554.

Henkin, T.M., Grundy, F.J., Nicholson, W.L., Chambliss, G.H. (1991) Catabolite repression of alpha-amylase gene expression in *Bacillus subtillis* involves a trans-acting gene product homologous to the *Escherichia coli lacl* and *galR* repressors. Molecular Microbiology **5**, 575-584.

Heuer, O.E., Pedersen, K., Jensen, L.B., Madsen, M., Olsen, J.E. (2002) Persistence of vancomycin-resistant enterococci (VRE) in broiler houses after the avoparcin ban. Microbiology Drug Resistance **8**, 355-361.

Hew C.M., Korakli M., Vogel R.F. (2007) Expression of virulence-related genes by *Enterococcus faecalis* in response to different environments. Systematic and Applied Microbiology **30**, 257-267.

Hidron, A.I., Edwards, J.R., Patel, J., Horan, T.C., Sievert, D.M., Pollock, D.A., Fridkin, S.K., for National Healthcare Safety Network Team and Facilities (2008) Antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. Infection Control and Hospital Epidemiology **29**, 996-1011.

Horvath P., Romero D.A., Coute-Monvoisin A.C., Richards M., Deceau H., Moineau S., Boyaval P., Fremaux C., Barrangou R. (2008) Diversity, activity, and evolution of CRISPR loci in *Streptococcus themophilus*. Journal of Bacteriology **190**,1401-1412.

Horvath, P., Barrngou, R. (2010) CRISPR/Cas, the Immune System of Bacteria and Archaea. Science **327**, 167-170.

Hubble, T.S., Hatton, J.F., Nallapareddy, S.R., Murray, B.E., Gillespie, M.J. (2003) Influence of *Enterococcus faecalis* proteases and the collagen-binding protein, Ace, on adhesin to dentin. Oral Microbiology and Immunology **18**, 121-126.

Hufnagel, M., Koch, S., Creti, R., Baldassarri, L., Huebner, J. (2004) A putative sugar-binding transcriptional regulator in a novel gene locus in *Enterococcus faecalis* contibutes to production of biofilm and prolonged bacteremia in mice. Journal of Infectious Diseases **189**, 420-430.

Hulse, M.I., Smith, S., Chi, E.Y., Pham, A., Rubens, C.E. (1993) Effect of type III group streptococcal capsular polysaccharide on invasion of respiratory epithelial cells. Infection and Immunity **61**, 4835-4831.

Huycke, M.M., Abrams, V., Moore, D.R. (2002) *Enterococcus faecalis* produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA. Carcinogenesis **23**, 529-536.

Huycke, M.M., Joyce, M., Wack, M.F. (1996) Augmented production of extracellular superoxide by blood isolates of *Enterococcus faecalis*. The Journal of Infectious Diseases **173**, 743-746.

Huycke, M.M., Gilmore, M.S., Jett, D.B., Booth, J.L. (1992) Transfer of pheromone-inducible plasmids between *Enterococcus faecalis* in the Syrian hamster gastrointestinal tract. The Journal of Infetious Diseases **166**, 1188-1191.

Ike, Y., Clewell, D.B. (1992) Evidence that the hemolysin/bacteriocin phenotype of *Enterococcus faecalis* subsp. *zymogenes* can be determined by plasmids in different incompatibility groups as well as by the chromosome. Journal of Bactriology **174**, 8172-8177.

Ike Y., Hashimoto H., Clewell D.B. (1984) Hemolysin of *Streptococcus faecalis* subspecies *zymogenes* contributes to virulence in mice. Infection and Immunity **45**, 528-530.

Jacob, A.E., Hobbs, S.J. (1974) Conjugal transfer of plasmid-borne multiple antibiotic resistance in *Streptococcus faecalis* var. *zymogenes*. Journal of Bactreiology **117**, 360-372.

Jansen, R., Embden, J.D., Gaastra, W., Schouls, L.M. (2002) Identification of genes that are associated with DNA repeats in prokaryotes. Molecular Microbiology **43**, 1565-1575.

Jansen, G., Deddens, B., Wilkinso, M., van der Waaij, D. (1993) Significant decrease of titres of circulating IgG after oral intake of a preparation of *Enterococcus faecalis* in a group of ten healthy volunteers. Infection **21**, 193-194.

Jett, B.D., Atkuri, R.V., Gilmore, M.S. (1998) *Enterococcus faecalis* localization in experimental endophthalmitis: role of plasmid-encoded aggregation substance. Infection and Immunity **66**, 843-848.

Jett B.D., Jensen H.G., Nordquist R.E., Gilmore M.S., (1992) Contribution of pAD1-encoded cytolysin to the severity of experimental *Enterococcus faecalis* endophthalmitis. Infection and Immunity **60**, 2445-2452.

Johansson, M.E.V., Holmén Larsson, J.M., Hansson, G.C. (2011) The two mucus layers of the colon are organized by MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. Proceedings of the National Academy of Sciences **108**, 4659-4665.

Johnson, J.R., Clabots, C., Hirt, H., Waters, C., Dunny, G. (2004) Enterococcal aggregation substance and binding substance are not major contributors to urinary tract colonization by *Enerococcus faecalis* in a mouse model of ascending unobstrcuted urinary tract infection. Infection and Immunity **72**, 2445-2448.

Jones, M.E., Lipmann, F. (1960) Chemical and enzymatic synthesis of carbamyl phosphate. Biochemistry **46**, 1194-1205.

Kalina, A.P. (1970). The Taxonomy and Nomenclatur of Enterococci. International Journal of Systematic Bacteriology **20**, 185-189.

Kanemitsu, K., Nishino, T., Kunishima, H., Okamura, N., Takemura, H., Yamamoto, H., Kaku, M. (2001) Quantitative determination of gelatinase activity among enterococci.

Journal of Microbiological Methods **47**, 11-16.

Kang, S., Denman, S.E., Morrison, M., Yu, Zhongtang, McSweeney, C.S. (2009) An efficient RNA extraction method for estimating gut microbial diversity by polymerase chain reaction. Current Microbiology **58**,464-471.

Katakura, K., Lee, J., Rachmilewitz, D., Li, G., Eckmann, L., Raz, E. (2005) Toll-like receptor 9-induced type I IFN protects mice from experimental colotis. Journal of Clinical Investigation **115**, 695-702.

Klare, I., Konstabel., C., Badstübner, D., Werner, G., Witte, W. (2003) Occurrence and spread of antibiotic resistances in *Enterococcus faecium*. International Journal of Food Microbiology **88**, 269-290.

Kleessen B., Bezirtzoglou, Mättö, J. (2000) Culture-based knowledge on biodiversity, development and stability of human gastrointestinal microflora. Microbial Ecology in Health and Disease **2**, 53-63.

Klein, G. (2003) Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinl tract. International Journal of Food Microbiology **88**, 123-31.

Kobayashi, R. (1940) Studies concerning hemolytic streptococci: typing of human hemolytic streptococci and their relation to diseases and their distribution on mucous membranes. The Kitasato Archives of Experimental Medicine **17**, 218-241.

Koide, N., Muramatsu, T (1974) Endo-beta-N-acetylglucosaminidase acting on carbohydrate moieties of glycoproteins: purification and properties of the enzyme from *Diplococcus pneumoniae*. The Journal of Biological Chemistry **249**, 4897-4904.

Koonin E.V., Makarova K.S. (2009) CRISPR-Cas: an adaptive immunity system in prokaryotes. F1000 Biology Reports **1**:95.

Kreft, B., Marre, R., Schramm, U., Wirth, R. (1992) Aggregation substance of *Enterococcus faecalis* mediates adhesion to cultered renal tubular cells. Infection and Immunity **60**, 25-30.

Krueger, W.A., Krueger-Rameck, S., Koch, S., Carey, V., Pier, G.B., Huebner, J. (2004) Assessment of the role of antibiotics and enterococcal virulence factors in a mouse model of extraintestinal translocation. Critical Care Medicine **32**, 467-471.

Kurokawa, K., Itoh, T., Kuwahara, T., Oshima, K., Toh, H., Toyoda, A., Takami, H., Morita, H., Sharma, V.K., Srivastava, T.P., Taylor, T.D., Noguchi, H., Mori, H., Ogura, Y., Ehrlich, D.S., Itoh, K., Takagi, T., Sakaki, Y., Hayashi, T., Hattori, M. (2007). Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. DNA Research **14**, 169-181.

Lancefield, R.C. (1933). A serologicl differentiaton of human and other groups of hemolytic streptococci. The Rockefeller University Press **57**, 571-595.

Lauková, A., Fraqueza, M.J., Strompfová, V., Simonová, M.P., Elias, M., Barreto, A. (2011). Bacteriocinogenic activity of *Enterococcus faecalis* srains from Chourico, traditionl sausage produced in Southern Portugal. African Journal of Microbiology

Research **5**, 334-339.

Lay, C., Rigottier-Gois, L., Holmstrøm, Raijlic, M., Vaughan, E.E., deVos, W.M., Collins, M.D., Thiel, R., Namsolleck, P., Blaut, M., Doré, J. (2005). Colonic microbiota signatures across five northern European countries. Applied and Environmental Microbiology **71**, 4153-4155.

Leboeuf, C., Leblanc, L, Auffray, Y., Hartke, A. (2000) Characterization of the *ccpA* gene of *Enterococcus faecalis*: identification of starvation-inducible proteins regulated by CcpA. Journal of Bacteriology **182**, 5799-5806.

Leclerq, R., Derlot, E., Duval., J., Courvalin, P. (1988). Plasmid-mediated vancomycin and teicoplanin resistance in *Enterococcus faecium*. The New England Journal of Medicine **319**, 157-161.

Litopoulou-Tzanetaki, E., Tzanetakis, N., Vafopoulou-Mastrojiannaki, A. (1993) Effect of the type of lactic acid starter on microbiological chemical and sensory characteristics of Feta cheese. Food Microbiology **10**, 31-41.

Litopoulou-Tzanetaki, E. (1990) Changes in numbers and kinds of lactic acid bacteria during ripening of Kefalotyri cheese. Journal of Foos Science **55**, 111-113.

Low, Y.L., Jakubovics, N.S., Flatman, J.C., Jenkinson, H.F., Smith, A.W. (2003) Manganese-dependent regulation of the endocarditis-associated virulence factor EfaA of *Enterocccus faecalis*. Journal of Medical Microbiology **52**, 113-119.

Lowe, A.M., Lambert, P.A., Smith, A.W. (1995) Cloning of an *Enterococcus faecalis* endocarditis antigen: homology with adhesins from some oral streptococci. Infection and Immunity **63**, 703-706.

Macovei, L., Zurek, L. (2006) Ecology of antibiotic resistance genes: characterization of enterococci from houseflies collected in food settings. Applied and Environmental Microbiology **72**, 4028-4035.

Maekawa S., Yoshioka, M., Kumamoto Y. (1992) Proposal of a new scheme for the serological typing of *Enterococcus faecalis* strains. Microbiology and Immunology **36**, 671-681.

Mäkinen P.L., Mäkinen, K.K. (1994) The *Enterococcus faecalis* extracellular metalloendopeptidase (EC 3.4.24.30; coccolysin) inactivates human endothelin at bonds involving hydrophobic amino acid residues. Biochemical and Biophysical Research Communications **200**, 981-985.

Mäkinen P.L., Clewell D.B., An F., Makinen K.K. (1989) Purification and substrate specificity of a strongly hydrophobic extracellular metalloendopeptidase ("gelatinase") from *Streptococcus faecalis* (strain OG1-10). Journal of Biological Chemistry **264**, 3325-3334.

Makarova, K.S., Grishin, N.V., Shabalina, S.A., Wolf, Y.I., Koonin, E.V. (2006) A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic

RNAi, and hypothetical mechanisms of action. Biology Direct **1**:7.

Mannu, L., Paba, A. (2002) Genetic diversity of lactococci and enterococci isolated from home-made Pecorino Sardo ewe's milk cheese. Journal of Applied Microiology **92**, 55-62.

Mannu L., Paba A., Daga E., Comunian R., Zanetti S., Dupre I., Sechi L.A. (2003) Comparison of the incidence of virulence determinants and antibiotic resistance between *Enterococcus faecium* strains of dairy, animal and clinical origin. International Journal of Food Microbiology **88**, 291-304.

Manson, J.M., Hancock, L.E., Gilmore, M.S. (2010) Mechanism of chromosomal transfer of *Enterococcus faecalis* pathogenicity island, capsule, antimicrobial resistance, and other traits. Proceedings of the National Academy of Science of the USA **107**, 12269-12274.

Mantini D., Petrucci F., Pieragostino D., Del Boccio P., Di Nicola M., Di Ilio C., Federici G., Sacchetta P., Comani S., Urbani A., 2007. LIMPIC: a computational method for the separation of protein MALDI-TOF-MS signals from noise. BMC Bioinformatics 8:101.

Marchesini, B., Buttin, A., Romailler, N., Moreton, R.S., Stucchi, C., Sozzi, T. (1992) Microbiological events during commercial meat fermentation. Journal of Applied Microbiology **73**, 203-209.

Marquis, R.E., Bender, G.R., Murray, D.R., Wong, A. (1987) Arginine deiminase system and bacterial adaptation to acid environments. Applied and Environmental Microbiology **53**, 198-200.

Martin B., Garriga M., Hugas M., Aymerich T. (2005) Genetic diversity and safety aspects of enterococci from slightly fermented sausages. Journal of Applied Microbiology **98**, 1177-1190.

Martin-Platero, A.M., Valdivia, E., Ruiz-Rodriguez, M., Soler, J.J., Martin-Vivaldi, M., Maqueda, M., Martinez-Bueno, M. (2006) Characterization of antimicrobial substances produced by *Enterococcus faecalis* MRR 10-3, isolated from the uropygial gland of the hoopoe (*Upupa epops*). Applied and Environmental Microbiology **72**, 4245-4249.

Matsuki, T., Watanabe, K., Fujimoto, J., Takada, T., Tanaka, R. (2004) Use of 16S sRNA gene-target group-specific primers for real-time PCR analysis of predominant bacteria un human feces. Applied and Environmental Microbiology **70**, 7220-7228.

Matsuki, T., Watanabe, K., Fujimoto, J., Miyamoto, Y., Takada, T., Matsumoto, K., Oyaizu, H., Tanaka, R. (2002). Development of 16S rRNA-gene-target group-specific primers for the detection and identification of predominant bacteria in human feces. Applied and Environmental Microbiology **68**, 5445-5451.

McBride, S.M., Fischetti, V.A., LeBlanc, D.J., Moellering Jr, R.C., Gilmore, M.S. (2007) Genetic diversity among *Enterococcus faecalis*. PLoS One **2**, e582.

Metaxopoulos, J., Samelis, J., Papaedlli, M. (2001) Technological and microbiological evaluation of traditional processes as modified for the industrial manufacturing of dry

fermented sausages in Greece. Italian Journal of Food Science **13**, 3-18.

Miyazaki, S., Ohno, A., Kobayashi, I., Uji, T., Yamaguchi, K., Goto, S. (1993) Cytotoxic effect of hemolytic culture supernatant from *Enterococcus faecalis* on mouse polymorphonuclear neutrophils and macrophages. Microbiology and Immunology **37**, 265-270.

Molander, A., Reit, C., Dahlen, G., Kvist, T. (1998) Microbiological status of root-filled teeth with apical peridontitis. International Endodontic Journal **21**, 1-7.

Monnet, C., Nardi, M., Hols, P., Gulea, M., Corrieu, G., Monnet, V. (2003) Regulation of branched-chain amino acid biosynthesis by alpha-acetolactate decarboxylase in *Streptococcus thermophilus*. Letters Applied Microbiology **36**, 399-405.

Mortazavi, A., Williams, B.A., McCue, K., Schaeffer, L., Wold, B. (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nature Methods **5**, 621-628.

Murray, B.E., Singh, K.V., Ross, R.P., Heath, J.D., Dunny, G.M., Weinstock, G.M. (1993) Generation of restriction map of *Enterococcus faecalis* OG1 and investigation of growth requirements and regions encoding biosynthetic function. Journal of Bacteriology **175**, 5216-5233.

Murray, B.E., Mederski-Samaroj, B. (1983) Transferable beta-lactamase. A new mechanism for *in vitro* penicillin resistance in *Streptococcus faecalis*. The Journal of Clinical Investigation **72**, 1168-1171.

Nachin, L., Loiseau, L., Expert, D., Barras, F. (2003) SufC: an unothodox cytoplasmic ABC/ATPase required for [Fe-S] biogenesis under oxidative stress. The EMBO Journal **22**, 427-437.

Nallapareddy S.R., Qin X., Weinstock G.M., Hook M., Murray B.E. (2000) *Enterococcus faecalis* adhesion, ace, mediates attachment to extracellular matrix proteins collagen type IV and laminin as well as collagen type I. Infection and Immunity **68**, 5218-5224.

Nallapareddy, S.R., Singh, K.V., Sillanpää, J., Garsin, D.A., Höök, M., Erlandsen, S.L., Murray, B.E. (2006). Endocarditis and biofilm-associated pili of *Enterococcus faecalis*. Journal of Clinical Investigation **116**, 2799-2807.

Nannini, E.C., Teng, F., Singh, K.V., Murray, B.E. (2005) Decreased virulence of a *gls24* mutant of *Enterococcus faecalis* OG1RF in an experimental endocarditis model. Infection and Immunity **73**, 7772-7774.

Nieto-Arribas, P., Sesena, S., Poveda, J.M., Chicón, R., Cabezas, L., Palop, L. (2011). *Enterococcus* population in artisanal Manchego cheese: Biodiversity, thenological and safety aspects. Food Microbiology **28**, 8991-899.

Noble, W.C., Virani, Z., Cree, R.G. (1992) Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus*. FEMS Microbiology Letters **72**, 195-198.

Olmsted, S.B., Kao, S.M., van Putte, L.J., Gallo, J.C., Dunny, G.M. (1991) Pole of the pheromone-inducible surfcase protein Asc10 in mating aggregate formation and

conjugal transfer of the *Enterococcus faecalis* plasmid pCF10. Journal of Bacteriology **173**, 7665-7672.

Palmer K.L., Gilmore M.S. (2010) Multidrug-resistant enterococci lack CRISPR-cas. MBio 1:4 10 e00227-10.

Palmer, K.L., Carniol, K., Manson, J.M., Heiman, D., Shea, T., Young, S., Zeng, Q., Gevers, D., Feldgarden, M., Birren, B., Gilmore, M.S. (2010) High-quality draft genome sequences of 28 *Enterococcus* sp. isolates. Journal of Bacteriology **192**, 2469-2470.

Patti, J.M., Allen, B.L., McGavin, M.J., Höök, M. (1994) MSCRAMM-mediated adherence of microorganisms to host tissues. Annual Reviews of Microbiology **48**, 585-617.

Paulsen, I.T., Banerjei, L., Myers, G.S.A., Nelson, K.E., Seshadri, R., Read, T.D., Fouts, D.E., Eisen, J.A., Gill, S.R., Heidelberg, J.F., Tettelin, H., Dodson, R.J., Umayam, L., Brinkac, L., Beanan, M., Daugherty, S., DeBoy, R.T., Durkin, S., Kolonay, J., Madupu, R., Nelson, W., Vamathevan, J., Tran, B., Upton, J., Hansen, T., Shetty, J., Khouri, H., Utterback, T., Radune, D., Ketchum, K.A., Dougherty, B.A., Fraser, C.M. (2003) Role of mobile DNA in the evolution of Vancomyci-resistant *Enterococcus faecalis*. Science **299**, 2071-2074.

Perkins, D.N., Pappin, D.J., Creasy, D.M., Cottrell, J.S. (1999) Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis **20**, 3551-3567

Pignogne, M., Greth, K.M., Cooper, J., Emerson, D., Tang, J. (2006) Identification of mycobacteria by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. Journal of Clinical Microbiology **44**,1963-1970.

Pribil, P., Fenselau, C. (2005) Characterization of enterobacteria using MALDI-TOF mass spectrometry. Analytical Chemistry **77**, 6092-6095.

Portenier, I., Watimo T.M.T., Haapasalo, M. (2003). *Enterococcus faecalis* -the root canal survivor and 'star' in post-treatment disease. Endodontic Topics **6**, 135-159.

Portillo, A., Ruiz-Larrea, F., Zarazaga, M., Alonso, A, Martinez, J.L., Torres, C. (2000) Macrolide resistance genes in *Enteroococcus* spp. Antimicrobial Agents an Chemotherapy **44**, 967-971.

Qin X., Singh K.V., Weinstock G.M., Murray B.E. (2000) Effects of *Enterococcus faecalis fsr* genes on production of gelatinase and a serine protease and virulence. Infection Immunity **68**, 2579-2586.

Rakita, R.M., Vanek, N.N., Jacques-Palaz, K., Mee, M., Mariscalco, M.M., Dunny, G.M., Snuggs, M., van Winkle, W.B. Simon, S.I. (1999) *Enterococcus faecalis* bearing aggregation substance is resistant to killing by human neutrophils despite phagocytosis and neutrophil activation. Infection and Immunity **67**, 6067-6075.

Reid, K.C., Cockerill III, F.R., Patel, R. (2001) Clinical and epidemiological features of *Enterococcus casseliflavus/flavescens* and *Enterococcus gallinarum* bacteremia: a

report of 20 cases. Clinical Infectious Diseases **32**, 1540-1546.

Riboldi, G.P., Verli, H., Frazzon, J. (2009) Structural studies of the *Enterococcus faecalis* SufU [Fe-S] cluster protein. BMC Biochemistry **10**:3

Rice, L.B., Eliopoulos, G.M., Wennersten, C., Goldmann, D., Jacoby, G.A, Moellering Jr. , R.C. (1991) Chromosomally mediated beta-lactamase production and gentamicin resistance in *Enterococcus faecalis*. Antimicrobial Agents and Chemotherapy **35**, 272-276.

Rich, R.L., Kreikemeyer, B., Owens, R.T., LaBrenz, S., Narayana, S.V.L., Weinstock, G.M., Murray, B.E., Höök, M. (1999) Ace is a collagen-binding MSCRAMM from *Enterococcus faecalis*. The Journal of Biological Chemistry **274**, 26939-26945.

Roberts, G., Homer, K.A., Tarelli, E., Philpott-Howard, J., Devriese, L.A., Beighton, D. (2001) Distribution of endo-beta-N-acetylglucosaminidase amongst enterococci. Journal of Medical Microbiology **50**, 620-626.

Sahm, D.F., Kissinger, J., Gilmore, M.S., Murray, P.R., Mulder, R., Solliday, J., Clarke, B. (1989) *In vitro* susceptibility studies of vancomycin-resistant *Enterococcus faecalis*. Antimicrobial Agents and Chemotherapy **33**, 1588-1591.

Salminen, S., Isolauri, E., Salminen, E. (1996) Clinical uses of probiotics for stabilizing the gut mucosal barrier: successful strains and future challenges. Antonie van Leeuwenhoek **70**, 347-358.

Sarantinopoulos, P., Kalantzopoulos, Tsakalidou, E. (2002) Effect of Enterococcus faecium on microbiological physicochemical and sensory characteristics of Greek Feta cheese. International Journal of Food Microbiology **76**, 93-105.

Sarantinopoulos, P., Kalantzopoulos, Tsakalidou, E. (2001) Citrate metabolism by *Enterococcus faecalis* FAIR-E 229. Applied and Environmental Microbiology **67**, 5482-5487.

Sartingen, S., Rozdzinski, E., Muscholl-Silberhorn, A., Marre, R. (2000) Aggregation substance increases adherence and internalization, but not translocation, of *Enterococcus faecalis* through different intestinal epithelial cells *in vitro*. Infection and Immunity **68**, 6044-6047.

Schilling, S.J., Birkhaug, K.E. (1927) Rheumatic fever: bacteriologic studies of a non-methemoglobin-forming *Streptococcus* with special reference to its soluble toxin production. Journal of Infectious Diseases **40**, 549-569.

Schleifer, K.H., Kilpper-Bälz, R. (1984). Transfer of *Streptococcus faecalis* and *Streptococcus faecium* to the genus *Enterococcus* nom. rev. as *Enterococcus faecalis* Bacteriology **34**, 31-34.

Schlievert, P.M., Gahr, P.J., Assimacopoulos, A.P., Dinges, M.M., Stoehr, J.A., Harmala, J.W., Hirt, H., Dunny, G.M. (1998) Aggregation and binding substances enhance pathogenicity in rabbit models of *Enterococcus faecalis* endocarditis. Infection and Immunity **66**, 218-223.

Semedo, T., Santos, M.A., Martins, P., Lopes, M.F.S., Figueiredo Marques, J.J., Tenreiro, R., Barreto Crespo, M.T. (2003) Comparative study using type strains and clinical and food isolates to examine hemolytic activity and occurrence of the *cyl* operon in enterococci. Journal of Clinical Microbiology **41**, 2569-2576.

Shankar, N., Coburn, P., Pillar, C., Haas, W., Gilmore, M.S. (2004) Enterococcal cytolysin: activities and association with other virulence traits in a pathogenicity island. International Journal of Medical Microbiology **293**, 609-618.

Shankar, N., Lockatell, C.V., Baghdayan, A.S., Drachenberg, C., Gilmore, M.S., Johnson, D.E. (2001) Role of *Enterococcus faecalis* surface protein Esp in the pathogenesis of ascending urinary tract infection. Infection and Immunity **69**, 4366-4372.

Shankar, V. Baghdayan, A.S., Huycke, M.M., Lindahl, G., Gilmore, M.S. (1999) Infection-derived *Enterococcus faecalis* strains are enriched in *esp*, a gene encoding a novel surface protein. Infection and Immunity **67**, 193-200.

Sherman, J.H. (1937). The Streptococci. Bacteriological Reviews **1**, 3-97.

Shevchenko, A., Sunyaev, S., Loboda, A., Bork, P., Ens, W., Standing, K.G. (2001) Charting the proteomes of organisms with unsequenced genomes by MALDI-quadrupole time-of-flight mass spectrometry and BLAST homology searching. Analyticyl Chemistry **73**,1917-1926

Sifri C.D., Mylonakis E., Singh K.V., Qin X., Garsin D.A., Murray B.E., Ausubel F.M., Calderwood S.B. (2002) Virulence effect of *Enterococcus faecalis* protease genes and the quorum-sensing locus *fsr* in *Caenorhabditis elegans* and mice. Infection and Immunity **70**, 5647-5650.

Singh, K.V., Nallapareddy, S.R., Nannini, E.C., Murray, B.E. (2005) Fsr-indipendent production of protease(s) may explain the lack of attenuation of an *Enterococcus faecalis* fsr mutant versus a *gelE-sprE* mutant in induction of endocarditis. Infection and Immunity **73**, 4888-4894.

Singh, K.V., Qin, X., Weinstock, G.M., Murray, B.E. (1998) Generation and testing of mutants of *Enterococcus faecalis* in a mouse peritonitis model. Journal of Infectious Diseases **178**, 1416-1420.

Snoep, J.L., Teixeira de Mattos, M.J., Starrenburg, M.J., Hugenholtz, J. (1992) Isolation, characterization, and physiological role of the pyruvate dehydrogenase complex and alpha-acetolactate synthase of *Lactococcus lactis* subsp. *lactis* bv. diacetylactis. Journal of Bacteriology **174**, 4838-4841.

Sorek, R., Kunin, V., Hugenholtz, P. (2008) CRISPR—a widespread system that provides acquired resistance against phages in bacteria and archaea. Nature Reviews Microbiology **6**, 181-186.

Steck, N., Hoffmann, M., Sava, I.G., Kim, S.C., Hahne, H., Tonkonogy, S.L., Mair, K., Krueger, D. Pruteanu, M., Shanahan, F., Vogelmann, R., Schemann, M., Kuster, B., Sartor, R.B., Haller, D. (2011) *Enterococcus faecalis* metalloprotease comprises epithelial barrier and contributes to intestinal inflammation. Gastroenterology **141**,

959-971.

Sutrina, S.L., McGeary, T., Bourne, C.A. (2007) The phosphoenolpyruvate:sugar phosphotransferase system and biofilms in Gram-positive bacteria. Journal of Molecular Microbiology and Biotechnology **12**, 269-272.

Süssmuth, S.D., Muscholl-Silberhorn, A., Wirth, R., Susa, M., Marre, R., Rozdzinski, E. (2000) Aggregation substance promotes adherence, phagocytosis, and intracellular survival of *Enterococcus faecalis* within human macrophages and suppresses respiratory burst. Infection and Immunity **68**, 4900-4906.

Teng, F., Nannini, E.C., Murray, B.E. (2005) Importance of gls24 in virlunce and stress response of *Enterococcus faecalis* and use of the Gls24 protein as a possible immunotherapy target. The Journal of Infectious Diseases **191**, 472-480.

Templer, S.P., Baumgartner, A. (2007) Enterococci from Appenzeller nd Schabziger raw milk cheese: antibiotic resistance, virulence factors, and persistence of particular strains in the products. Journal of Food Protection **79**, 450-455.

Thiercelin, M.E. and Jouhaud, L. (1903). Reproduction de l'entérocoque; taches centrales; granulations péripheriques et microblastes. C.R. **55**, 686 -688.

Thiercelin, M.E. (1899). Sur un diplocoque saprophyte de l'intestin susceptible de devenir pathogène. *C.R.* Séances Société Biologie **5**, 269 -271.

Toledo-Arana, A., Valle, J., Solano, C., Arrizubieta, M.J., Cucarella, C., Lamata, M., Amorena, B., Leiva, J., Penades, J.R., Lasa, I., (2001) The enterococcal surface protein, ESP, is involved in *Enterococcus faecalis* biofilm formation. Applied and Environmental Microbiology **67**, 4538-4545.

Uttley, A.H.C., Collins, C.H., Naidoo, J., George, R.C. (1988) Vancomycin-resistant enterococci. The Lancet **331**, 57-58.

Vebø, H.C., Solheim, M., Snipen, L., Nes, I.F., Brede, D.A. (2010) Comparative genomic analysis of pathogenic and probiotic *Enterococcus faecalis* isolates, and their transcriptional responses to growth in human urine. PLoS One **5**(8): e12489.

Vebø, H.C., Snipen, L., Nes, I.F., Brede, D.A. (2009) The transcriptome of nosocomial pathogen *Enterococcus faecalis* V583 reveals adaptive responses to growth in blood. PLoS One **4**(11): e7660

Vergis, E.N., Shankar, N., Chow, J.w., Hayden, M.K., Snydman, D.R., Zervos, M.J., Linden, P.K., Wagener M.M., Muder, R.R. (2002). Association between the presence of enterococcal virulence factors gelatinase, hemolysin, and enterococcal surface protein and mortality among patients with bacteremia due to *Enterococcus faecalis*. Clinical Infectious Diseases **35**, 570-575.

Wang, X., Huycke, M.M. (2007) Extracellular superoxide production *by Enterococcus faecalis* promotes chromosomal instability in mammalian cells. Gastroenterology **132**, 551-561.

Ward, D.E., van der Weijden, C.C., van der Merwe, M.J., Westerhoff, H.V., Claiborne, A.,

Snoep, J.L. (2000) Journal of Bacteriology **182**, 3239-3246.

Ward, D.E., Ross, R.P., van der Weijden, C.C., Snoep, J.L., Claiborne, A. (1999) Catabolism of branched-chain alpha-keto acids in *Enterococcus faecalis*: the *bkd* gene cluster, enzymes, and metabolic route. Journal of Bacteriology **181**, 5433-5442.

Weigel, L.M., Clewell, D.B., Gill, S.R., Clark, N.C., McDougal, L.K., Flannagan, S.E., Kolonay, J.F., Shetty, J., Killgore, G.E., Tenover, F.C. (2003) Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. Science **302**, 1569-1571.

Wells, C.L., Moore, E.A., Hoag, J.A., Hirt, H., Dunny, G.M., Erlandsen, S.L. (2000) Inducible Expression of *Enterococcus faecalis* aggregation substance surface protein facilitates bacterial internalization by cultured enterocytes. Infection and Immunity **68**, 7190-7194.

Wells, C.L., Jechorek, R.P., Erlandsen, S.L. (1990) Evidence for the translocation of *Enterococus faecalis* across the mouse intestinal tract. The Journal of Infectious Diseases **162**, 82-90.

Whitehead, R.H., Robinson, P.S., Williams, J.A., Bie, W., Tyner, A.L., Franklin, J.L. (2008) Conditionally immortalized colonic epithelial cell line from a Ptk6 null mouse that polarizes and differentiates *in vitro*. Journal of Gastroenterology and Hepatology **23**, 1119-1124.

Whitehouse, N.L., Olson, V.M., Schwab, C.G., Chesbro, W.R., Cunningham, K.D., Lykos, T. (1994) Improvement techniques for dissociating particle-associated mixed ruminal microorganisms from ruminal digesta solids. Journal of Animal Science **72**, 1335-1343.

WHO/FAO joint expert consolidation on evaluation of health nutritional properties of probiotics in food including powder milk with live lactic acid bacteria, October 2001 (http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf)

Wunderlich, P.F., Braun, L., Fumagalli; I., D'Apuzzo, V., Heim, F., Karly, M., Lodi, R., Politta, G., Vonbank, F., Zeltner, L. (1989) Double-blind report on the efficacy of lactic acid-producing *Enterococcus* SF68 in the prevention of antibiotic-associated diarrhoe and in the treatment of acute diarrhoea. Journal of International Medical Reasearch **17**, 333-338.

Xiao, J., Höök, M., Weinstock, G.M., Murray, B.E. (1998) Conditional adherence of *Enterococcus faecalis* to extracellular matrix proteins. FEMS Immunology & Medical Microbiology **21**, 287-295.

Xiong, T., Guan, Q., Song, S., Hao, M., Xie, M. (2012) Dynamic changes of lactic acid bacteria flora during Chinese sauerkraut fermentation. Food Control **23**, 178-181.

Xu, Y., Singh, K.V., Qin, X., Murray, B.E., Weinstock, G.M. (2000) Analysis of a gene cluster of *Enterococcus faecalis* involved in polysaccharide biosynthesis. Infection and Immunity **68**, 815-823.

Xu, Y., Murray, B.E., Weinstock, G.M. (1998) A cluster of genes involved in

polysaccharide biosynthesis from *Enterococcus faecalis* OG1RF. Infection and Immunity **66**, 4313-4323.

Zareba, T.W., Pascu, C. Hryniewicz, W., Wadström, T. (1997) Binding of extracellular matrix proteins by enterococci. Current Microbiology **34**, 6-11

Zeng, J., Teng, F., Weinstock, G.M., Murray, B.E. (2004) Translocation of *Enterococcus faecalis* strains across a monolayer of polarized human enterocyte-like T84 cells. Journal of Clinical Microbiology **42**, 1149-1154.

Zervos. M.J., Kauffman, C.A., Therasse, P.M., Bergman, A.G., Mikesell, T.S., Schaberg, D.R. (1987) Nosocomial infection by gentamicin-resistant *Streptococcus faecalis*. An epidemiologic study. Annals of Internal Medicine **106**, 687-691.

8 Appendix

Prolab® RMH 3500, Autoclavable 5P04*

DESCRIPTION

Prolab® Rat/Mouse/Hamster 3500 is an autoclavable diet formulated for growth and reproduction. It is the companion product of Prolab® RMH 3000. Protein, mineral and vitamin levels have been fortified and carefully balanced so that maximum food values are maintained after sterilization. It can also be pasteurized or sterilized without clumping. This diet is formulated using the unique and innovative concept of Constant Nutrition®, paired with the selection of highest quality ingredients to assure minimal inherent biological variation in long-term studies.

Features and Benefits

- Constant Nutrition® formula helps minimize nutritional variables
- Processed and packaged for autoclaving
- Supports optimum growth and efficient reproduction performance of rats, hamsters and mice
- Fortified with extra nutrients to compensate for losses during autoclaving
- Processed with silicon dioxide to reduce sticking and clumping

Product Forms Available

- Oval pellet, 10 mm x 16 mm x 25 mm length (3/8"x5/8"x1")

GUARANTEED ANALYSIS

Crude protein not less than	22.0%
Crude fat not less than	5.0%
Crude fiber not more than	5.0%
Ash not more than	6.0%
Added minerals not more than	2.5%

AUTOCLAVING SUGGESTIONS

To autoclave the pellets, place on trays, in small bags, or in larger bags, to a depth of no more than 3 inches. When steam autoclaved, the pellets swell and exert force on adjacent pellets. Confinement by a bag or container creates additional pressure, which may result in sticking. ***Assay before and after autoclaving:*** Conditions of sterilization must be determined for each autoclaving unit. Microbiological evaluation should be done to insure sterilization is achieved. It is best to assay the diet before and after sterilization to determine nutrient losses.

INGREDIENTS

Ground wheat, soybean meal, wheat middlings, ground yellow corn, fish meal, soybean hulls, soybean oil, alfalfa meal, calcium lignin sulfonate, calcium carbonate, brewers dried yeast, salt, dicalcium phosphate, silicon dioxide, DL-methionine, L-lysine, manganous oxide, magnesium oxide, ferrous sulfate, zinc oxide, copper sulfate, calcium iodate, cobalt carbonate, vitamin A acetate, cholecalciferol, dl-alpha tocopheryl acetate, vitamin B_{12} supplement, riboflavin, nicotinic acid, calcium pantothenate, menadione dimethylpyrimidinol bisulfite (source of vitamin K), folic acid, pyridoxine hydrochloride, thiamin mononitrate, biotin, choline chloride.

FEEDING DIRECTIONS

Prolab® RMH 3500, Autoclavable is designed for growth and reproduction of rodents. It contains all the nutrients that are required for growth, lactation, and reproduction. This diet should be fed free choice in a self-feeder. Keep a constant supply of fresh water available.

Rats- All rats will eat varying amounts of feed depending on their genetic origin. Larger strains will eat up to 30 grams per day. Smaller strains will eat up to 15 grams per day. Feeders in rat cages should be designed to hold two to three days supply of feed at one time.

Mice-Adult mice will eat up to 5 grams of pelleted ration daily. Some of the larger strains may eat as much as 8 grams per day per animal. Feed should be available on a free choice basis in wire feeders above the floor of the cage.

Hamsters-Adults will eat up to 14 grams per day.

Important: A feeding program is only as effective as the management practices followed.

Caution: Store in a dry, well ventilated area, free of pests and insects. Do not use moldy or insect-infested feed.

12/11/09

CHEMICAL COMPOSITION[1]

Nutrients[2]	
Protein, %	**22.5**
Arginine, %	1.32
Cystine, %	0.29
Glycine, %	1.12
Histidine, %	0.53
Isoleucine, %	1.16
Leucine, %	1.66
Lysine, %	1.35
Methionine, %	0.51
Phenylalanine, %	1.01
Tyrosine, %	0.61
Threonine, %	0.83
Tryptophan, %	0.30
Valine, %	1.15
Serine, %	1.16
Aspartic Acid, %	2.31
Glutamic Acid, %	5.07
Alanine, %	1.20
Proline, %	1.63
Taurine, %	0.03
Fat (ether extract), %	**5.9**
Fat (acid hydrolysis), %	**6.7**
Cholesterol, ppm	265
Linoleic Acid, %	2.72
Linolenic Acid, %	0.34
Arachidonic Acid, %	0.00
Omega-3 Fatty Acids, %	0.65
Total Saturated Fatty Acids, %	1.19
Total Monounsaturated Fatty Acids, %	1.29
Fiber (Crude), %	**4.8**
Neutral Detergent Fiber[3], %	15.0
Acid Detergent Fiber[4], %	6.4
Nitrogen-Free Extract (by difference), %	**50.1**
Starch, %	31.3
Glucose, %	0.1
Fructose, %	0.1
Sucrose, %	0.8
Lactose, %	0.0
Total Digestible Nutrients,%	**79.4**
Gross Energy, kcal/gm	**4.10**
Physiological Fuel Value[5], kcal/gm	**3.43**
Metabolizable Energy, kcal/gm	**3.19**

Minerals	
Ash, %	**6.7**
Calcium, %	1.17
Phosphorus, %	0.76
Phosphorus (non-phytate), %	0.51
Potassium, %	0.82
Magnesium, %	0.21
Sulfur, %	0.24
Sodium, %	0.27
Chlorine, %	0.47
Fluorine, ppm	20
Iron, ppm	400
Zinc, ppm	120
Manganese, ppm	130
Copper, ppm	13
Cobalt, ppm	0.27
Iodine, ppm	0.99
Chromium, ppm	1.5
Selenium, ppm	0.28

Vitamins	
Carotene, ppm	2.4
Vitamin K (as menadione), ppm	1.8
Thiamin Hydrochloride, ppm	90
Riboflavin, ppm	15
Niacin, ppm	80
Pantothenic Acid, ppm	31
Choline Chloride, ppm	1500
Folic Acid, ppm	2.9
Pyridoxine, ppm	6.4
Biotin, ppm	0.33
B_{12}, mcg/kg	73
Vitamin A, IU/gm	30
Vitamin D_3 (added), IU/gm	2.2
Vitamin E, IU/kg	75
Ascorbic Acid, mg/gm	—

Calories provided by:	
Protein, %	26.201
Fat (ether extract), %	15.459
Carbohydrates, %	58.340

*Product Code

1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly.
2. Nutrients expressed as percent of ration except where otherwise indicated. Moisture content is assumed to be 10.0% for the purpose of calculations.
3. NDF = approximately cellulose, hemi-cellulose and lignin.
4. ADF = approximately cellulose and lignin.
5. Physiological Fuel Value (kcal/gm) = Sum of decimal fractions of protein, fat and carbohydrate (use Nitrogen Free Extract) x 4,9,4 kcal/gm respectively.

LabDiet
www.labdiet.com

Figure 1: Mouse feed, standard autoclavable diet Prolab® RMH 3500 for LabDiet® PMI Nutrition International, Purina Mills, Inc Richmond, Indiana, USA

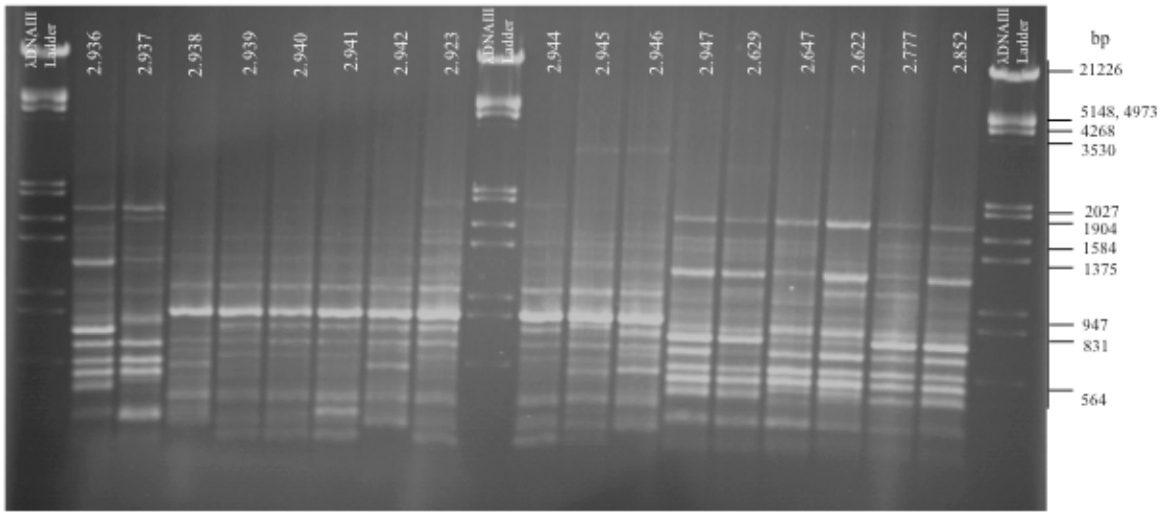


Figure 2: RAPD-PCR pattern of *Enterococcus* isolates 2.936, 2.937, 2.938, 2.939, 2.940, 2.941, 2.942, 2.923, 2.944, 2.945, 2.946, 2.947, 2.629, 2.647, 2.622, 2.777, 2.852

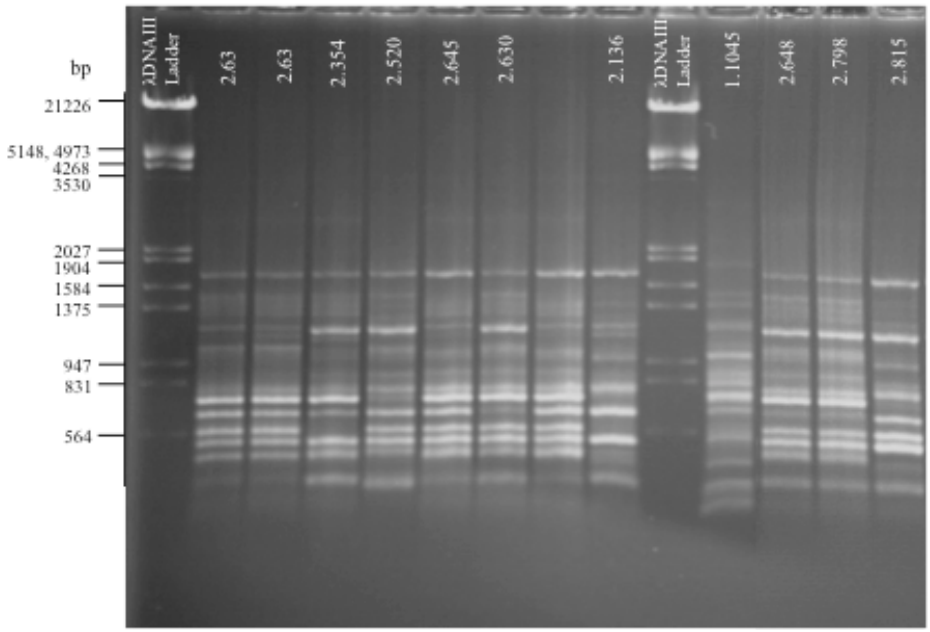


Figure 3: RAPD-PCR pattern of *Enterococcus* isolates 2.63, 2.354, 2.520, 2.645, 2.630, 2.136, 1.1045, 2.648, 2.798, 2.815

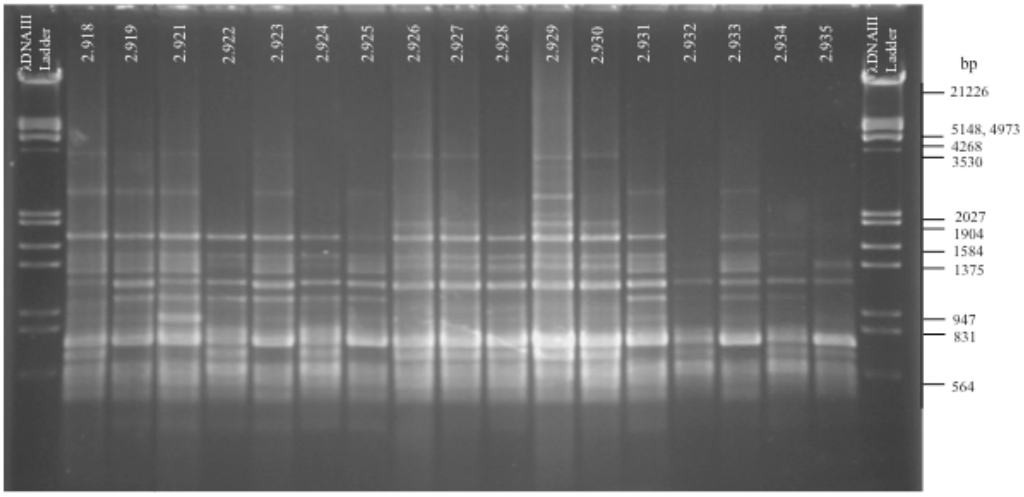


Figure 3: RAPD-PCR pattern of Enterococcus isolates 2.918, 2.919, 2.921, 2.922, 2.923, 2.924, 2.925, 2.926, 2.927, 2.928, 2.929, 2.930, 2.931, 2.932, 2.933, 2.934, 2.935

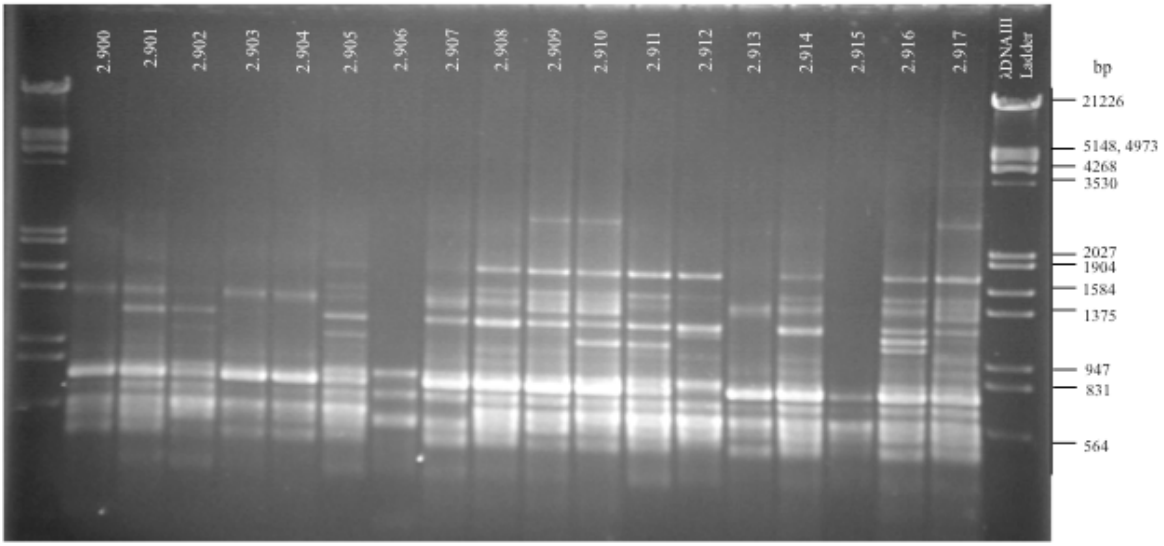


Figure 3: RAPD-PCR pattern of Enterococcus isolates 2.900, 2.901, 2.902, 2.903, 2.904, 2.905, 2.906, 2.907, 2.908, 2.909, 2.910, 2.911, 2.912, 2.913, 2.914, 2.915, 2.916, 2.917