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Insights in translocation through the intestinal barrier and mechanisms of persistance in the intestinal tract of *Enterococcus faecalis* OG1RF

Zhen Peng

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Abbreviations

3-D	3-dimensional
°C	Degree
A	Ampere
ADI	Arginine deiminase
API	Analytical profile index
APP.	Appendix
ATCC	American type culture collection, Manassas, Virginia, USA
BHI	Brain heart infusion broth
BLAST	Basic local alignment search tool
Bromophenol blue	3',3",5',5"-tetrabromophenolsulfonphthalein
BSA	Bovine serum albumin
CcpA	Catabolite control protein A
CCR	Carbon catabolite repression
cDNA	Complementary DNA
Cre	Catabolite repression element
CFU	Colony-forming unit
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
DMEM	Dulbecco´s modified Eagle´s medium
DNA	Desoxyribonucleic acid
DTT	Dithiothreitol
E.	*Enterococcus*/ *Escherichia*
EI	Enzyme I
EII	Enzyme II
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
E_m^R	Erythromycin resistance
FCS	Fetal calf serum
Fig.	Figure

g	Gram
GFP	Green fluorescent protein
h	Hour (s)
HPr	Histidine-containing phosphocarrier protein
INF-γ	Interferon gamma
IPTG	Isopropyl β-D-1-thiogalactopyranoside
k	Kilo (10^3)
L	Liter
L.	Lactobacillus
LCCM	L929-cell conditioned medium
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LSCM	Laser scanning confocal microscope
m	Milli (10^{-3}), meter
M	Mol/L
M-CSF	Macrophage colony stimulating factor
MRS	de Man, Rogosa and Sharpe
min	Minute (s)
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
Mw	Molecular weight
NCBI	National center for Biotechnology Information
OD	Optical density
p.a.	Pro analysis (chemical purity grade)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PTS	Phosphotransferase system
qRT-PCR	Quantitative reverse transcription PCR
rpm	Rounds per minute
SDS	Sodium n-dodecylsulfate
T	Time
Tab.	Table

TE	Tris-EDTA
TEMED	N,N,N´,N´-tetramethyl-ethylendiamine
TMW	Technische Mikrobiologie Weihenstephan
Tris	Tris (hydroxymethyl) aminomethan
U	Unit(s)
V	Volt
v/v	Volume/volume
W	Watt
w/v	Weight/volume
w/w	Weight/weight
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside
µ	Micro

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1 Introduction

1.1 *Enterococcus faecalis*

Enterococci are a genus of lactic acid bacteria of the phylum firmicutes. Prior to that enterococci were categorized to group D streptococci. There were four varieties of streptococci (Sherman, Stark, & Mauer, 1936): *S. fecalis* (hemolysis-, proteolysis-), *S. faecalis* var. hemolyticus (hemolysis+, proteolysis-), *S. fecalis* var. liquefaciens (hemolysis-, proteolysis+), and *S. faecalis* var. zymogenes (hemolysis+, proteolysis+). Later on, Schleifer and Bälz suggested *Streptococcus* (*S.*) *faecalis* and *S. faecium* should be transferred to the genus "*Enterococcus*", of which *S. faecalis* refers to *Enterococcus* (*E.*) *faecalis* (Kilpper-bälz, 1984). It is one of the common species that ranks top three causations of nosocomial infection. *E. faecalis* was described as non-hemolytic, having strong reducing action on neutral red, and coagulating milk (Andrewes & Horder, 1906). *E. faecalis* is a gram-positive, facultative anaerobic bacterium that is able to cope with complicated growing environments. For example, it maintains growth ability in following conditions: temperature ranging from 0 to 50 °C; after the treatment of 60°C for 30 min in a neutral medium (Gardin et al., 2001); in a broad range of pH value (pH 5.5-pH 9.6). The ability to survive in broth with 6.5% NaCl and pH 9.6 is commonly used as physiological criterion for distinguishing *E. faecalis* from other streptococci (Sherman, 1937). It displays resistance to 40% bile salts owning to the esculin hydrolase (Gilmore, 2002). Biophysical profiles of *E. faecalis* are characterized. Under normal culture condition, *E. faecalis* is single, or forms typically in chain or in pairs and under certain growth conditions appears to be coccobacillary, some are known to be motile (Fig. 1). Certain enterococci display the group D lancefield antigen and can be detected by monoclonal antibody-based agglutination tests (Sherman, 1937). Besides, *E. faecalis* is pyrrolidonyl-β-naphthylamide hydrolyzable, chemoorganotroph, metabolism fermentative, benzidine negative and usually catalase negative. The predominant end product of glucose fermentation is L-lactic acid (Kilpper-bälz, 1984). At least genomes of 45 strains of *E. faecalis* are currently established. The genomes of two typical strains OG1RF and V583 are most frequently studied and cited. The genomes of *E. feacalis* OG1RF and *E. faecalis* V583 are estimated to be 2,740 kb and 3,218 kb with identical G+C content (38%). Several important features in V583 like pathogenicity island, *van*B (vancomycin B) transposon, 25% mobile DNA and prophages are missing in OG1RF (Giridhara Upadhyaya, Ravikumar, & Umapathy, 2009). The diverse strains make it not categorical to define *E. faecalis* as pathogen or probiotic. It is known that some strains of *E. faecalis* is an everlasting disturbing fiend to the medical cure because of its stubborn survival ability e.g. resistance to multiple antibiotics,

and virulence genes, which are able to transfer in horizontal level by making use of plasmid as vector. To thoroughly investigate the pathogenesis of enteorcocci in epidemiology, *E. faecalis* OG1RF was developed as the relatively safe and often used experimental strain, owing to that it is not resistant to commonly used antibiotics (except for gentamicin, penicillin G, rifampicin and fusidic acid) and it carries no plasmid, which makes it amenable for genetic manipulations. *E. faecalis* OG1RF is often considered as commensal, but its original strain OG1X could be detected in clinical infections (Bourgogne et al., 2008). *E. faecalis* V583 is frequently detected in clinical disease outbreaks and is therefore classified as pathogen (Vebø, Snipen, Nes, & Brede, 2009). *E. faecalis* Symbioflor® is however a probiotic, commercially used for treatment of respiratory infections and training of the immune system (Christoffersen et al., 2012).

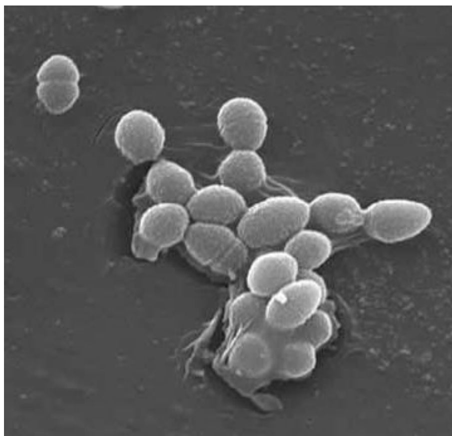


Fig. 1 *Enterococcus faecalis*.
Photo by: Pete Wardell/CDC

1.2 Distribution ecology of *E. faecalis*

E. faecalis usually inhabits in the gastrointestinal tract, vaginal and oral tracts (R Beargie, 1975; Smyth, Halpenny, & Ballagh, 1987) of human beings. A few were also found in insects and warm-blooded animals like cattle, dog, chicken, sheep, swine, wild bird and rabbit, which indicates that *E. faecalis* is peculiar to certain internal environments (Van Tyne, Martin, & Gilmore, 2013; Wheeler, Hartel, Godfrey, Hill, & Segars, 1991). In the human intestine, the density of enterococci is up to 10^6 CFU per gram of small intestinal content (Goldin, 2005) and typical density of enterococci in stool ranges from 10^5 to 10^7 CFU per gram of feces, accounting for less than 0.01% of the total bacterial flora in intestine, which is normal for healthy organisms (Jett, Huycke, & Gilmore, 1994). The persistence of *E. faecalis* in the aquatic environment is high (Bonjoch, García-Aljaro, & Blanch, 2011). American water works association use enterococci as a bacterial indicator to estimate fecal contamination in surface waters. Water quality guidelines based on enterococcal density have been proposed for recreational waters (Clesceri, 1998). 100 mL marine water containing less than 35 (CFU) enterococci is defined as not polluted (Agency, 2012). Many bacteria-carriers like patients, medical apparatusses and instruments could harbor and transport *E. faecalis*, which makes *E. faecalis* a stubborn hospital-derived pathogeny. It is on record that *E. faecalis* could be isolated from harsh environments owing to the resistance to physical and chemical agents such as UV radiation, heavy metals, ethanol, azide, detergents, bile salts, and sodium hypochlorite (Noble, Virani, & Cree, 1992; De Niederhäusern et al., 2013; J. C. Giard et al., 2001; Kakinuma, 1998). There are evidences that they could even settle down in sewage, soil, sea water, plants, poultry, dairy and meat products (Foulquié Moreno, Sarantinopoulos, Tsakalidou, & De Vuyst, 2006). Enterococci merely survive in the harsh environment and await opportunities to propagate and infect underlying host.

Distribution of enterococcal strains is geography-dependent (Rivas et al., 1997) and the presence of enterococcal strains varies in different hosts and environments (Wheeler et al., 1991). Even in the same host, the inner environmental modifications could lead to increase or decrease of the bacteria. For example, *E. faecalis* increased with age in the intestine of chicken (Devriese, Kerckhove, Kilpper-balz, & Schleifer, 1987).

1.3 Opportunistic pathogens

Human beings are surrounded and inhabited by a variety of bacteria from birth to death. Unlike probiotics, which sustainably endow their host with beneficial effects, or obligate pathogens, which instantly attempt to develop undesirable diseases, opportunistic pathogens can colonize and propagate in healthy humans or animals without doing any harm as long as they do not exceed the resistance of the host. This character makes opportunistic pathogens not be neglected in disease prevention and precaution. Generally, opportunistic pathogens refer to bacteria, which cause diseases in compromised hosts that follow a perturbation (e.g. disease, wound, medication, prior infection, immune deficiency and ageing) but normally do not occur in the healthy hosts (Brown, Cornforth, & Mideo, 2012). A relatively complete and mature hypothesis about how opportunistic pathogens evolved to cope with gross stimuli is the plasticity theory: it is only the challenge of frequent exposure to distinct environments that selects for adaptive phenotypic plasticity, in which case the benefits of adaptive plasticity outweigh the likely costs of the machinery necessary to generate such plasticity (Brown et al., 2012; Dewitt, Sih, & Wilson, 1998). The endogenous symbiotic bacteria and external environmental acquired microbes could be the origins of opportunists. Once opportunists reside in their host, they remain dormant and play an anonymous role for the most part of their lives. However, opportunistic pathogens are not loyal to their hosts. They learn to face tough defiance in hosts (e.g. low pH, oxidative stress) during dwelling by stimulating survival mechanisms and virulence genes, a strategy that allows commensal growth with hosts but also starts riots in case of any possible chance.

E. faecalis OG1RF is a facultative pathogen of the human intestinal tract that can cause disorders when homeostasis is unbalanced. There are several conditions that could be used by enterococci as the opportunity to intrude organisms: 1, dysfunction of immune system. Colonization of *E. faecalis* OG1RF ($10^{9.6}$ CFU) in the cecal contents was found to induce severe colitis (histology score: 3.6±0.2) in IL-10 deficient mouse, but not in wild-type mouse (histology score: 0.5±0.2) (Hoffmann, Kim, Sartor, & Haller, 2009; Ruiz, Shkoda, Kim, Sartor, & Haller, 2006). The underlying diseases such as cancer, diabetes, side effects of certain medical therapies, age (young and old) and malnutrition could cause dysfunction of immune system; 2, intestinal microbes out of balance. The use of broad-spectrum antibiotics could result in the decrease of most microbes and surviving microbes will overpopulate the system. A broad-spectrum antibiotic beneficial to dominant growth of enterococci resulted in its distribution in mesenteric lymph nodes, liver, and spleen whereas cecum and colon were still home courts (Jett et al., 1994; Wells & Erlandsen, 1991); 3, trauma formation. Skin

damages and surgical wounds introduce microbes to an area of the body that is axenic or that they do not normally emerge (Brown et al., 2012). Damaged gut is inclined to develop mucosal inflammation due to the loose junction, which allows *Bacillus, Staphylococcus* and *Phenylobacterium* to pass the barrier (T. Chen et al., 2013). An example of enterococci switching from commensal to pathogen in *Manduca sexta* was displayed by Mason and his coworkers. Larvae of *Manduca sexta* were reared on antibiotic food to clear the midgut microbiota prior to all experiments. *E. faecalis* OG1RF induced no morbidity or death when early-5th-instar larvae were forced to orally administrate *E. faecalis* (10^8 CFU), but induced death in a dose-dependent manner when *E. faecalis* OG1RF was injected into the hemocoel of larvae (Mason, Stepien, & Blum, 2011). In another case, 1-day-old chickens orally fed with *E. faecalis* isolates did not result in any pathological changes, but were found to develop growth retardation and arthritis when *E. faecalis* (10^6 CFU) were inoculated into muscle (Landman, Veldman, Mevius, & Doornenbal, 2000). These examples indicate that to conquer intestinal barrier and translocate into blood tissues, muscles or other organs is crucial for successful infection.

1.4 Virulence of *E. faecalis*

Enterococci are the leading cause of surgical sites infection (17.1%), second foremost origin of nosocomial bacteremia (11.5%) and third leading cause of urinary tract infections (14.3%) in the United States (Hancock & Gilmore, 2006; Pillar & Gilmore, 2004; Poulsen, Bisgaard, Son, Trung, & An, 2012). Enterococci could cause disease like endocarditis, meningitis, urinary tract infection and so on. Approximately 85 to 90% of enterococcal infections attribute to *E. faecalis* and 5 to 10% attribute to *E. faecium*. Infections caused by other enterococcus species (*E. durans*, *E. avium*, *E. raffinosus*, *E. gallinarum*, and *E. casseliflavus*) occasionally emerge and have gained attention (Simjee, Manzoor, Fraise, & Gill, 2000). Once bacteria conquer the intestinal barrier in host, many attributes with regard to survival and persistence are engaged to achieve advantage in the niche by self-protection, accompanying offensive defending mechnisms against other bacterial species because the "battlefield" is much more harsh than that of colonization sites (Giridhara Upadhyaya et al., 2009). There are more than 15 virulence genes reported in enterococci, which were isolated from food of animal origin (Diarra et al., 2010; Valenzuela et al., 2009). Most-cited virulence factors in *E. faecalis* are aggregation substance, surface adhesin, sex pheromones, lipoteichoic acid, extracellular superoxide production, the lytic enzymes gelatinase and hyaluronidase, and the toxin cytolysin (Kayaoglu & Orstavik, 2004). In the genome of *E. faecalis* V583, a

consecutive DNA as large as 150 kb was found to embrace the majority of virulence genes, which is named pathogenicity island. Pathogenicity island is a term to describe a large section (20 kb to 200 kb and up) of genome, where virulence factors, colonization-promoting factors, and virulence delivery factors congregate (Pillar & Gilmore, 2004). Pathogenicity islands are found in many other pathogenic bacteria such as *Escherichia* (*E.*) *coli*, *Shigella* (*S.*) *flexneri*, and *Salmonella* (*S.*) *enterica* (Hacker & Kaper, 2002; Marcus, Brumell, Pfeifer, & Finlay, 2000). Genomic study reveals that no such pathogenicity island was found in *E. faecalis* OG1RF. Virulence genes in *E. faecalis* OG1RF distribute dispersedly along the genome (Bourgogne et al., 2008).

1.5 Resistance to antibiotics and bacteriocins

A profound factor that contributes to the survival of bacteria in a complex environment is the resistance to a variety of antibiotics and bacteriocins. The enhanced capability of survival in tough niches raises the risk of bacterial infection. *E. faecalis* M20 being administrated to streptomycin-treated mice was found to overgrow in intestine and cause infections in mesenteric lymph nodes, liver, and spleen (Wells, Jechorek, & Erlandsen, 1990). The use of antimicrobials in livestock rearing environments to prevent zoonosis has the potential to sift out certain antibiotic-resistant pathogens and leave over latent danger afterwards. It has been speculated that the extensive use of virginiamycin (analogue of quinupristin-dalfopristin) in animal husbandry may have contributed to the 14% quinupristin-dalfopristin-resistant *E. faecium* human carrier (Hayes et al., 2001; Werner et al., 2000). Similarly, quinupristin-dalfopristin-resistant enterococci were also isolated from fecal samples of chickens and pigs at slaughterhouses in korea due to misuse of antibiotics (Hwang et al., 2009). To avoid further misgivings, virginiamycin was forbidden to be used as excipient in forage in Europe since 1999. Although virginiamycin was banned in 1998 in Denmark, persistence of virginiamycin-resistant enterococci in pigs was still up to 22.5% after two years of ban. This could derive from enterococci that subjected to other antimicrobial agents like erythromycin, tetracycline, and streptomycin, which could co-select for virginiamycin-resistant bacteria (Aarestrup, Seyfarth, Pedersen, Hendriksen, & Emborg, 2001; Simjee et al., 2002). Enterococci become the third most common pathogenic bacteria after *E. coli* and *Staphylococcus* (*S.*) *aureus* at hospitals partly owing to the acquired multi-drug-resistance, which causes serious problem in the management of patients (Gilmore, 2002). Previously, the sensitive enterococcal strains could be killed by vancomycin whereas nowadays a catalogue of vancomycin-resistant enterococci developed: vanA, vanB, and vanC, of which the former two are most threatening

for the strong resistance and readily transfer on a plasmid (Smith, Skilling, Cherry, Mead, & Matson, 1998). It is therefore suggested that misapplication of multiple antibiotics against enterococci as a quick and routine solution should stop.

Some enterococci maintain dominant growth by producing bacteriocins against competitive bacteria in a micropopulation (Hata et al., 2009; Toit, Franz, Dicks, & Holzapfel, 2000; Yamamoto, Togawa, Shimosaka, & Okazaki, 2003). In return, to prevent the inhibition effects of bacteriocin produced by competitive bacteria, enterococci learned to impede the approach of bacteriocin by modifying (mutating) the receptor for bacteriocins (Opsata, Nes, & Holo, 2010). Mannose PTS as the target receptor for class II bacteriocin (pediocin, lactococcin, microcin) was characterized one after another, indicating that PTS is involved in bacterial bacteriocin-resistance and persistence in niches (Bieler, Silva, Soto, & Belin, 2006; Diep, Skaugen, Salehian, Holo, & Nes, 2007; Ramnath, Arous, Gravesen, Hastings, & Héchard, 2004).

1.6 Aggregation of yeast mediated by bacteria

Binding to a mannose-containing receptor of epithelial cells is one important step for pathogens to colonize the gastrointestinal tract. Thus the ability of bacteria to generate agglutination of mannose-producing yeast is one criterion for virulence evaluation. Species of yeast produce more than 180 polysaccharides with mannose as the major constitute. Methyl α-D-mannoside as an analogue of mannose could inhibit yeast agglutination, which in return attests to the mechanism of agglutination to be due to mediation by mannose. Some specific adhesins in bacteria targeting mannose in yeast were identified and characterized such as lectin in *E. coli*, MSA (encoded by lp_1229) in *L. plantarum* WCFS1, and SasA in *S. aureus* (Ofek, Mirelman, & Sharon, 1977; Pretzer, Snel, Molenaar, Wiersma, Bron, Lambert, Vos, Meer, A., et al., 2005; Roche, 2003). These mannose-binding adhesins however are not present in all bacteria. *E. faecalis* OG1RF as an opportunistic pathogenic bacterium is presumed to own ability to bind mannose. Mannose/fructose/sorbose phosphotransferase system as the transporter of mannose, whose influence on mannose-binding, i.e., yeast agglutination remains to be investigated.

1.7 Adhesion mediators and moonlighting proteins

The essential ability of pathogenic bacteria to adhere to cellular surface is the basic premise to colonize a host before any further activities such as propagation, toxic substances secretion,

resistance to the response of host system. The tactic of pathogens to anchor host cellular surface is diverse. Similar to Gram-negative microorganisms, which utilize adhesin at the tip of pili to enhance contact with target, some enterococci also own the ability to generate pili as the driving force to orient to objects (Nallapareddy et al., 2006). Moreover, *E. faecalis* possesses abundant adhesins in cell membrane, which are in charge of host-binding. These adhesins comprise moonlighting proteins interpreted below. In turn, host cell as bacterial target provides receptors like fibrinogen, collagen and vitronectin for bacteria to land (Gailit & Clark, 1996). Integral host membrane is constructed by integrins, cadherins, selectins, and antigen-related adhesion molecules (CEACAMs), which are responsible for the integrality of cell structure meanwhile represent the receptor for bacterial adhesion and invasion (Linke & Goldman, 2011). The strategy of bacterial invasion: invasion can be achieved by direct interaction with receptors on the surface of host cells or by transporting bacterial virulence factors into the host-cell cytosol that will promote rearrangements of the plasma membrane architecture, and induce pathogen engulfment (Pizarro-Cerdá & Cossart, 2006). Three (Fss1, Fss2 and Fss3) of the predicted MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) in *E. faecalis* V583 were identified to bind fibrinogen (Sillanpää et al., 2009). A putative collagen-binding protein (Ace) in *E. faecalis* isolates was identified by bioinformatics (Rich et al., 1999). Protein EfbA in *E. faecalis* JH2-2 was found to be fibronectin-binding and important for virulence in a mouse model of urinary tract infection (Torelli et al., 2012). Binding of host cell superficies depends on C-terminal WxL domain in *E. faecalis* and in other gram positive bacteria (Brinster, Furlan, & Serror, 2007).

One gene one protein was for a long time the dogma for gene annotation. Piatigorsky and his coworker in year 1988 first found that one gene in ducks encoded two different functions, an enzyme and a structural protein and this phenomena was called gene sharing (Piatigorsky et al., 1988). As the concept "gene sharing" could be misunderstood as horizontal gene transfer, the term moonlighting protein invented by Jeffery in year 1999 was comprehensively used, which stands for more than one biological function with the second being done at night (Jeffery, 1999). The mechanism how moonlighting proteins take effect is determined by several factors: differential localization (inside or outside of the cell, in the plasma membrane or in the cytoplasm within the cell); expression in different cell types; oligomerization (monomer or multimer); ligand/substrate concentration. Moonlighting proteins are found in both eukaryote and prokaryote and enable modifications and adaptions in organic sphere. The identified moonlighting proteins assist cellular function in many ways. One very important and common feature of moonlighting proteins is to promote bacteria to bind receptors in host

such as host epithelia, extracellular matrices (ECMs), mucin, actin and host proteolytic plasminogen system. Besides, moonlighting proteins play a role in modulation of host immune responses (Henderson & Martin, 2011). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and enolase in *Lactobacillus* (*L.*) *crispatus* were found to bind human plasminogen (Hurmalainen et al., 2007). Many other moonlighting proteins identified in lactic acid bacteria include the heat shock protein GroEL and elongation factor Tu (EF-Tu) in *L. johnsonii* La1 capable of binding mucins and intestinal epithelial cells; DnaK in *Bifidobacterium* (*B.*) *animalis* capable of binding human plasminogen; EF-Tu, GAPDH and triose phosphate isomerase in *L. plantarum* 423 capable of binding Caco-2 (Granato et al., 2004; Bergonzelli et al., 2006; Candela et al., 2010; Katakura, Sano, & Hashimoto, 2010; Ramiah, van Reenen, & Dicks, 2008). It is incontrovertible that moonlighting proteins exist not only in commensal but also in pathogen. Major groups of proteins that moonlight in bacterial virulence include the following (Henderson & Martin, 2011): 1, the metabolic enzymes of the glycolytic pathway; 2, enzymes of other metabolic pathways such as the glyoxylate cycle; 3, molecular chaperones and protein-folding catalysts. Triose phosphate isomerase is an enzyme in glycolytic pathway, which was found to mediate adhesion of *S. aureus* to *Cryptococcus* (*C.*) *neoformans*. Phosphoglycerate kinase in glycolytic pathway in streptococci functioned additionally as adhesin to bind plasminogen. Owing to the more and more moonlighting proteins detected in pathogens, researchers have recently paid attention to the role of moonlighting proteins playing in host-invasion and persistence.

1.8 Bacterial translocation

Bacterial translocation is defined as the passage of viable bacteria from the gastrointestinal tract through the mucosal epithelia to other tissues, such as the mesenteric lymph nodes, spleen, liver and blood (Berg, 1985). Translocation may result in bacteremia, peridonitis, liver abscess and multiple organ failure because organs cannot tolerate these bacteria. Thus, harmful complications like inflammatory bowel diseases would occur as a consequence of gastrointestinal disorders, which alter intestinal integrity and attenuate the tightness of intestinal barrier. Transcellular and paracellular migration are the two common and important routes for bacterial translocation. Transcellular migration means endocytosis while paracellular migration implies extracellular metastasis. To be more specific, transcellular migration refers to invading epithelial or specialised M cells at the apical surface, followed by intracellular trafficking and exiting these cells at the basolateral membrane. Paracellular migration is to break the tight junction and E-cadherin-based adherens junction between

adjacent epithelial cells and cross the epithelial barrier without entering cells. Despite the majority of translocated bacteria are Gram-negative (from the normal gut flora), which cause no injury due to lack of virulence, *E. faecalis* OG1RF as a facultative pathogen was also reported to translocate polarized human enterocyte-like T84 cells (Zeng, Teng, & Murray, 2005). At least two factors (gelatinase and enterococcal polysaccharide antigen) are important for translocation of *E. faecalis* OG1RF (Singh, Lewis, & Murray, 2009; Zeng et al., 2005). However, the precise mechanisms involved in translocation remain unknown, i.e., whether *E. faecalis* OG1RF steps across the epithelial layer mainly by way of transcellular migration or paracellular migration remains to be investigated.

1.9 Survival in macrophages and survival-related genes

The immune system senses the invasion of xenobiotics. Microbial stimuli as antigens are firstly recognized in innate immune system by pattern-recognition receptors, such as toll-like receptors, CD 14, lipopolysaccharide-binding protein and a range of non-opsonic receptors. These stimuli induce production of pro-inflammatory cytokines, such as interferon-α/β, and reactive oxygen species (ROS) and nitric oxide (NO), followed by a regulated anti-inflammatory response (P. J. Murray & Wynn, 2011). Therefore, very few microbes could survive in the harsh environment of host due to activated innate immune system and macrophages. There are however some strains of *E. faecalis*, which could sustain the hardness and survive in macrophage for a certain period. Some of the genes that play an important role in survival in macrophage were discovered. *E. faecalis* JH2-2 is able to tolerate oxidative stress owing to the hydrogen peroxide regulator (*hyp*R). The *hyp*R mutant resulted in a significant reduction of survival in mouse peritoneal macrophages comparing to the parent strain, suggesting *hyp*R contributes to the persistence (virulence) of *E. faecalis* JH2-2 (Verneuil et al., 2004). Absence of *spx* in *E. faecalis* OG1RF generated more sensibility to mouse-derived macrophage cell line J774A.1, which showed that *spx* is a major stress gene regulator and is implicated in the pathophysiology of *E. faecalis* OG1RF (Kajfasz et al., 2012). *E. faecalis* V583 lack of *csp*R exhibited a lower survival rate in peritoneal macrophages comparing with the wild type strain, illuminating *csp*R as a regulator in *E. faecalis* V583 is responsible for stress tolerance and survival regulation (Michaux et al., 2012). Inactivation of *efa*R impaired the ability of *E. faecalis* V583 to survive in macrophages J774A.1 and to tolerate oxidative stress, which reveals that *efa*R is an important modulator of persistence (virulence) in *E. faecalis* V583 (Abrantes, Kok, & Lopes, 2013). Multiple survival-related genes contribute to survival of *E. faecalis* in macrophages. Expression of these survival-

related genes is initiatively activated or passively regulated by a regulatory system when it encounters stressful environments. The mannose receptor and scavenger receptor-A promote the phagocytosis and endocytosis of host (Gordon, 2003). Therefore, mfs-PTS is assumed to regulate bacterial survival in macrophages. In this study, the role of mfs-PTS in regulation of survival-related genes is investigated based on the survival discrepancy between *E. faecalis* OG1RF and *pts* (encoding mannose/fructose/sorbose, EIICD) mutants in two types of macrophage.

1.10 Survival in the instestinal tract

In a former study (Lindenstrauß, 2012), germfree wild type mice 129 SvEv TAC (11-12 weeks old) mono-associated with *E. faecalis* OG1RF by gavage feeding were found to tolerate *E. faecalis* OG1RF and generate no lesion in murine colon, caecum or small intestine. The transcriptome of *E. faecalis* OG1RF recovered from these murine intestinal samples was analysed. The group of highly up-regulated genes consists of 97 genes, and the group of genes that were strongly down-regulated consists of 62 genes. Up-regulated genes were speculated to enhance survival and persistence in intestine. Functional categories in which the highly up-regulated genes could be classified are transport and binding proteins (32 genes), energy metabolism (31 genes), hypothetical proteins (13 genes), protein metabolism, amino acid biosynthesis, cellular processes, and regulation of transcription. Important changes in gene expression, which may be referred to adaptation to the intestinal tract, included upregulation of 21 genes encoding components of phosphoenolpyruvate-dependent sugar phosphotransferase systems (PTS) specific for mannose, fructose and sorbose and genes of the glycerol metabolism (e.g. the *glp* operon, which is responsible for the glycerol dissimilation). The distinctly down-regulated gene cluster, which encodes a complete set of enzymes responsible for fatty acid biosynthesis (*accABCD*, *fabD*, *acpP*, *fabH*, *fabG3*, *fabZ2*, *fab K*, *fabF2*), suggests a change in the cell envelope in *E. faecalis*.

This thesis focusses on two of the most significantly up-regulated mannose/fructose/sorbose *pts* genes. This is because apart from their annotated metabolic function the cell surface located components (EIIC and EIID) of PTS systems may also express additional "moonlighting" functions such as adhesion, tolerance to oxidative stress, long term persistence in the gut, bacteriocin recognition or global sugar metabolism regulation.

1.11 Phosphotransferase system in *E. faecalis* OG1RF

E. faecalis utilizes sugars as the main energy source. It ferments not only monosaccharides like glucose, fructose and mannose, but also substances like salicin, and mannitol. The fermentation of mannitol is considered to be very characteristic. Besides, It catabolizes a variety of energy sources including glycerol, lactate, malate, citrate, arginine, agmatine, and many keto acids (Gilmore, 2002). Aside from permeases, phosphotransferase systems (PTSs) are the major uptake systems for sugars in bacteria. Phosphotransferase system is ubiquitous in both gram positive and negtive bacteria, which generally catalyzes the transportation and phosphorylation of sugars meant for bacterial energy. PTS constituents act upon the availability of substrates in the enviroment to accomplish the regulatory function. The complete function of phosphotransferase system including: chemoreception, transportation, sugar/protein phosphorylation, regulation of non-PTS sugar transportation and metabolism, regulation of carbon metabolism, regulation of carbon storage, regulation of fermentation versus respiration, regulation of cellular motility, coordination of nitrogen and carbon metabolism, regulation of non-carbon-compound transport, regulation of gene expression, regulation of pathogenesis, regulation of cell physiology, regulation of cell division (Barabote & Saier, 2005). Interestingly, *L. monocytogenes* has the most abundant PTSs (30 complete PTS) followed by *E. faecalis* (25 complete PTS) and the presence of *pts* genes is “moonish”, i.e., *pts* genes gained and lost frequently in bacterial phylogeny (Barabote & Saier, 2005). Sugar transportation is concomitant with phosphorylation conducted by a chain of catalyzing enzymes in PTS. These enzymes belong to two general proteins: 1, Enzymes I (EIs) and Histidine-containing phosphocarrier protein (HPr). Both HPr and EI are cytoplasmic PTS constituents in charge of the uptake of all PTS sugars. 2, A series of more than 15 substrate-specific enzymes II (EIIs). EIs in different bacteria share common ancestry while EIIs vary. Some important loci of sugar-binding sites are characterized in EIIs. A highly conserved motif ([Gn]-[Iv]-[Tsn]-E) in EIIs is speculated to be part of carbohydrate binding site and the consensus sequence [Flm]-[Gn]-[Iv]-[Tsn]-E-P-[Aiv]-[ILmv]-[Fy]-G-[Vilma]-Npt]-[Li] is suggested to be binding site for disaccharides (Kotrba, Inui, & Yukawa, 2001). These motifs enable a general model constructed by EIIs to function as carbohydrate transportation systems, phosphotransferases, chemoreceptors in chemotaxis (Lengeler, Titgemeyer, Vogler, & Wohrl, 1990). Each part of PTS components executes a successive assignment of responsibility. As shown in Fig. 2, PTS components form a phosphorylation cascade beginning with EI, which autophosphorylates at the N-ε3 position of a conserved histidyl

residue at the expense of phosphoenolpyruvate (PEP). Phosphorylated EI (P~His-EI) transfers the phosphoryl group to the N-δ1 position of His-15 and Ser-46 in HPr, then histidyl-phosphorylated HPr (P~His-HPr) passes the phosphoryl group on to one of several sugar-specific EIIAs at the N-ε3 position of a histidyl residue. P~EIIAs phosphorylate their cognates EIIBs at a cysteyl residue (except the EIIBs of the mannose PTS family, which are phosphorylated at the N-δ1 position of a conserved histidyl). Finally, P~EIIBs transfer phosphoryl group to a carbohydrate molecule bounded to the cognates EIICs, or EIIDs designated in mannose PTS (Deutscher et al., 2014). The hyperspace distribution is common for intracellular EIIs. For example, the EIICB (Glc) subunit spans the membrane eight times (Siebold, Flu, Beutler, & Erni, 2001). One EII could sometimes have two forms. There are two forms of EIIAB (Man) in *S. salivarius*: $EIIAB_L$ (Man) and $EIIAB_H$ (Man). $EIIAB_L$ (Man) is the much more often mentioned type of EIIAB (Man), which phosphorylates readily consumable sugars such as fructose, glucose, and mannose while the $EIIAB_H$ (Man) also receives phosphoryl from HPr but with a yet undefined function in the cascade of phosphoryl transportation (Jacqueline, Yi-Ywan, & Robert, 2003). As shown in Fig. 2, except for phosphoryl transfer, EIIA and EIIB are involved in bacterial biofilm formation (Lazazzera, 2010). Disrupted EIIA, EIIB, EIIAB (Man), and EIIABC (Fru) in *E. faecalis* V583, *Streptococcus* (*S.*) *pneumonia*, *S. mutans*, and *S. gordonii* resulted in decreased biofilm formation (Abranches, Candella, Wen, Baker, & Burne, 2006; Cook, 2012; Ernesto J, Joan, & Andrew, 2008; Loo, Mitrakul, Voss, Hughes, & Ganeshkumar, 2003). Some intermediate products in PTS associate with virulence regulation (Fig. 2). For example, P~His-HPr and P~Ser-HPr associate with virulence regulator PrfA and *mga* in *L. monocytogenes* and *S. pyogenes*, respectively (Poncet, Abdallah, Deghmane, & Bolle, 2009).

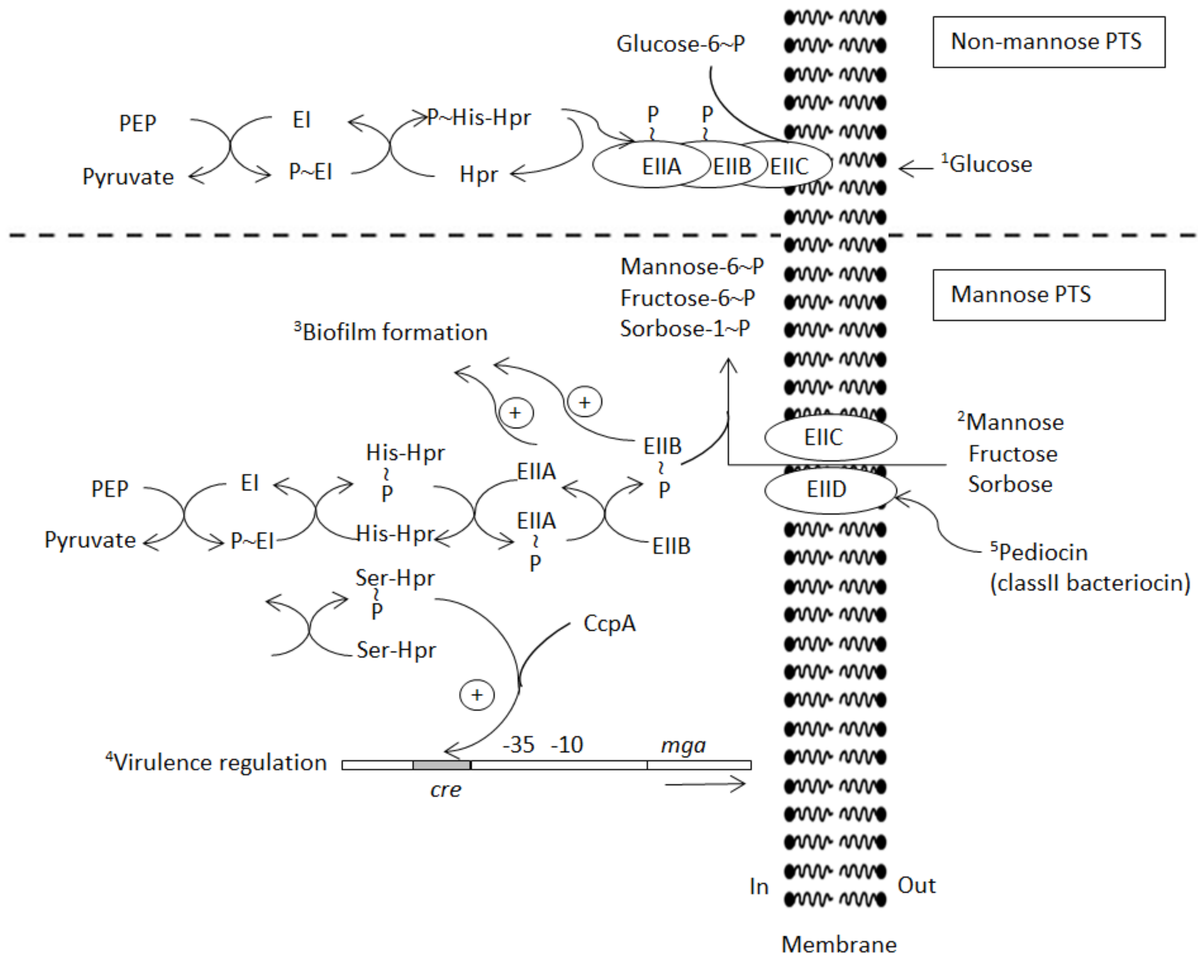


Fig. 2 Schematic presentation of the phosphorylation cascade formed by the PTS components necessary for the uptake of Mannose, fructose, sorbose and glucose in gram-positive bacterium. Two forms of phosphorylated HPr (P~His-HPr and P~Ser-HPr) are generated during phosphoryl transfer.

1, Glucose PTS is the system for glucose uptake.

2, Mannose/fructose/sorbose PTS is the system for uptake of mannose, fructose, and sorbose. This system contains IID as a specific component to differentiate from other PTSs.

3, EIIA and EIIB in streptococci positively regulate bacterial biofilm formation

4, P~Ser-HPr:CcpA complex positively regulate virulence regulator (*mga*) by binding operator site *cre* , which locates upstream from the *mga* in streptococci

5, IID is specific in mannose PTSs (in this context is mannose/fructose/sorbose PTS), which is the receptor for class II bacteriocins.

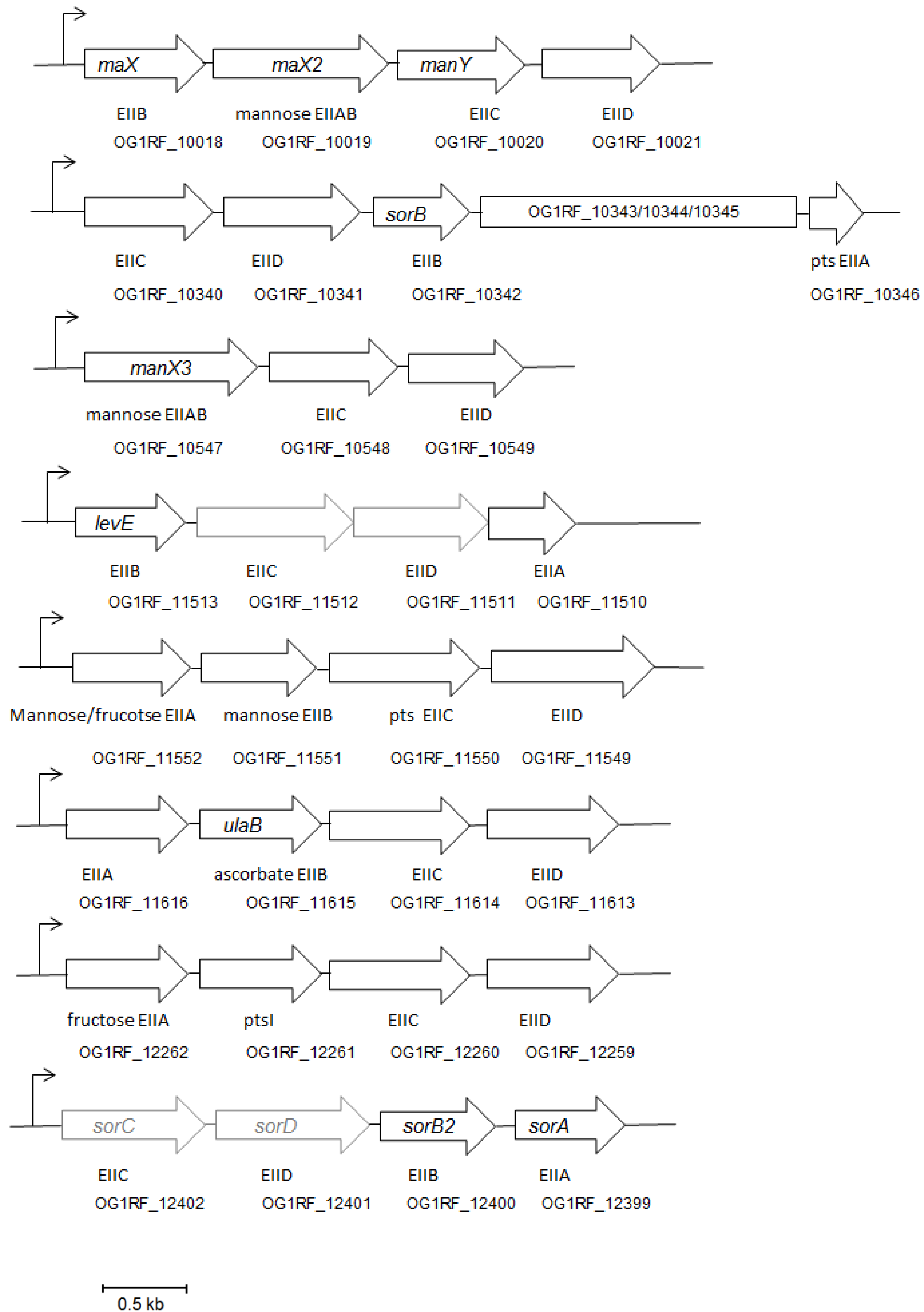


Fig. 3 Genetic organization of all the operons predicted as mannose/fructose/sorbose PTS operons in *E. faecalis* OG1RF. Grey squares stand for genes to be deleted later in this work. Mannose/fructose/sorbose – EIIA/EIIB/EIIC/EIID is abbreviated as EIIA/EIIB/EIIC/EIID

E. faecalis OG1RF possesses 25 complete and more than 25 incomplete phosphotransferase systems, more than 80% of which are EIIs, enabling transportation and phosphorylation of different PTS sugars (Fig. 4). Mannose/fructose/sorbose phosphotransferase system (mfs-PTS) in *E. faecalis* OG1RF is reported to be of importance for the survival in murine intestine by systemic regulation (Lindenstrauß, 2012). Analysis by BLAST in NCBI revealed that 8 fragments of the genome are annotated as mfs-PTS with DNA length ranging from around 2,000 to 6,000 bp (Fig. 3). Mfs-PTS compromises three sugar transporters in one system due to the high homology of mannose and L-sorbose PTSs in enteric bacteria and fructose PTSs in *Bacillus* (*B.*) *subtilis* (45 to 65% identical residues) (Postma, Lengeler, & Jacobson, 1993). The porter members of mfs-PTS usually show compatible intake of several sugars (Plumbridge & Vimr, 1999). Mannose and L-sorbose PTSs can phosphorylate fructose in addition to its major substrate (Postma & Lengeler, 1985). Besides, mannose PTSs in many Gram-positive bacteria are responsible for glucose phosphorylation and transportation (Yebra, Monedero, Zuniga, Deutscher, & Perez-Martınez, 2006).

Bacteria respond to changing environments by regulating expression of global genes. PTS as a transport system could catalyze the uptake of numerous carbohydrates and convert into respective phosphoesters during transportation. Gene regulation by phosphotransferase system acts mainly upon the diversity of sugars, which are usually divided into preferential sugars (like glucose and fructose) and non-preferential sugars (like lactose and galactose). Two important regulatory mechanisms of PTS considering preferential sugar and non-preferential sugar uptake are carbon catabolite repression (CCR) and inducer exclusion. CCR and inducer exclusion use different mechanisms to delay consumption of non-preferential sugars due to the prior utilization of preferential sugars. Sugar uptake is the most important way to gain energy source whereas it also provokes or attenuates the virulence of bacteria. For example, disrupted EII (Man) in *L. monocytogenes* made a small contribution to upregulation of virulence gene transcription in the presence of glucose (Vu-Khac & Miller, 2009). Some *in vitro* and *in vivo* studies have revealed that the transcription of virulence genes is temporally related to those genes encoding carbohydrate utilization proteins in group A streptococci (Iii et al., 2008). Therefore, phosphotransferase systems may play a role in bacterial virulence by regulation of sugar metabolism.

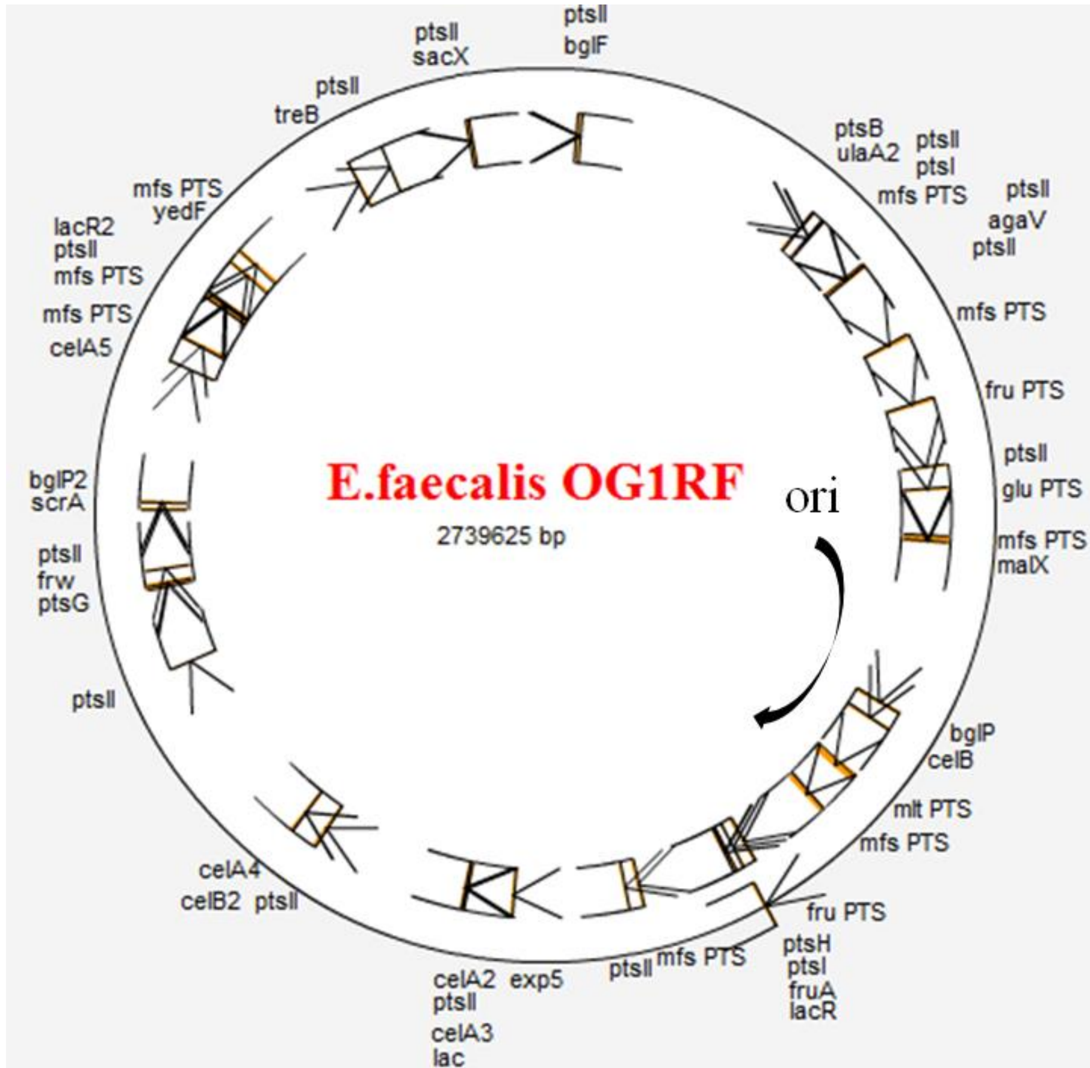


Fig. 4 Distribution of all predicted PTSs in *E. faecalis* OG1RF

1.12 Aim of the study

In this work insights should be generated in mechanisms involved in the translocation through the intestinal barrier and its persistance in the intestinal tract of *E. faecalis* OG1RF. Therefore, translocation of *E. faecalis* should be investigated in a Ptk6 cell monolayer model displaying different barrier functions. Factors should be identified, which are involved in the adhesion and translocation process or facilitate survival in the intestinal tract. Initially, actin binding proteins and their possible role in translocation should be identified. As former studies on the *in vivo* gene expression in *E. faecalis* in the mouse intestinal tract suggested an important role of predicted mannose/fructose/sorbose phosphotransferase systems in *E. faecalis* OG1RF, some of which were up to 7-fold up-regulated as compared to the bacterium residing in culture media. As PTSs are described as playing important roles not only in bacterial metabolism but also in virulence, aggregation ability, stress tolerance and survival in macrophages, such functions should be probed by comparison of the wild type strain with selected PTS knock out mutants. To achieve these aims, knock out mutants lacking the EIIC and EIID components should be generated in two predicted mannose/fructose/sorbose PTSs, which were the strongest upregulated ones in the *in vivo* gene expression study. Their biochemical transport function should be determined as well as their possible role in aggregation, tolerance to acid and oxidation, bacteriocin resistance, and survival in macrophages. Moreover, regulation of antioxidant and energy-related genes by *pts* (encoding mannose/fructose/sorbose, EIICD) genes in *E. faecalis* OG1RF should be investigated.

2 Material and methods

2.1 Material

2.1.1 Devices

Devices used in this work are listed in Tab. 1 in alphabetical order.

Device	Model	Manufacturer
Agitator	Certomat R	B. Braun Biotech International, Melsungen, Germany
	Vortex 2 Genie	Scientific Industries Inc., Bohemia, NY, USA
Agarose gel chamber	Easy Cast electrophoresis system (25 x 20 cm)	Owl Separation Systems, Portsmouth, NH, USA
	Easy Cast electrophoresis system (13.8 x 12 cm)	Owl Separation Systems, Portsmouth, NH, USA
	Owl A2	Owl Separation Systems, Portsmouth, NH, USA
Autoclaves	2540 ELV	Systec GmbH, Wettenberg, Germany
	VX-150	Systec GmbH, Wettenberg, Germany
	VE-40	Systec GmbH, Wettenberg, Germany
Clean bench	BioChemGARD e3	Heraeus instruments GmbH, Hanau, Germany
Drying oven	Venticell	MMM group, Munich ,Germany
Electrophoresis supplies	MPP 2 × 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
	2197 Supply PPS 200-1D	MWG Biotech AG, Ebersberg, Germany
Electrophoresis chamber	Mini-PROTEAN® Tetra Cell	Bio rad, California, USA
Electroporatoration system	Gene pulser II/pulser controller plus 165-2110	Bio rad, California, USA
Fermentation control system	ICinac™, AFL-W15A	Westco scientific instruments, Inc. Unity Scientific (NIR), Brookfield, USA
Fluorescence and absorbance microplate reader	SpectraFluor	TECAN, Maennedorf, Switzerland

Device	Model	Manufacturer
Fridge	KIR41AF30G	Bosch GmbH, Stuttgart, Germany
Fume hood	Trox technik FH1	Trox GmbH, Düsseldorf, Germany
Freezer	ES series	Thermo scientific, Waltham, Massachusetts, USA
	MDF-U700VX-PE Ultra Low Temperature Freezer	Panasonic healthcare, Co., Ltd, Tokyo, Japan
	Platinum 500	Angelantoni Industries s.p.a, AS biomedical division, Massa Martana, Italy
Ice machine	AF100	Scotsman Europe-Frimont S.p.A.
Incubation and shaking cabinet	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
	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
	Hermle Z513 K	Hermle Labortechnik, Wehningen, Germany
Laminar flow sterile work bench	HERA safe	Heraeus Instruments, Hanau, Germany
Scanner	Epson Expressiom 1600pro	Seiko Epson Corporation, Tokyo, Japan
Magnetic stirrer	RCT-Basic	Mettler-Toledo, Gießen, Germany
	Wisestir MSH-20A	Witeg labortechnik GmbH, Wertheim, Germany
Microscope	Axiolab	Carl Zeiss MicroImaging GmbH, Germany
Microwave oven	Intellowave MS-2342G	LG. Seoul, South Korea

Device	Model	Manufacturer
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
Pipettes	Pipetman	Gilson-Abomed, Langenfeld, Germany
	Multipette Stream	Eppendorf AG, Hamburg, Germany
	Multichannel pipette	Eppendorf AG, Hamburg, Germany
Pure water system	Euro 25 and RS 90-4/UF	SG Wasseraufbereitung GmbH, Barsbüttel, Germany
RT-qPCR device	PikoReal 24 Real-Time PCR System	Thermo scientific, Waltham, Massachusetts, USA
SpectroPhotometer	NovaspeIIq	Pharmacia Biotech, Cambridge, England
	Pharmacia LKB-Biochrom 4060	Pharmacia, Stockholm, Sweden
Sample Preparation System	Fast-Prep®-24	MP Biomedical, Irvine CA, USA
Scale	Kern 572	KERN & SOHN GmbH
	SBA 52/SPO61	Scaltec instruments GmbH
	SI-234	Dennver instrument
Steam box	Varioklav, dampftopf	HP Medizintechnik GmbH
Thermo block	Techne DRI-Block DB3	Thermo-Dux Gesellschaft für Laborgerätebau GmbH, Wertheim, Germany
Ultra sonic cell disruptor	SMS73/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
Washer	G7883	Miele professional
Water-bath	Lauda BD	LAUDA Dr. D. Wobser GmbH & Co., Lauda-Königshofen, Germany
	E103 type	LAUDA Dr. D. Wobser GmbH & Co., Lauda-Königshofen, Germany

Tab. 1 Devices used in this work

2.1.2 Chemicals

Chemicals used in this work are listed in Tab. 2 in alphabetical order.

Chemicals and media	Purity	Manufacturer
Acetic acid	100% , p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Acrylamide/bis solution	(19:1); 30% (w/v)	SERVA, Heidelberg, Germany
Actin	≥85 % by SDS-PAGE	SIGMA-Aldrich, Steinheim, Germany
Agar	Molecular biology grade	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Agarose	For electrophoresis	Biozym Scientific GmbH, Oldendorf, Germany
Amino acids	Research grade	SERVA, Heidelberg, Germany
Ammonium persulfate (APS)	Electrophoresis grade	SERVA, Heidelberg, Germany
Ammonia sulfate	>99%, p.a.	Merck, Darmstadt, Germany
Antibiotic/antimycotic solution	Molecular biology grade	Invitrogen Life Technologie GmbH, Darmstadt, Germany
Antibody to rabbit IgG (Fluorescein-labeled)	Affinity purified	Gaithersburg MD, USA
β-mercaptoethanol	99%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Biotin	Research grade	SERVA, Heidelberg, Germany
Bovine Serum Albumin	≥96%	SIGMA-Aldrich, Steinheim, Germany
Brain heart infusion (BHI) broth	For microbiology	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Bromphenol blue	For electrophoresis	SIGMA-Aldrich, Steinheim, Germany
Bromocresol purple	Laboratory reagent grade	Merck, Darmstadt, Germany
Calcium pantothenic acid	Research grade	SERVA, Heidelberg, Germany
Catalase	2,000-5,000 units/mg protein	SIGMA-Aldrich, Steinheim, Germany

Chemicals and media	Purity	Manufacturer
Cell culture plate (12, 24 well)	FALC353047	Omnilab, Munich ,Germany
Chloramphenicol	Research grade	SERVA, Heidelberg, Germany
Coomassie brilliant blue R250	p.a.	Bio-Rad, Hercules, USA
Crystal violet solution	IVD grade	SIGMA-Aldrich, Steinheim, Germany
Cysteinhydrochloride.H_2O	For microbiology	Merck, Darmstadt, Germany
di-Ammonium hydrogen citrate	≥98%, p. a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Diethylpyrocarbonate (DEPC)	p.a.	Merck, Darmstadt, Germany
Dimethyl sulfoxide (DMSO)	Research grade	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Di-sodium hydrogen phosphate trihydrat ($Na_2HPO_4 . 3H_2O$)	≥98%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Di-potassium hydrogen phosphate ($K_2HPO_4 .$ 3H2O)	p. a.	Merck, Darmstadt, Germany
1,4 Dithio-D,L-Threitol (DTT)	p. a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
6 x DNA loading dye	High purity	GERBU Biotechnik, GmbH, Gaiberg, Germany
Dulbecco's phosphate buffered saline (DPBS)	For cell culture	SIGMA-Aldrich, Steinheim, Germany
Erythromycin	-	Fermentas GmbH, St. Leon-Rot, Germany
Ethanol, denatured	For molecular biology	SIGMA-Aldrich, Steinheim, Germany
Ethanol, absolute	99% with 1% methylethylketone	Chemikalien und Laborbedarf Nierle, Freising, Germany
Ethylendiaminetetraacetic acid (EDTA)	≥99.8%	VWR, Prolabo, Foutenay-sous-Bois, France
Ferrous chloride	p.a.	Merck, Darmstadt, Germany
Ferrous sulfate	p.a.	Merck, Darmstadt, Germany
Fetal bovine serum (FBS)	For molecular biology	SIGMA-Aldrich, Steinheim, Germany
Fibronctin	>95%, for cell culture	SIGMA-Aldrich, Steinheim, Germany
Folic acid	≥96%, for	Carl Roth GmbH & Co. KG,

Chemicals and media	Purity	Manufacturer
	biochemistry	Karlsruhe, Germany
Formaldehyde	37% (w/v) in H_2O	SIGMA-Aldrich, Steinheim, Germany
Formic acid	>99.5%	Fisher Scientific, Fairlawn, NJ, USA
Fructose	>99.5%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Galactose	>98.5%	GERBU Biotechnik, GmbH, Gaiberg, Germany
Gentamicin	~600 µg gentamicin per mg	SIGMA-Aldrich, Steinheim, Germany
Glucose	>99.7%	SERVA, Heidelberg, Germany
Gluconic acid sodium salt	p. a.	Merck, Darmstadt, Germany
Glycerol	>99.5%	GERBU Biotechnik, GmbH, Gaiberg, Germany
Glycine	>99.5%	GERBU Biotechnik, GmbH, Gaiberg, Germany
HCl (37 %)	Molecular biology grade	Merck, Darmstadt, Germany
Hydrogen peroxide (30%)	p.a.	Merck, Darmstadt, Germany
IPTG	≥99%,	Promega, madison, USA
Insulin-transferrin-selenium A GIBCO	≥99%, for HPLC	SIGMA-Aldrich, Steinheim, Germany
Interferon-gamma (IFN-γ)	≥98% by SDS-PAGE	SIGMA-Aldrich, Steinheim, Germany
Isopropanol	>98.5%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Kanamycin sulfate	Residue <0.2%	SIGMA-Aldrich, Steinheim, Germany
Lactose	For biochemistry analyse	Merck, Darmstadt, Germany
L-cystin	>98.5%	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Lysozyme	≥90%, ≥40,000 units/mg	SIGMA-Aldrich, Steinheim, Germany

Chemicals and media	Purity	Manufacturer
Mannose	>98.5% by HPLC	SERVA, Heidelberg, Germany
Malt extract	For microbiology	AppliChem GmbH, Darmstadt, Germany
Maltose	>95%, by HPLC	SERVA, Heidelberg, Germany
Magnesium sulfate heptahydrat ($MgSO_4$ * 7 H_2O)	p.a.	Merck, Darmstadt, Germany
Manganese(II) sulphate tetrahydrate	p.a.	Merck, Darmstadt, Germany
Meat extract	For microbiology	Merck, Darmstadt, Germany
Methanol	For microbiology	Merck, Darmstadt, Germany
Mineral oil	For microbiology	SIGMA-Aldrich, Steinheim, Germany
Mucin	>98%	SIGMA-Aldrich, Steinheim, Germany
Nicotinic acid	≥98%	SIGMA-Aldrich, Steinheim, Germany
p-Cl-Phe	≥99,8%	SIGMA-Aldrich, Steinheim, Germany
Paraffin oil	p.a.	SIGMA-Aldrich, Steinheim, Germany
Peptone from casein	For microbiology	Merck, Darmstadt, Germany
Penicillin G	>98%, by HPLC	SIGMA-Aldrich, Steinheim, Germany
Phosphoric acid	99%	SIGMA-Aldrich, Steinheim, Germany
Potassium chloride (KCl)	>99%, cellpure	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	p.a.	Merck, Darmstadt, Germany
Primer	p.a.	Merck, Darmstadt, Germany
Pyridoxine	For cell culture	MWG-BiotechAG, Ebersberg, Germany
Riboflavin	For cell culture	SIGMA-Aldrich, Steinheim, Germany
RLT buffer	-	Thermo scientific, Waltham, Massachusetts, USA
RPMI Media 1640 + L-Glutamine	-	Qiagen, Hilden, Germany

Chemicals and media	Purity	Manufacturer
Roti®-Blue 5 x concentrated	-	Invitrogen Life Technologie GmbH, Darmstadt, Germany
Saponin from quillaja bark	-	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Silver nitrate	p.a.	SERVA, Heidelberg, Germany
Sodium acetate	>99.9%, p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium azide (NaN_3)	>99.5%	SIGMA-Aldrich, Steinheim, Germany
Sodium carbonate (Na_2CO_3)	p.a.	Merck, Darmstadt, Germany
Sodium chloride (NaCl)	>99.5%, p.a.	VWR, Prolabo, Foutenay-sous-Bois, France
Sodium citrate	p.a.	Merck, Darmstadt, Germany
Sodium dodecyl sulfate (SDS)	p.a.	Merck, Darmstadt, Germany
Sodium gluconate	p.a.	SERVA, Heidelberg, Germany
Sodium hydroxide (NaOH)	p.a.	Merck, Darmstadt, Germany
Sodium hydroxide solution	50%, for HPLC	SIGMA-Aldrich, Steinheim, Germany
Sodium dihydrogen phosphate	p.a.	Merck, Darmstadt, Germany
Sorbose	>98% by HPLC	Merck, Darmstadt, Germany
Sorbitol	>99.5% by GC	SIGMA-Aldrich, Steinheim, Germany
Streptomycin	~95%	SIGMA-Aldrich, Steinheim, Germany
Sucrose	>99.5% by GC	SIGMA-Aldrich, Steinheim, Germany
Tertamethylethylenediamine (TEMED)	~99%	SIGMA-Aldrich, Steinheim, Germany
Thiamine	>99.5%	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Thiosulfate	p.a.	Merck, Darmstadt, Germany
Tris base	>99%	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Tris-HCl	>99%	Carl Roth GmbH + Co. KG, Karlsruhe, Germany

Chemicals and media	Purity	Manufacturer
Triton×100	Ultra pure	ICN Biomedicals, Inc., Ohio, USA
Trypsin-EDTA	p.a.	Merck, Darmstadt, Germany
Tween 80	-	Invitrogen Life Technologie GmbH, Darmstadt, Germany
Urea	-	Invitrogen Life Technologie GmbH, Darmstadt, Germany
X-gal	-	Promega, madison, USA
Yeast extract	For microbiology	Carl Roth GmbH + Co. KG, Karlsruhe, Germany

Tab. 2 Chemicals used in this work

2.1.3 Media and buffer

Media and buffer used in this study is listed in Tab. 3-Tab. 22

Ingredients	Content
BHI	37.5 g
Glycine	60 g (vary with regard to bacterial strains)
Sucrose	0.5 M
dH_2O	to 1 L
Note: SGBHI is always freshly prepared, sterilize at 121°C for 15 min (sucrose should be sterilized separately)	

Tab. 3 SGBHI medium (1 L)

Ingredients	Content
Peptone from casein	10 g
Meat extract	2 g
Yeast extract	7 g
Glucose	5 g
Gluconic acid sodium salt	2 g
Sodium acetate trihydrate	5 g
Di-ammonium hydrogen citrate	5 g
Dipotassium hydrogen phosphate	2.5 g
Magnesium sulfate heptahydrate	0.2 g
Manganese sulfate tetrahydrate	0.1 g
Ferrous sulfate heptahydrate	0.05 g
Cystein-HCL	0.5 g
Tween 80	1 g
Agar	15 g

dH_2O	to 1 L
Note: pH adjust to 5.4, sterilize at 121°C for 15 min (sugars should be sterilized separately)	

Tab. 4 MRS medium (1 L)

Ingredients	Content
Malt extract	130 g
Chloramphenicol	0.1 g
dH_2O	to 1 L
Note: adjust pH to 6.0±0.2, sterilize at 121°C for 15 min	

Tab. 5 Malt broth medium (1 L)

Ingredients	Content
Peptone from casein	10 g
Yeast extract	5 g
NaCl	5 g
dH_2O	to 1 L
Note: adjust pH to 7.0, sterilize at 121°C for 15 min	

Tab. 6 LB medium (1 L)

Ingredient	Content
Yeast extract	0.25 g
p-Cl-Phe	199.6 mg
dH_2O	89 mL
Autoclave at 121°C for 30 min, mix thoroughly to fully dissolve *p*-Cl-Phe, cool to 55°C, and add following substances	
10×sterile M9 salts	10 mL
Sterile 50% glucose	0.5 mL
X-gal	150 mg
dH_2O	to 1 L
Note: separately sterilize then mix solutions	

Tab. 7 MM9YEG agar medium (1 L)

Ingredients	Content
Na_2HPO_4	60 g
KH_2PO_4	30 g
NaCl	5 g
NH_4Cl	10 g

dH$_2$O	to 1 L
Note: sterilize at 121°C for 15 min	

Tab. 8 10×sterile M9 salts (1 L)

Ingredients	Content
K_2HPO_4	7 g
KH_2PO_4	2 g
Sodium citrate	0.5 g
MgSO4	0.1 g
NH_4SO4	1 g
Thiamine	100 µg/mL
Biotin, calcium pantothenic acid, and pyridoxine	each 20 µg/mL
Nicotinic acid and riboflavin	each 2 µg/mL
Folic acid	0.2 µg/mL
20 amino acids	each 20 µg/mL
dH$_2$O	to 1 L
Note: Davis medium is sterized by 0.2 µm filter	

Tab. 9 Davis medium (1 L)

Solution name	Solution ingredients and contents
Fix	40% ethanol, 10% acetic acid
Wash	30% ethanol
Thiosulfate solution	0.02% Na2S2O3
Silver nitrate solution	0.2% AgNO3
Developing	3% Na2CO3, 0.05% H2CO, 0.0004% Na2S2O3
Stop solution	0.5% Glycine

Tab. 10 Silver stain solutions

Ingredients	Content
$Na_2HPO_4 \cdot 2H_2O$	14.4 g
$NaH_2PO_4 \cdot H_2O$	2.62 g
NaCl	8.77 g

Tab. 11 PBS buffer (0.1 M, 1 L)

Ingredients	Content
Tris pH 6.8 (1 M)	2.4 mL
SDS	0.8 g
100% glycerol	4 mL

Bromophenol blue	0.01% (w/v)
β-mercaptoethanol	1mL
dH_2O	2.8 mL

Tab. 12 Laemmli buffer for SDS-PAGE

Ingredients	Content
Tris powder	15.1 g
Glycine	94 g
SDS	5 g
dH_2O	to 1 L

Tab. 13 5×Tris glycine (1 L)

Ingredients	Content
Coomassie brilliant blue R250	1.25 g
Ethonal or methonal	250 mL
dH_2O	300 mL
Acetic acid	80 mL
dH_2O	1 L
Note: substances above should be added in the right order and finally decontamination with filter paper soon after the solution is thoroughly mixed	

Tab. 14 Staining solution for SDS-PAGE (1 L)

Ingredients	Content
Methonal or ethonal	250 mL
Acetic acid	80 mL
dH_2O	670 mL

Tab. 15 Destaining solution (1 L)

Ingredients	Content (v/v)
Glycerol	12%
Ethonal	30%
dH_2O	58%

Tab. 16 Solution for SDS-PAGE gel

Ingredients	Content (v/v)
Acetic acid	10%
Ethonal	40%
dH_2O	50%

Tab. 17 Solution for gel sequencing storage

Ingredients	Content
1 M Tris-HCL (pH 8.0)	5 mL
0.5 M EDTA (pH 8.0)	1 mL
dH_2O	to 500 mL
Note: 0.5 M EDTA (pH 8.0) 7.36 g EDTA add to 50 mL dH_2O, adjust pH to 8.0	

Tab. 18 1×TE buffer

Ingredients	Content
Sucrose	0.5 M
Glycerol	10%
dH_2O	to desired volume
Note: adjust pH to 7.0	

Tab. 19 Electroporation buffer

Ingredients	Content
Polypeptone	10 g
Yeast extract	5 g
Tween 80	1 mL
K_2HPO_4	2 g
$CH_3COONa·3H_2O$	5 g
Di-ammonium hydrogen citrate	2 g
$MgSO_4·7H_2O$	0.2 g
$MnSO_4·4H_2O$	0.05 g
Bromocresol purple	0.17 g
dH_2O	to 1 L
Note: pH adjust to 6.7-7.1, sterilize at 121°C for 15 min	

Tab. 20 API 50 CHL media (1 L)

Ingredients	Content
DEPC	0.1%
dH_2O	to desired volume
Note: DEPC is highly toxic, mix gently and stay overnight, then sterile by autoclaving	

Tab. 21 DEPC solution

Solutions	Preparation

1 M Tris pH 6.8	6.05 g Tris add to 50 mL dH_2O, adjust pH to 6.8
1 M Tris pH 8.8	6.05 g Tris add to 50 mL dH_2O, adjust pH to 8.8
10% SDS (w/v, g/mL)	5 g SDS add to 50 mL dH_2O, mix gently
10% APS (w/v, g/mL)	5 g APS add to 50 mL dH_2O

Tab. 22 Reagents for SDS-PAGE

2.1.4 Expendable materials

Expendables used in this work are listed in Tab. 23 in alphabetical order.

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
Electroporation cuvettes	Diameter: 1 cm, 2 cm	Biozym scientific GmbH, Oldendorf, Germany
Microtiter plates	Multi well plate 96-well flat bottom with lid	Sarstedt, Nümbrecht, Germany
Reaction tubes	200 µl, 1.5 ml, 2 ml	Eppendorf, Hamburg, Germany
Sterile ml tubes	15 ml, 50 ml	Sarstedt, Nümbrecht, Germany
Sterile filter	Filtropur S 0.2 (0.2 µm)	Sarstedt, Nümbrecht, Germany

Tab. 23 Expendables used in this work

2.1.5 Kits

Kits used in this work are listed in Tab. 24 in alphabetical order.

Kit	Type	Manufacturer
E.Z.N.A. Bacterial DNA Kit	DNA isolation	Omega Bio-Tek Inc., Norcross, GA, USA
Maxima SYBR Green/ROX qPCR master mix (2×)	#K0221	Thermo scientific, Waltham, Massachusetts, USA
peqGOLD Gelextraction Kit	Gel extraction	PEQLAB Biotechnologie GmbH, Erlangen, Germany
peqGOLD plasmid miniprep kit	Plasmid miniprep kit	PEQLAB Biotechnologie GmbH, Erlangen, Germany
QIAquick PCR purification Kit	PCR purification Kit	Qiagen GmbH, Hilden, Germany
RNeasy Midi Kit	RNA isolation	Qiagen AG, Hilden, Germany

Kit	Type	Manufacturer
Fast digest enzyme		Thermo Scientific, Waltham, Massachusetts, USA
T4 DNA ligase	DNA ligation	New England Biolabs GmbH, Frankfurt am Main, Germany
Taq Core Kit	DNA polymerase	MP Biomedicals Solon, Ohio, USA
Reverse transcription supermix for RT-qPCR	170-8841	BIO-RAD
TURBO DNA-free^{TM} Kit	DNA degradation from RNA samples	Applied Biosystems Inc., Foster City, California, USA

Tab. 24 Kits used in this work

2.1.6 DNA and protein markers

DNA markers used for size comparison are: GeneRuler 1 kb DNA Ladder (ready-to-use, 250 to 10,000 bp) purchased from Thermo Scientific and 1 kb DNA Ladder (Ready-to-load, 500 to 10,000 bp) purchased from Bioneera. Protein marker used for size estimation is PageRuler Plus Prestained Protein Ladder (10-250 KDa) purchased from Thermo Scientific.

2.1.7 Bacterial strains

All enterococcal strains used in this work were obtained from the TMW culture collection and are listed in numerical order in Tab. 25.

Species	TMW strain No.	Origin
E. coli	2.580	*Escherichia coli* strain Top10 (Invitrogen)
E. coli	2.1363	*Escherichia coli* strain EC 1000
E. coli	2.1365	*Escherichia coli* strian EC 1000 with pCJK47 plasmid
E. durans	1.1590	*Enterococcus durans* isolated from baby feces
E. faecalis	2.622	*Enterococcus faecalis* strain isogenic with OG1RF, a rifampicin and fusidic acid resistant derivative of the human oral isolate OG1 (Dunny et al. 1978; Gold et al. 1975) donated by Barbara Murray, University of Texas, USA
E. faecalis	2.630	*Enterococcus faecalis* strain isolated from sheep's milk cheese
E. faecalis	2.777	*Enterococcus faecalis* strain isogenic with symbioflor®1, SymbioPharm (Domann et al. 2007), Herborn, Germany

Species	TMW strain No.	Origin
E. faecalis	2.852	*Enterococcus faecalis* strain isogenic with strain V583 (Sahm et al. 1989), Ingolf Nes, Norwegian University of Life Sciences, Norway
E. faecalis	2.902	*Enterococcus faecalis* strain from Klinikum rechts der Isar, Roger Vogelmann, Technische Universität München*
E. faecalis	2.905	*Enterococcus faecalis* clinical isolate , Klinikum rechts der Isar, Roger Vogelmann, Technische Universität München*
E. faecalis	2.911	*Enterococcus faecalis* strain clinical isolate , Klinikum rechts der Isar, Roger Vogelmann, Technische Universität München*
E. faecalis	2.912	*Enterococcus faecalis* strain clinical isolate , Klinikum rechts der Isar, Roger Vogelmann, Technische Universität München*
E. faecalis	2.922	*Enterococcus faecalis* strain clinical isolate , Klinikum rechts der Isar, Roger Vogelmann, Technische Universität München*
E. faecalis	2.934	*Enterococcus faecalis* strain clinical isolate , Klinikum rechts der Isar, Roger Vogelmann, Technische Universität München*
E. faecalis	2.1142	*Enterococcus faecalis* strain SL5, Nanchang University, China
E. faecalis	2.1143	*Enterococcus faecalis* strain ATCC19433, Nanchang University, China
E. faecalis	2.1451	*Enterococcus faecalis* OG1RF lack of OG1RF_12401 and OG1RF_12402 genes, designated as $\Delta pts1$
E. faecalis	2.1452	*Enterococcus faecalis* OG1RF lack of OG1RF_11511 and OG1RF_11512 genes, designated as $\Delta pts2$
E. faecalis	2.1453	*Enterococcus faecalis* OG1RF lack of OG1RF_12401, OG1RF_12402, OG1RF_11511 and OG1RF_11512 genes, designated as $\Delta pts1/2$
E. faecium	2.938	*Enterococcus faecium* clinical isolate , Klinikum rechts der Isar, Roger Vogelmann, Technische Universität München*
L. animals	1.972	*Lactobacillus animals* isolated from duck P078 by Petra Kurzak
L. brevis	1.317	*Lactobacillus brevis* isolated from beer
L. brevis	1.313	*Lactobacillus brevis* isolated from beer
L. brevis	1.240	*Lactobacillus brevis* isolated from beer
L. brevis	1.465	*Lactobacillus brevis* isolated from softdrink
L. brevis	1.485	*Lactobacillus brevis* isolated from beer
L. paracasei	1.1434	*Lactobacillus paracasei* strain Medipharma F19
L. plantarum	1.1565	*Lactobacillus plantarum* strain isolated from chidren´s feces St7 by Rima Albesharat in Syria

Species	TMW strain No.	Origin
L. plantarum	1.1609	*Lactobacillus plantarum* strain isolated from chidren´s feces B0s10 by Rima Albesharat in Syria
L. plantarum	1.1628	*Lactobacillus plantarum* strain isolated from chidren´s feces B1m9 by Rima Albesharat in Syria
L. plantarum	1.1733	*Lactobacillus plantarum* strain isolated from food O2 by Rima Albesharat in Syria
L. plantarum	1.1734	*Lactobacillus plantarum* strain isolated from food K9 by Rima Albesharat in Syria
L. sanfranciscensis	1.1304	*Lactobacillus sanfranciscensis* strain BRS1 (Id. 07.03.06), isolated from sour dough
S. aureus subsp. aureus	2.421	*Staphylococcus aureus subsp. aureus* isogenic with strain DSM 799, ATCC 6538, WS 2286
S. carnosus	2.850	*Staphylococcus carnosus* isogenic with strain TH 1123, pLipPS1 (Cmr, lip gene from *Staphylococcus hyicus* under control of an *S. carnosus* promoter, 5.4 kbp)
Sacchromyces cerevisiae	3.0170	Isolated from baking environment
Pediococcus acidiactici	2.1338	Isolate from contaminated *Staphylococcus carnosus* LTH 59, Hohenheim

Tab. 25 Bacterial strains used in this study

2.1.8 Primers used in this study

Oligonucleotides for screening and sequencing purposes are listed in Tab. 26-28 and were synthesized by Eurofins MWG-Operon (Ebersberg, Germany). Primers developed in this study were designed on bases of public available sequences.

No.	Name of primer	Sequence of primer	Annotation
1	F_{pts1U} (5´-3´)	GGAATTCCCTTGGCAGATTCAGAGTATC EcoR I	Forward primer of *pts*1 up arm
2	R_{pts1U}(5´-3´)	GGGGTACCCCCGGTGACAATCATCGCTT Kpn I	Reverse primer of *pts*1 up arm
3	F_{pts1D}(5´-3´)	GGGGTACCCCGCGGAATGCCACAGGTTT Kpn I	Forward primer of *pts*1 down arm
4	R_{pts1D}(5´-3´)	GCTCTAGAGCCCTGATTGTAAGGACTCGC Xba I	Reverse primer of *pts*1 down arm
5	F_{pts1I} (5´-3´)	TCTATGCCAGCAGAACCATC	Forward primer of *pts*1 inner fragment
6	R_{pts1I} (5´-3´)	ACCTGAGTCAATCGCATCAC	Reverse primer of *pts*1 inner fragment
7	F_{pts1O}(5´-3´)	CGTGAAAGTTGGACCGCAAT	Forward primer of *pts*1 outer fragment
8	R_{pts1O}(5´-3´)	CTTCAGCAATCGGTGTG	Reverse primer of *pts*1 outer fragment
9	P_{pts1S}(5´-3´)	GCCATCATAGACGCATT	Sequence primer of *pts*1 mutant
10	F_{pts2U} (5´-3´)	GGAATTCCTGACGCAATGGCAACAAT EcoR I	Forward primer of *pts*2 and *pts*1/2 up arm
11	R_{pts2U}(5´-3´)	GGGGTACCCCTGCTTGTTCAGGATTCAT Kpn I	Reverse primer of *pts*2 and

			*pts*1/2 up arm
12	F_{pts2D}(5´-3´)	GGGGTACCCCCATTGTCTGCGTTAGGTG Kpn I	Forward primer of *pts*2 and *pts*1/2 down arm
13	R_{pts2D}(5´-3´)	GCTCTAGAGCGGTGTTAAGCCAGATAGT Xba I	Reverse primer of *pts*2 and *pts*1/2 down arm
14	F_{pts2I} (5´-3´)	GATTCCAGTCTTCTTAGCAC	Forward primer of *pts*2 and *pts*1/2 inner fragment
15	R_{pts2I} (5´-3´)	ATTCAGGCGTATCATCC	Reverse primer of *pts*2 and *pts*1/2 inner fragment
16	F_{pts2O}(5´-3´)	AGGGTCGTGTCAATTAC	Forward primer of *pts*2 and *pts*1/2 outer fragment
17	R_{pts2O}(5´-3´)	TCTGGAATGCTATCTACG	Reverse primer of *pts*2 and *pts*1/2 outer fragment
18	P_{pts2S}(5´-3´)	GCGTCCATCTGATTGCTC	Sequence primer of *pts*2 and *pts*1/2 mutants

Tab. 26 Primer used in *pts* mutants construction

No.	Name of primer	Sequence of primer	Origin of amplified PGK fragment

1	Fp (5´-3´) Rp (5´-3´)	GGCTAAATTAATYGTTTCAG CAGAAATAGCWGCRATACC	*L. paracasei* TMW 1.1434 *L. plantarum* TMW 1.1733 *L. plantarum* TMW 1.1628 *L. plantarum* TMW 1.1609 *L. plantarum* TMW 1.1734
2	Fb (5´-3´) Rb (5´-3´)	GGCTAAATTAACAGTTTCCG CTTCAAGGTATTCCAGAGA	*L. brevis* TMW 1.313 *L. brevis* TMW 1.465 *L. brevis* TMW 1.317 *L. brevis* TMW 1.485 *L. brevis* TMW 1.240

Tab. 27 Primers used for PGK amplification in *Lactobacillus*

No.	Name of primer	Sequence of forward and reverse primer	Length of product (bp)
1	ahpC	Forward: AAACAGAGTAAACCTCACAA Reverse: ACACGAGTATCAACAGAAG	151
2	gor	Forward: CCGTATATTCTGTCCCATTG Reverse: TACGGGTTTGATGTTGAAAT	187
3	hypR	Forward: AGAACGGCTAGACAAAGGAT Reverse: CGGCTGCAGTAATAACATTT	158
4	katA	Forward: AGAAGCTGTTTGGGATTTTT Reverse: CGGCTGCAGTAATAACATTT	190
5	npr	Forward: CAGTAGGGGATGCTACGTTA Reverse: GTTAATCCCTGTTGAAGCAA	194
6	tpx	Forward: TATTAGAATTGACAGGTGAG Reverse: TATCAGGAACCACACTAA	135
7	rex	Forward: TCGGGGTTGGTAATTTAGGAAGT	120

		Reverse: TACCGTCAACAATGCGTCCC	
8	lysR	Forward: GAACGGCTAGACAAAGGATTATTAGA Reverse: ATCAGAGAGTTGGCTCGGAAC	219
9	CcpA	Forward: CTGAGCGTGTCCGTAACAGT Reverse: AGCATCCCATCTAATAAACCAATC	82
10	hprK	Forward: GGACATCGGCTAATCGCAGA Reverse: CCCACGCCTCTAAATAAACGA	199
11	gyrA	Forward: CAAATATTCCACCACACAATTTAAGT Reverse: TTCCCCATCACTAAACCGCC	139
12	23s rRNA	Forward: CCGCAAGGCTAAATACTCCC Reverse: CACGCCATCACTCATTAACG	143

Tab. 28 Primers used in qRT-PCR

2.2 Methods

2.2.1 Anti-*E. faecalis* antiserum preparation

Antiserum preparation was carried out according to the method of Hufnagel with minor modifications (Hufnagel et al., 2004). The protocol was modified according to the guidelines of Charles River (China) and was approved by the ethic procedure of the department of animal science laboratory at Nanchang University (China). *E. faecalis* OG1RF cultured overnight in BHI were collected and inactivated by incubation with formalin for 24 h at room temperature, then concentrated to 2×10^9 CFU/mL with PBS buffer. Immunization with whole bacterial cells was done by injecting bacteria into rabbits subcutaneously once a week, for a total of nine weeks. Rabbits were killed one week after the last injection, precipitated serums were collected, and the final titer was determined by indirect ELISA. After the addition of an equal volume of glycerol, antisera were stored at -20°C until use (for a longer term stored at -80°C).

2.2.2 Adhesion and translocation of bacteria in Ptk6 cell monolayers

Ptk6 cells (Whitehead et al., 2008) seeded into BD falcon dishes (diameter: 15 cm) were grown in RPMI 1640 supplemented with 5% fetal bovine serum (FBS; PAN Biotech), 1%

insulin-transferrin-selenium mix (Invitrogen), and 5 U/mL INF-γ (Invitrogen), and cells were incubated at 37°C under 5% CO_2. RPMI containing all the supplements is referred to as RPMIS in this manuscript. Adhesion and translocation by two-chamber assay were conducted according to the protocol of Zeng with slight alterations (Zeng et al., 2005; Zeng, Teng, Weinstock, & Murray, 2004). 10^5 Ptk6 cells were well distributed onto transwell filters (12-mm well, 3.0 m pore size, rat tail collagen coated polycarbonate membrane, Corning, USA) containing 0.5 ml RPMIS in the upper compartment (lower compartment filled with 1.5 ml RPMIS), and grew for 4 days to form tight junctions, i.e., confluent monolayers (Fig. 5). Prior to infection, Ptk6 cells on filters were washed twice with RPMIS, and both compartments were left medium-free. To achieve a multiplicity of infection (MOI) of 10, approximately 10^6 bacteria in 0.5 mL RPMIS were added into the upper chamber, 1.5 mL RPMIS was added to the lower chamber and incubated at 37°C for 4 h. To determine the total number of bacteria at 4 h, an additional 10^6 bacteria in 0.5 mL RPMIS were added to 1.5 mL of RPMIS in a well without filter and incubated under the same conditions to yield equal bacterial numbers as those in the infection groups. To quantify the adherent bacteria after 4-h infection, media in both chambers were removed and Ptk6 cells were washed 3 times with PBS buffer to remove non-specifically binding bacteria. Ptk6 cells and adherent bacteria were scraped off the filter into 1 mL dH_2O using a cell scraper. Serial dilutions of cell-bacteria suspension were plated on BHI agar plates. Meanwhile, to determine bacterial translocation numbers, viable bacteria in the lower chambers were counted after 4-h incubation by serial dilutions and plating on agar plates. Experiments were repeated at least 3 times. Adhesion and translocation in monolayer treated with EDTA buffer for 20 min (open barrier) are performed likewise.

The effect of cytochalasin D on bacterial translocation was examined using the same system. Ptk6 cell monolayers were pretreated with 5 µg/mL or 10 µg/mL cytochalasin D before infection. Ptk6 cell monolayers without treatment was used as control.

To visualize adherent bacteria in a parallel group, filters with infected Ptk6 cells were stained and observed by laser scanning confocal microscope (LSCM). For immunofluorescence staining, medium on transwell filters was removed by suction pipette and filters were washed twice with PBS buffer, then bacteria and Ptk6 cells were fixed by 2% paraformaldehyde (PFA) in 100mM sodium phosphate buffer (pH 7.4) for 15 min. Following fixation, infected cells were washed three times with PBS buffer, and filters were cut and transferred into a humidified chamber on parafilm. Antibody access into infected Ptk6 cells was achieved via permeabilisation. To accomplish this, cells were incubated with PBS buffer containing 3% BSA, 1% saponin, 0.1% triton X-100, and 0.05% NaN_3 for 10 min at room temperature and

stained with primary antibody (anti-*E. faecalis* antiserum, 1:1000) in PBS buffer containing 3% BSA and 1% saponin, then incubated for 2 h. Thereafter, cells were washed three times with PBS buffer containing 3% BSA and 1% saponin, followed by incubation with secondary antibody and cellular dyes (Alexa Fluor 594-coupled phalloidin, Alexa Fluor 488-coupled goat anti-rabbit IgG, Invitrogen; Vectashield® mounting medium with DAPI, Vector Laboratories, Inc.) in PBS buffer containing 3% BSA and 1% saponin for 40 min in the dark. Prior to mounting, cells were washed five times with PBS buffer containing 3% BSA and 1% saponin, followed by washing with PBS buffer. Filters were sealed on glass slides with nail polish and stored in the dark at 4°C until microscopy.

Samples were imaged with a laser scanning confocal microscope (Nikon), and z-stacks were projected onto three-dimensional reconstructions by Volocity 4.1 software (PerkinElmer Life Sciences). The images were assembled with Photoshop CS (Adobe Systems, magnification: 60×1.5).

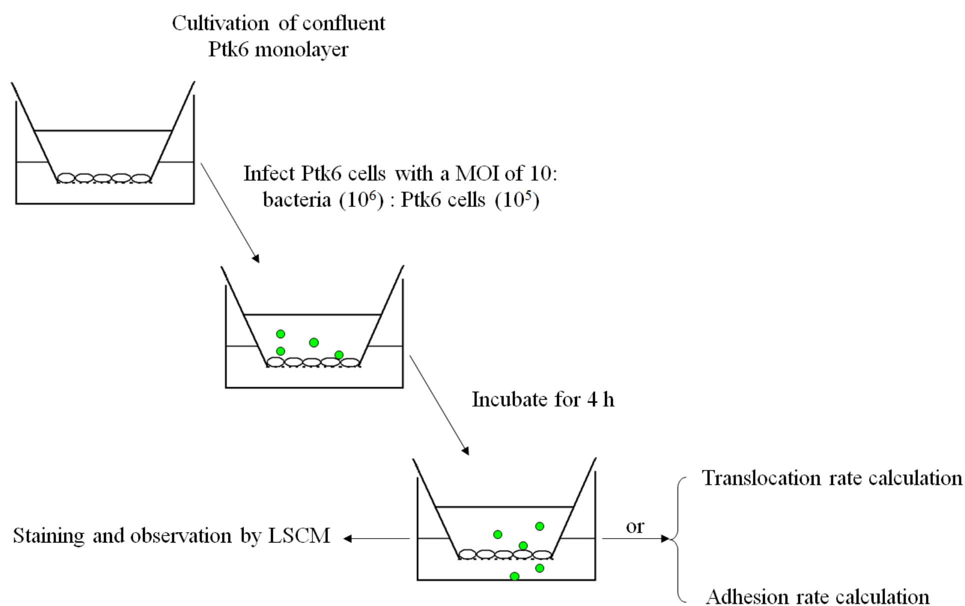


Fig. 5 Model of bacterial adhesion and translocation in two-chamber assay

2.2.3 Adhesion of enterococci to actin

The actin adhesion assay was performed as described in Hwang´s work with minor alterations (J. H. Hwang, Smith, Salhia, & Rutka, 2008). Briefly, 100 µL actin solution (1 µg/µL, Sigma Aldrich) was immobilized onto 96-well plate (Sarstedt, Newton, NC, USA) by

incubation at 37°C for 2 h then at 4°C overnight. The unbound sites were blocked by incubation with 1% BSA in PBS buffer for 2 h at 37°C. Plates were washed three times with PBS buffer, and *E. faecalis* with an OD_{590} of 0.4 was added into each well and incubated at 37°C for 2 h. One blocked well without bacteria was maintained as negative control. Plates were washed three times to remove unbound bacteria, followed by crystal violet (0.5%, w/v) staining at room temperature for 1 min. Crystal violet was then dissolved by acetic acid (5%, v/v), and Abs_{570} was measured on a microtiter plate reader.

2.2.4 Isolation of proteins in enterococci that bind to actin

To isolate adhesion proteins from *E. faecalis* OG1RF, Symbioflor® and V583, bacteria were cultured in BHI media. Bacterial whole proteins were obtained by ultrasonication (70 W, 10 min). Proteins from supernatants were extracted according to the protocols of Sánchez (Sánchez, Schmitter, & Urdaci, 2009) and stored at -20°C until use.

Actin was immobilized to 96-well plates as described above. Unbound sites were blocked by incubation with 1% BSA at 37°C for 2 h. Afterwards, 100 µL sonication-treated bacteria or supernatant proteins were added into each pretreated well and incubated at 37°C for 2 h. Plates were washed twice, 1% SDS buffer was added to each well, and the plates were then incubated at 37° C for 2 h with gentle agitation. Following incubation, wells were dried and adherent proteins were dissociated from wells with Laemmli buffer. The solutes were analyzed by SDS-PAGE, followed by silver staining. Corresponding bands were sequenced via LC–MS/MS (ZfP Zentrallabor für Proteinanalytik, München, Germany).

2.2.5 Specificity evaluation of actin as enterococcal receptor

To determine the specificity of actin as the receptor for enterococcal adhesion, ten strains of lactobacilli were applied to immobilized actin. Adhesion ability of ten lactobacilli to actin was evaluated as described in chapter 2.2.3. Moreover, isolation of actin-binding proteins in lactobacilli was performed as described in chapter 2.2.4.

Besides actin, other host receptors like mucin (two concentrations: 100 µg/well, 1 mg/well) and fibronectin (two concentrations: 1 µg/well, 10 µg/well) were also immobilized to 96-well plates to evaluate bacterial (ten strains of lactobacilli and three strains of enterococci) ability to adhere as indicated in chapter 2.2.3.

2.2.6 PGK-actin binding domain analyse

PGK was isolated from lactobacilli as an actin-binding protein and the specific binding site in PGK was investigated. To amplify *pgk* from ten lactobacilli, genomes from overnight-cultured lactobacilli were extracted with E.Z.N.A.® bacterial DNA kit (Omega, Biotek) and used as templates for polymerase chain reaction. Two pairs of primers were designed for three genera of lactobacilli (Tab. 27). Amplified *pgk* was purified with E.Z.N.A. cycle pure kit (Omega, Biotek) and sent to sequence (GATC Biotech). Obtained *pgk* sequences were aligned with reported PGK-actin binding domain in group B *Streptococcus* (GBS) (Boone & Tyrrell, 2012) by software GENtle.

2.2.7 *Pts* mutants construction

Competent *E. faecalis* was prepared according to Holo *et al* (Holo & Nes, 1989). Briefly, 2 mL *E. faecalis* OG1RF was grown still in 100 mL fresh SGBHI for 24-36 h to reach a minimum optical density of 0.3. Bacteria were then washed twice with 50 mL pre-cooling electroporation buffer without vortex, then resuspended cells in 500 µL electroporation buffer. Aliquots of 40 µL cell suspension were instantly frozen by liquid nitrogen and stored at -80°C till use.

Derived OG1RF strain lack of OG1RF_12401 (encoding mfs-PTS, EIID) and OG1RF_12402 (encoding mfs-PTS, EIIC) genes is designated as *pts*1 mutant (Δ*pts*1); derived OG1RF strain lack of OG1RF_11511 (encoding mfs-PTS, EIID) and OG1RF_11512 (encoding mfs-PTS, EIIC) genes is designated as *pts*2 mutant (Δ*pts*2); derived OG1RF strain lack of OG1RF_12401, OG1RF_12402, OG1RF_11511 and OG1RF_11512 genes is designated as *pts*1/2 mutant (Δ*pts*1/2), i.e., Δ*pts*1/2 combined absence of *pts*1 and *pts*2 genes (Fig. 3). To generate Δ*pts*1 by homologous recombination, the DNA fragments (upstream of OG1RF_12402 and downstream of OG1RF_12401) was PCR-amplified from *E. faecalis* OG1RF genomic DNA with primers F_{pts1U}, R_{pts1U} (for upstream DNA) and F_{pts1D}, R_{pts1D} (for downstream DNA). The two amplified DNA fragments were fused by ligase at Kpn I site. Integrated product was inserted into *E. coli*–enterococcus shuttle vector pCJK47 (Fig. 6) from *E. coli* TMW 2.1365. Reconstructed pCJK47 was electro-transformed (25 µF, 0.2-cm cuvettes, 200 Ω, ~10000 V/cm) into *E. coli* TMW 2.1363 (Em^R) and propagated on BHI agar plates supplemented with erythromycin (300 µg/mL), IPTG (24 µg/mL) and X-gal (40 µg/mL) till blue colonies appeared (Kristich, Chandler, & Dunny, 2007). The reconstructed vectors pCJK47 confirmed by PCR with primers F_{pts1U} and R_{pts1D} were extracted from culture of blue

colony then electroporated into competent *E. faecalis* OG1RF and then grew on BHI agar plates supplemented with erythromycin, IPTG and X-gal with the same concentrations as indicated above (Dunny, Lee, & Leblanc, 1991). Blue colonies were screened for single cross-over mutants by PCR with primers F_{pts1U}-R_{pts1O} and F_{pts1O}-R_{pts1D} (principle of single cross-over as shown in Fig. 7). Single cross-over mutants were inoculated to BHI broth without antibiotic then plated serial dilutions on MM9YEG-agar plates with p-chlorophenylalanine (200 μg/mL). Appeared white colonies were restreaked on MM9YEG-agar plates with p-chlorophenylalanine and verified by PCR with primers F_{pts1I}-R_{pts1I} (inner fragment check) and F_{pts1O}-R_{pts1O} (outer fragment check) for double cross-over mutants. DNA of *pts*1 and its surrounding genes was amplified from reconstructed plasmid and sent to sequence with primer P_{pts1S}. The construction of Δ*pts*2 and Δ*pts*1/2 was performed likewise. All primers used in this section are listed in Tab. 26.

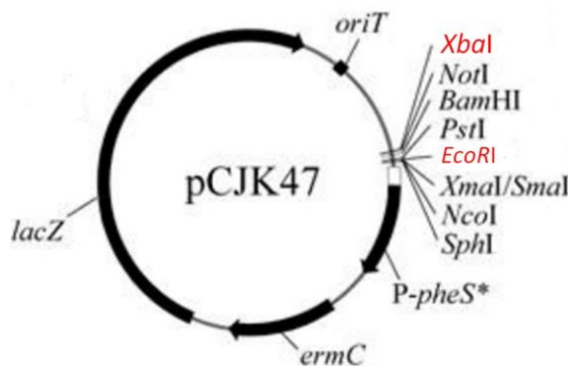


Fig. 6 Sketch of plasmid pCJK47. Red ones are cleavage sites used in this study

This sketch is cited from: Kristich CJ, Chandler JR, Dunny GM. Development of a host-genotype-independent counterselectable marker and a high-frequency conjugative delivery system and their use in genetic analysis of Enterococcus faecalis. Plasmid. 2007 Mar;57(2):131-44.

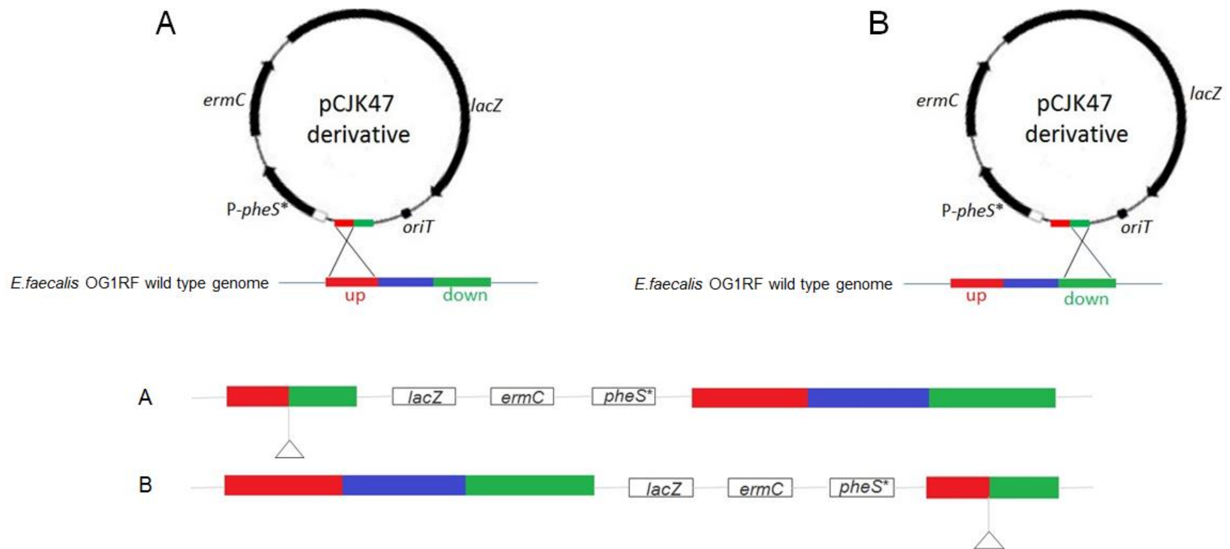


Fig. 7 Principle of single cross-over in homologous recombination

2.2.8 Preferential utilization of different sugars

API test (Bio-Mérieux, France) was applied to examine briefly which sugars could be utilized by *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, and Δ*pts*1/2. Later on, carbon source selection was performed based on two media, both of which were modified to contain galactose (5 g/L) as non-preferential carbon source and one hypothetic preferential sugar (5 g/L). One is developed from Davis minimal medium (B. E. Murray et al., 1993), the other is MRS medium. *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, and Δ*pts*1/2 were grown in both media with glucose, mannose, fructose, and sorbose (5 g/L) individually as the putative preferential carbon source with a starting OD_{590} of 0.05 and OD_{590} values were measured every two hours.

The amount of glucose consumed by *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, and Δ*pts*1/2 in MRS-based media was estimated by HPLC. To prepare HPLC samples, 750 µL each of *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, and Δ*pts*1/2 grew to mid-exponential phase were collected by centrifugation, and individually treated with 450 µL $ZnSO4 \cdot 7H_2O$ (10%, w/v), vortex and let stand for 20 min. Subsequently, supernatant was achieved by centrifugation and then sterilized by 0.2 µm filter. MRS medium without fermentation treated in the same method was used as baseline control. Samples were applied to ion chromatography to quantify glucose (Thermo Scientific™, Dionex™).

2.2.9 Yeast agglutination assay

Enterococci were pre-cultured in MRS media with different sugars (glucose, mannose, fructose) to induce mannose-sensitive strains. Coaggregation assay was performed according to Pretzer *et al* with modifications (Pretzer, Snel, Molenaar, Wiersma, Bron, Lambert, Vos, Meer, Smits, et al., 2005). In brief, bacteria were grown in MRS overnight, washed and suspended with PBS buffer, then OD_{590} of suspension was adjusted to 0.3. Fifty microliter bacterial suspension in PBS buffer was transferred into microtiter plates (96-well U-shaped; Greiner bio-one, Alphen a/d Rijn, The Netherlands). *Saccharomyces* (*S.*) *cerevisiae* was grown overnight in malt broth medium and washed three times by PBS buffer. To each well of bacterial suspension, 50 µL PBS buffer or PBS with methyl-α-D-mannopyranoside (inhibitor, final concentration: 25 mM; Sigma-Aldrich) was added as well as 100 µL *S. cerevisiae* suspension (1%, w/v). Turbidity of *S. cerevisiae* and bacteria mixture was measured every 10 minutes.

The ability of each strain to induce visible yeast cell agglutination was determined by microscopy in three independent experiments.

2.2.10 Resistance to pediocin

Agar-drop-test was used to survey the resistance of enterococci to bacteriocin (Fleming, Etchells, & Costilow, 1975). Ten microliter *Pediococcus* (*P.*) *acidiactici* overnight culture was dropped onto the MRS agar plate till dry. The MRS medium in this assay was specified (containning only 2 g/L glucose) to reduce the acid production by *P. acidiactici*, to avoid false negtive due to the possibility that these acids would inhibit the growth of enterococci. One hundred microliter enterococci overnight culture (OD_{590}=0.8) was innoculated into 7 mL 5% agar BHI medium (heated to liquid and incubated in 50°C water bath prior to innoculation) with or without 1 mg/mL catalase. Catalase protects enterococci from being killed by the hydrogen peroxide produced by *P. acidiactici*. Then the mixture were poured onto the MRS agar plates with dried drops of *P. acidiactici*. Plates were incubated at 37°C, inhibition zones were measured after 20-h incubation.

2.2.11 SOD-fluorescence activation by enterococci in nematote

Caenorhabditis (C.) elegans with GFP reporter in SOD were grown on LB agar plates with *E. coli* lawn as food source. Around 20 *C.elegans* with equal size and growth phase were

transferred onto BHI agar plates spread uniformly with 100 µL *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, or Δ*pts*1/2. Plates were incubated at 37°C for around 20 h.

For agar pad preparation (Fig. 8): place the two taped slides with a clean slide sandwiched between them on a flat surface. Using the Pasteur pipette, place a drop of agar onto the clean slide. Cover the agar with another clean slide placed on top of the three slides in a perpendicular fashion. Press gently so the agar drop is flattened to a circle about 0.4 mm thick (the thickness of the tape spacers). Avoid getting bubbles in the agar since worms will get stuck in them. After the agar solidifies, gently pull out the taped slides, then separate the remaining two slides by sliding one relative to the other. The agar pad should adhere to one of the slides (usually the bottom one). Rest the slide, agar side up, on the bench top.
Place a 1–2 µL drop of 10–25 mM sodium azide (NaN3) onto the center of the agar pad. NaN3 anesthetizes the worms so that they will not move. Transfer *C. elegans* to be observed with a worm pick into the drop then put on top the cover glass. Pictures of GFP fluorescence were taken on an Leica DMRE fluorescence microscope.

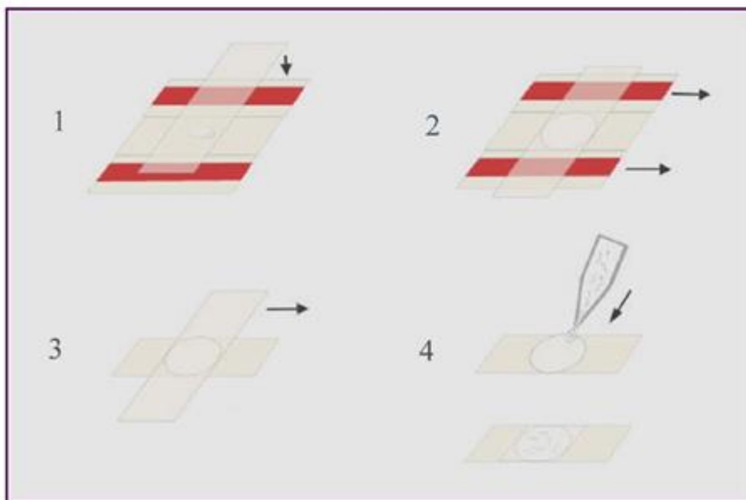


Fig. 8 General view of agar pad preparation

2.2.12 Tolerance to acid and hydrogen peroxide

Impacts of acid and hydrogen peroxide on *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, and Δ*pts*1/2 were verified by growth behavior and survival assay. BHI media were modified to low pH (pH 5.5) or with 0.5 mM hydrogen peroxide. *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, and Δ*pts*1/2 were innoculated into modified BHI above to achieve growth curves.

To investigate the tolerance of *E. faecalis* to acid and hydrogen peroxide, *E. faecalis* grew in BHI till exponential phase was washed by PBS buffer then incubated in buffers with final pH

of 4.1 or in the presence of 25 mM hydrogen peroxide at 37°C (Baureder, Reimann, & Hederstedt, 2012; Lenz, Hew Ferstl, & Vogel, 2010). Aliquots were taken at a certain interval, serially diluted and plated on BHI agar plates. Plates were incubated at 37°C for 18 h before colonies were counted.

2.2.13 Invasion and survival of enterococci in macrophages

The macrophage infection assay was performed according to Kajfasz (Kajfasz et al., 2012). Briefly, the murine macrophage cell line J774A.1 (Institut für Med. Mikrobiologie, Immunologie und Hygiene. Technische Universität München) were grown to confluent in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with GlutaMAX (Invitrogen, Grand Island, New York), 10% fetal calf serum, 50 U/ml penicillin G, and 50 µg/ml streptomycin at 37°C under 5% CO_2 atmosphere. J774A.1 cells were seeded into 12-well tissue culture plates (tissue culture treated) 12 h before infection at a density of 1×10^6 macrophages per well. Overnight cultures of *E. faecalis* OG1RF and all *pts* mutational strains in BHI media were washed and resuspended in equal volume of Dulbecco's Phosphate Buffered Saline (DPBS) buffer and diluted to 2×10^7 CFU/mL with DMEM lack of antibiotics. For infection assay, 1 milliliter bacterial suspension was added to the macrophage monolayers, yielding an approximate multiplicity of infection (MOI) of 20. Bacteria-macrophage contact was enhanced by centrifugation at 500×g for 5 min (4°C) followed by further incubation at 37°C under 5% CO_2 atmosphere for 90 min. Extracellular bacteria were killed by incubation with DMEM containing 300 µg/mL gentamicin and 50 µg /mL penicillin G for 90 min. Macrophage cells were lysed with sterile ice cold 0.1% triton X-100 buffer, serially diluted and plated on BHI agar plates for enumeration of viable internalized bacteria. For post-infection in parallel groups, macrophage J774A.1 infected with enterococci were refreshed every other day with DMEM supplemented with GlutaMAX, 10% fetal bovine serum, 50 U/mL penicillin G, and 50 µg/mL streptomycin and 30 µg/mL gentamicin, and incubated at 37°C under 5% CO_2 atmosphere for 48 h and 72 h. Experiments were repeated independently as least three times.

Survival of enterococci in bone marrow-derived macrophages (BMMs) was performed likewise. Praparation of BMMs was conducted by the guidelines of Marim, *et al* with modifications (Marim, Silveira, Lima, & Zamboni, 2010). The protocols for animal handling were previously approved by animal ethics committee in the institute. Femurs were obtained by dissection of 6-week old BALB/c mice euthanized and then sterilized by 70% ethanol. Take care that bones should be kept intact (from tibia below the knee joints to pelvic bone

close to the hip joint) so inner cells are axenic. Consequent bones were kept in petri dish containing sterile PBS buffer for further operations. In a tissue culture hood, bones were opened at the epiphysis sites using sterile scissors and forceps. Bone marrows were flushed through 70 µm cell strainer into 50 mL falcon tube with a syringe filled with DMEM (4.5 g/L glucose) supplimented with 10% FCS, 1% penstrep, and 0.1% β-mercaptoethanol (following mentioned DMEM always contained these additives). Bone marrows were resuspended in 10 mL DMEM and then went halves into two culture dishes (non-tissue culture treated) with 10 mL DMEM containning 10% M-CSF (LCCM), and incubated at 37°C under 5% CO_2 atmosphere. Semi-adherent cells were collected by washing and resuspending on the next day. Plated 6×10^6 cells in DMEM containning 10% M-CSF to culture dish and incubated at 37°C under 5% CO_2 atmosphere. Culture media with M-CSF were refreshed on the third day. The homogenous, proliferative, transfectable BMMs were ready on day 7, desired number of macrophage (1×10^6/well) in transwell should be prepared 12 h before infection experiments (on day 6).

2.2.14 Relative quantification of antioxidant and energy-related genes by qRT-PCR

This study is based on the observation that *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, and Δ*pts*1/2 displayed different tolerance to hydrogen peroxide and different survival abilities in two types of macrophages. PTS is speculated be involved in regulation of survival-related (antioxidant and energy-related) genes. Ten antioxidant or energy-related genes were selected for investigation (Tab. 28). *Gyr*A, 23s rRNA were used as internal control.

For RNA extraction, *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, and Δ*pts*1/2 were cultured in BHI media with or without 2.4 mM hydrogen peroxide till exponential phase. Bacteria were washed once with TE buffer, pellets were aliquot in EP tubes and instantly frozen with liquid nitrogen, stored at -80°C till use. RNA extraction was proceeded according to the instruction of RNeasy midi kit (QIAGEN, Germany). Additionally, RNA samples were digested by RNase-free DNase in RNA extraction process. The purity and concentration was verified by agarose gel electrophoresis and Nanodrop, RNA was stored at -80°C till use.

Before quantitative real time PCR, RNA was reverse transcribed into cDNA with IscriptTM reverse transcription supermix for RT-qPCR (BIO-RAD, USA). The amplification efficiency of each primer was confirmed by performing the standard curve assay. With the synthesized cDNA as template and maxima SYBR Green/ROX qPCR (Thermo scientific, USA) as

polymerase for quantitative real time PCR, detection of gene expression was carried out in PikoReal 24 Real-Time PCR system. Data were analysed with PikoReal Software 2.1 (Fig. 9).

$$\Delta Ct_{calibrator} = Ct_{calibrator} - Ct_{ref}$$

$$\Delta Ct_{sample} = Ct_{sample} - Ct_{ref}$$

$$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{calibrator}$$

$$\text{Relative quantity (quantity of sample/quantity of calibrator)} = 2^{-\Delta\Delta Ct}$$

Fig. 9 Formular of relative quantification in qRT-PCR

3 Results

3.1 Adhesion and translocation of lactic acid bacteria to epithelial cells

3.1.1 Adhesion and translocation of enterococci to epithelial cells

3.1.1.1 Binding and translocation in functional and dysfunctional Ptk6 cell monolayers

Adhesion and translocation of *E. faecalis* OG1RF, Symbioflor®, V583 and *E. coli* Top10 were studied in the Ptk6 cell monolayer model. Bacterial adhesion and translocation numbers were determined by colony counting in plates. As shown in Fig. 10A, all bacteria exhibited strong adhesion ability, which was in the same range for all strains tested. About 1 out of 1,000 bacteria in the close barrier could not be removed from the cells by the washing procedure. Adhesion in open barrier displayed no significant difference in comparison to the close barrier. Ability of bacteria to translocate the barrier varies (Fig. 10B). Open barrier significantly enhanced bacterial translocation to different degrees.

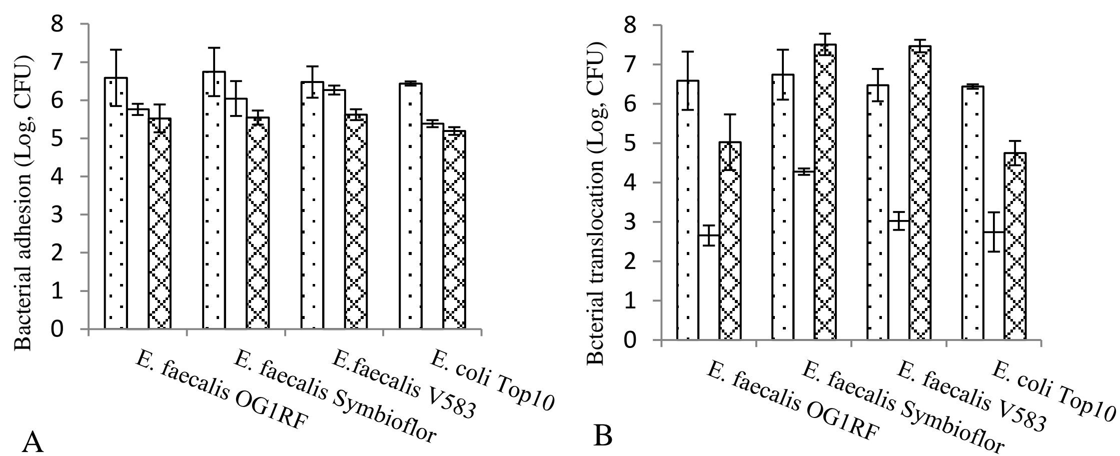


Fig. 10 Adhesion (A) and translocation (B) of enterococci and *E.coli* in close and open barrier. Statistics are average values ± standard deviation. ⊡ CFU of bacteria at 0 h; ☐ CFU of bacteria at 4 h in close barrier; ⊠ CFU of bacteria at 4 h in open barrier

3.1.1.2 Visualisation by laser scanning confocal microscope analysis

To visualize the location of the enterococci and *E. coli* in Ptk6 cell monolayers, filters with infected Ptk6 cells were mounted on glass slides. Actin cytoskeleton and nuclei in eukaryotic cells was stained with phalloidin and DAPI, respectively. Bacteria were stained with anti-*E. faecalis* antiserum (titer of antiserum was examined) and secondary antibody. Laser scanning confocal microscope analysis of stained samples was carried out to locate the bacteria in the

Ptk6 monolayers. Fig. 11 shows a 3-D reconstruction of the LSCM analyses. Enterococci were detected frequently within the Ptk6 cells in actin-rich areas. *E. coli* was also detected in

Part of the results in chapter “3.1” was published in: Zhen Peng, Viktoria Krey, Hua Wei, Qianglai Tan, Roger Vogelmann, Matthias A, Ehrmann, Rudi F. Vogel. Impact of actin on adhesion and translocation of *Enterococcus faecalis*. Arch Microbiol. 2014, 196: 109-117.

actin-rich areas within the Ptk6 cells, but in a limited number of visual fields. The x–z views clearly demonstrate that the bacteria are not just adhering to the cellular surface but are in peripheral actin along cellular skeleton within the monolayer.

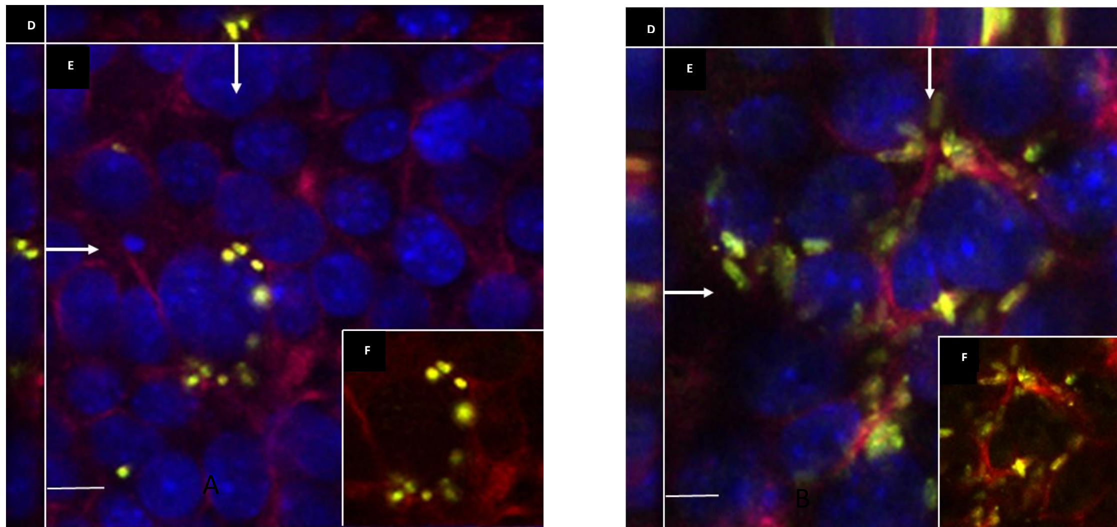


Fig. 11 3-D reconstruction of confocalimmunofluorescence images of confluent Ptk6 cells infected by *E. faecalis* Symbioflor® (A) and *E.coli* top10 (B). Bacteria (green and yellow), actin (red) and nuclei (blue). D are reconstructions of z-sections; arrows in E mark point where x-z view was taken. F represents a close up view of an attached colony. Scale bar: — 10 µm

3.1.2 Translocation of enterococci through Ptk6 monolayers

Translocation in confluent Ptk6 monolayer (without treatment of cytochalasin D) varied significantly among the strains (Fig. 12). *E. faecalis* Symbioflor® demonstrated the highest number of translocated cells (10^4 CFU), followed by strain V583 (10^3 CFU) and strain OG1RF (10^2 CFU). *E.coli* top10 in the control group displayed a similar translocation to OG1RF.

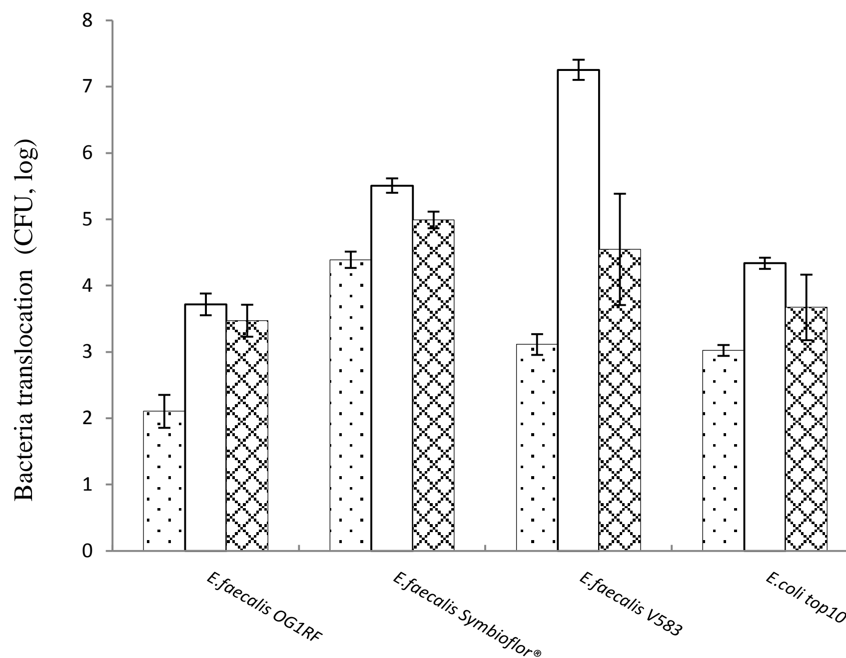
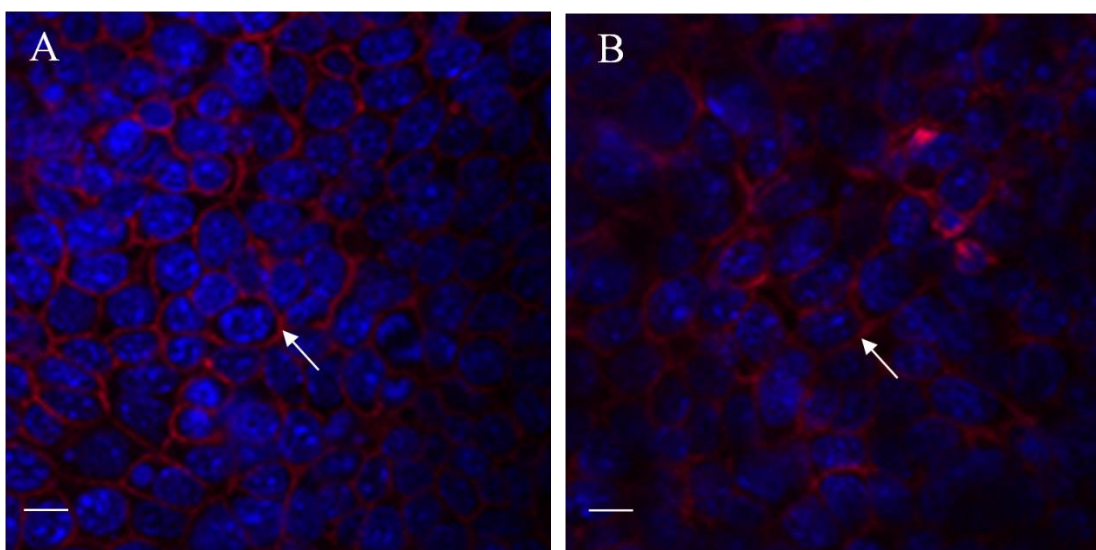


Fig. 12 Effect of cytochalasin D on enterococcal translocation in Ptk6 cell monolayers. Ptk6 cell monolayers were treated with 5 µg/mL and 10 µg/mL of cytochalasin D before infection (non-treated PTK6 cell monolayer as control), translocated bacteria in the lower chamber were calculated after 4 h incubation. Statistics are average values ± standard deviation. ☐ without cytochalasin D; ☐ 5 µg/mL cytochalasin D; ☒ 10 µg/mL cytochalasin D

3.1.3 Role of actin aggregation in bacterial translocation process

To gain a better understanding of the role of actin aggregation in bacterial attachment and translocation, Ptk6 monolayers were treated with cytochalasin D. Cytochalasin D is an actin polymerization inhibitor that works in an unconventional concentration-dependent manner: higher concentrations of cytochalasin D are less effective than lower concentrations in altering tight junction permeability and the normal organization of actin aggregation (Stevenson and Begg 1994). In the current study, Ptk6 cell monolayers were severely disrupted at a cytochalasin D concentration of 5 µg/mL, and actin was degraded to a critical degree as shown in Fig. 13C, resulting in an open barrier, which rendered bacterial translocation rates of all strains tested to maximal. At the concentration of 10 µg/mL, however, actin aggregation in Ptk6 cells were only partially disrupted, and a less compact meshwork with half open barrier was formed, which resulted in a decrease in bacterial translocation (Fig. 13B). Translocation of all strains was influenced by the degree of actin aggregation, namely the intactness/disruption of the Ptk6 monolayers (Fig. 14). While the same trends were detected for all strains, translocation of *E. faecalis* V583 was 10,000-fold increased by cytochalasin D treatment of Ptk6 monolayers (Fig. 12), and strain OG1RF was 100-fold increased while translocation of *E. faecalis* Symbioflor® was the least affected (10-fold increased). *E. faecalis* Symbioflor® exhibited the highest translocation rate among the strains tested when actin aggregation was permitted (namely, intact Ptk6 monolayers without treatment of cytochalasin D).



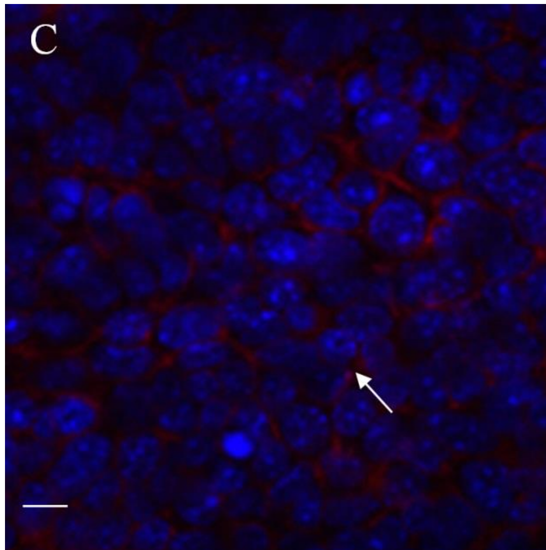


Fig. 13 Confocal immunofluorescence images of effect of cytochalasin D on confluent PTK6 cells.
A, without treatment, normal actin bundles; B, treated with 10 µg/mL cytochalasin D, actin network remains but partially disrupted; C, treated with 5 µg/mL cytochalasin D, actin severly disrupted.
actin (red) and nuclei (blue). Scale bar: — 10 µm

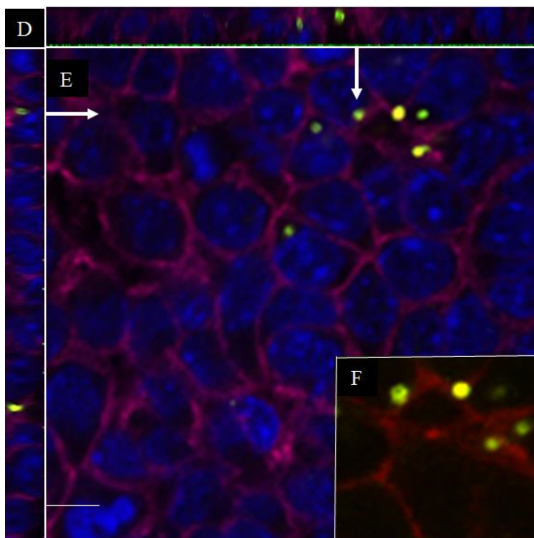


Fig. 14 3-D reconstruction of confocal immunofluorescence images of Cytochalasin D (5 µg/mL) treated confluent Ptk6 cells infected by *E. faecalis* V583 (MOI of 10).
Bacteria (green and yellow), actin (red) and nuclei (blue). D are reconstructions of z-sections; arrows in E mark point where x-z view was taken. F represents a close up view of an attached colony. Scale bar: — 10 µm

3.1.4 Adhesion of lactic acid bacteria to cell wall polymers

3.1.4.1 Binding of enterococci to actin

To further elucidate a possible role of actin in mediation of enterococci. *E. faecalis* OG1RF, *E. faecalis* Symbioflor®, and *E. faecalis* V583 were investigated for their adhesion abilities to actin. As shown in Fig. 15, the Abs_{570} of *E. faecalis* OG1RF, *E. faecalis* Symbioflor®, and *E. faecalis* V583 ranged from 0.1 to 0.12. All displayed similar, albeit not strong, adhesion to actin.

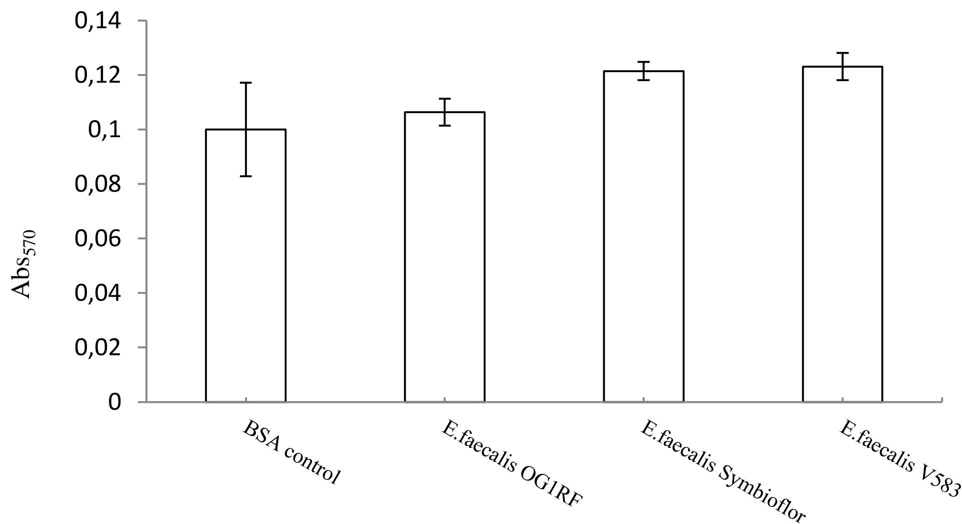


Fig. 15 Adhesion of enterococci and BSA to actin. Bacterial adhesion to actin in 96-well were measured by Abs_{570}. Statistics are average values ± standard deviation

3.1.4.1.1 Identification of actin-binding proteins

Actin-binding proteins were isolated from *E. faecalis* OG1RF, *E. faecalis* Symbioflor®, and *E. faecalis* V583. Bacterial extracts obtained by sonication and recovery of supernatant proteins were employed to isolate actin-binding proteins (Fig. 16). After sodium dodecyl sulfate polyacrylamide gel electrophoresis, bands were excised from gels and identified by LC–MS/MS (see Tab. 29 for peptide information). Four proteins from three strains of *E. faecalis* were identified as pyruvate formate lyase (95.7 kDa, arrow 1), enolase (55.3 kDa, arrow 3), glyceraldehyde-3-phosphate dehydrogenase (38.6 kDa, arrow 4), and GroEL (69.2 kDa, arrow 2). No adhesion protein was detected in supernatants (data not shown).

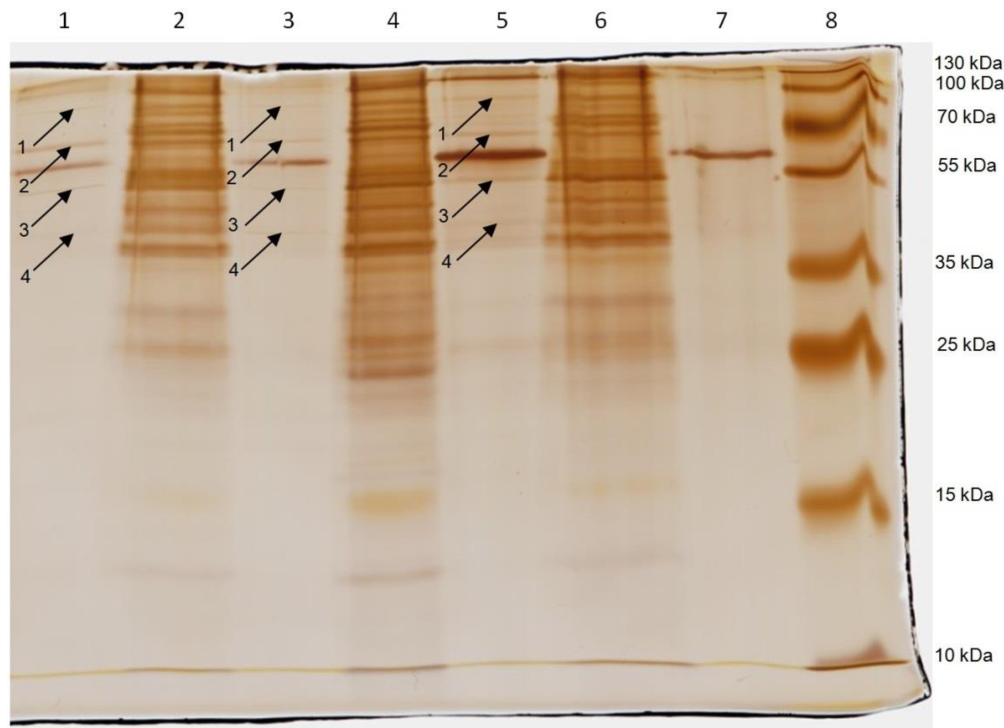


Fig. 16 Actin-binding proteins in enterococci separated by SDS-PAGE gel. Lane 1-8 are, actin-binding proteins isolated from *E. faecalis* OG1RF; ultrasonic cell debris of *E. faecalis* OG1RF; actin-binding proteins isolated from *E. faecalis* Symbioflor®; ultrasonic cell debris of *E. faecalis* Symbioflor®; actin-binding proteins isolated from *E. faecalis* V583; ultrasonic cell debris of *E. faecalis* V583; BSA binding to actin (negative control); PageRuler Plus Prestained Protein Ladder (10-250 KDa)

Mark number of band in the SDS-PAGE gel	Protein name	Peptides
1	pyruvate formate lyase	YAYEAPQL ALMDTDLQRT FATGIAGISH ATDSIMAIKH GEVEVIRDED GMAIDYVPTK
2	GroEL	SFGSPLITN DGVTIAKEIE LEDHFENMGA KLVSEVASKT NDIAGDGTTT ATVLTQAIVR
3	enolase	LTEVLGDKV QLVGDDLFVT NTTKLAEGIE KGIANSILIK
4	glyceraldehyde-3-phosphate dehydrogenase	VPVATG SLTELVTVLD KEVTVDEVNA VMEK

Tab. 29 Partial peptides of actin-binding protein in *E. faecalis*

3.1.4.2 Binding of lactobacilli to actin

In an effort to find out if actin is the receptor only for enterococci, ten lactobacilli were appllied to evaluate the ability to bind actin. Of the ten lactobacilli, *L. paracasei* TMW 1.1434, *L. plantarum* TMW 1.1734, and *L. brevis* TMW 1.485 displayed comparatively strong adhesion to actin in two conentrations, and the average Abs_{570} were 0.32, 0.25, 0.18, respectively (Fig. 17). BSA was the baseline set as a negtive control with Abs_{570} of 0.065 (not present in Figures). With regard to the more than 2.8 fold stronger adhesion ability of *L. paracasei* TMW 1.1434, *L. plantarum* TMW 1.1734, and *L. brevis* TMW 1.485 than that of BSA control, the three strains were therefore further applied to isolation of actin-binding proteins.

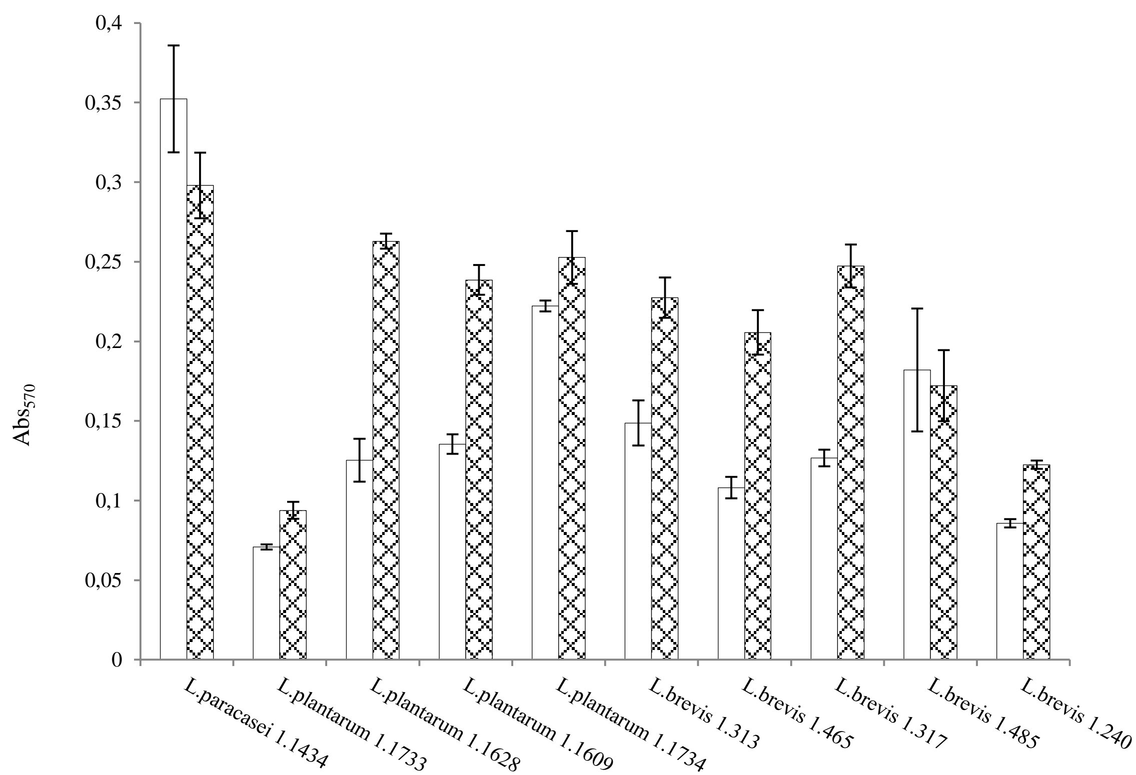


Fig. 17 Adhesion of lactobacilli to two concentrations of actin. Statistics are average values ± standard deviations. □10 µg/well; ▧100 µg/well

3.1.4.2.1 Identification of actin-binding proteins

Three highly adhesive lactobacilli were chosen for actin-binding protein isolation. *L. paracasei* TMW 1.1434, *L. plantarum* TMW 1.1734, and *L. brevis* TMW 1.485 all yielded actin-binding proteins (Fig. 18). The amount of band containning actin-binding protein for *L. paracasei* TMW 1.1434, *L. plantarum* TMW 1.1734, and *L. brevis* TMW 1.485 are 7, 5 and 6, respectively. Due to low concentration preventing from sequence, only six of the bands were sent to sequence by LC-MS/MS. These actin-binding proteins are pyruvate kinase (MW: 68 kDa), glucose-6-phosphate isomerase (MW: 49.5 kDa), and phosphoglycerate kinase (MW: 45 kDa) from *L. paracasei* TMW 1.1434; pyruvate kinase (MW: 68 kDa), chaperonin GroEL (MW: 60 kDa), and EF-Tu (MW: 47 kDa) from *L. plantarum* TMW 1.1734, and chaperonin GroEL (MW: 60 kDa) from *L. brevis* TMW 1.485. It is worth noting that, BSA could also be separated in this method, even though it shows a very negative adhesion ability to actin in the microtiter plate. We argue that this highly purified protein with a high concentration could promote its adehsion, this phenomena is also seen in Sanchez´s work (Sánchez, Schmitter, & Urdaci, 2009).

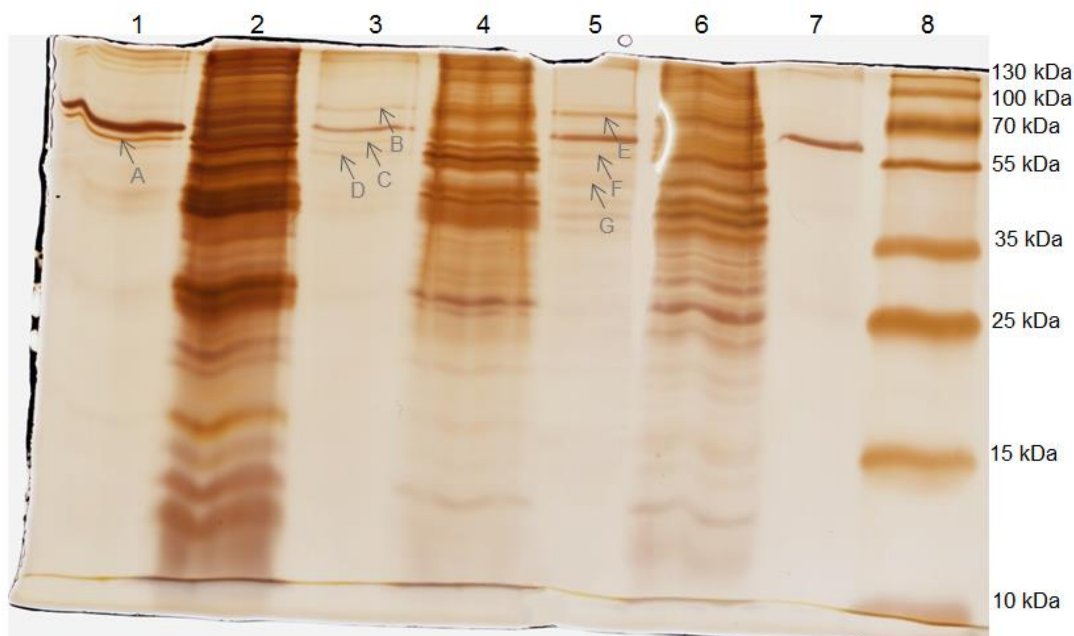


Fig. 18 Actin-binding proteins in lactobacilli separated by SDS-PAGE gel. Lane 1-8 are: actin-binding proteins isolated from *L. brevis* TMW 1.485; ultrasonic cell debris of *L. brevis* TMW 1.485; actin-binding proteins isolated from *L. plantarum* TMW 1.1734; ultrasonic cell debris of *L. plantarum* TMW 1.1734; actin-binding proteins isolated from *L. paracasei* TMW 1.1434; ultrasonic cell debris of *L. paracasei* TMW 1.1434; BSA binding to actin (negative control); PageRuler Plus Prestained Protein Ladder (10-250 KDa)

3.1.4.2.2 Identification of PGK-actin binding domain analysis

According to the PGK-actin domain KKESKNDEE in group B *Streptococcus* reported by Boone and Tyrrell (Boone & Tyrrell, 2012), the binding domain between actin and PGK (Phosphoglycerate kinase) from ten lactobacilli were identified and compared (Tab. 30). The PGK-actin binding domain in *L. paracasei* TMW 1.1434 was found out to be KKESGNDPE, and for the other nine lactobacilli are KRESGNDPE. The only difference in the binding site of PGK from ten bacterial strains is the lysine (K) in *L. paracasei* TMW 1.1434 being replaced by arginine (R) in other nine *Lactobacillus* strains.

Strains	binding domain	reference
Group B *Streptococcus*	KKESKNDEE	Boone, T. J., & Tyrrell, G. J. (2012)
L. paracasei TMW 1.1434	KKESGNDPE	this study
L. plantarum TMW 1.1733 TMW 1.1628 TMW 1.1609 TMW 1.1734 *L. brevis* TMW 1.313 TMW 1.465 TMW 1.317 TMW 1.485 TMW 1.240	KRESGNDPE	this study

Tab. 30 Identification of PGK-actin binding domain in lactobacilli

3.1.4.3 Binding of bacteria to mucin

To find out if there are other receptors for lactic acid bacteria, ten lactobacilli and three enterococci were appllied to evaluate the ability to bind mucin. Ten lactobacilli and three enterococci displayed adhesion to two concentrations of mucin (Fig. 19). *L. paracasei* TMW 1.1434, *L. plantarum* TMW 1.1609, *L. plantarum* TMW 1.1628, *L. plantarum* TMW 1.1734, and *L. brevis* TMW 1.485 displayed comparatively strong adhesion to mucin in two conentrations, and the average Abs_{570} were 0.22, 0.14, 0.16, 0.21, and 0.13, respectively while the Abs_{570} of BSA control was 0.065. In comparison with lactobacilli, enterococci displayed weak adhesion. Besides, mucin as receptor could retain less lactobacilli and enterococci than actin.

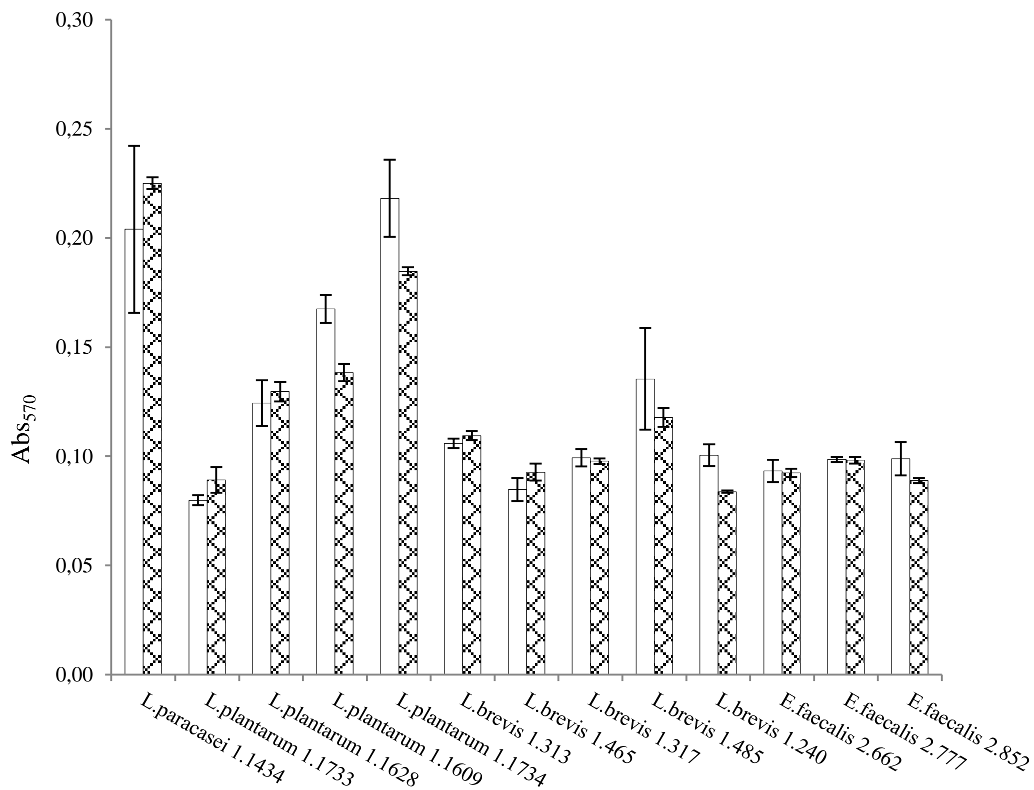


Fig. 19 Adhesion of ten lactobacilli and three enterococci to two concentrations of mucin. Statistics are average values ± standard deviations. □100 µg/well; ⊠1 mg/well

3.1.4.4 Binding of bacteria to fibronectin

To find out if there are other binding receptors apart from actin for lactic acid bacteria, ten lactobacilli and three enterococci were appllied to evaluate the ability to bind fibronectin. Ten lactobacilli and three enterococci displayed adhesion to two concentrations of fibronectin (Fig. 20). *L. paracasei* TMW 1.1434, *L. plantarum* TMW 1.1609, *L. plantarum* TMW 1.1734, and *L. brevis* TMW 1.465 displayed comparatively strong adhesion to fibronectin in two conentrations, and the average Abs_{570} were 0.18, 0.17, 0.25, and 0.15, respectively while the Abs_{570} of BSA control was 0.065. In comparison with lactobacilli, enterococci displayed weak adhesion. Besides, fibronectin as receptor could retain less lactobacilli and enterococci than actin.

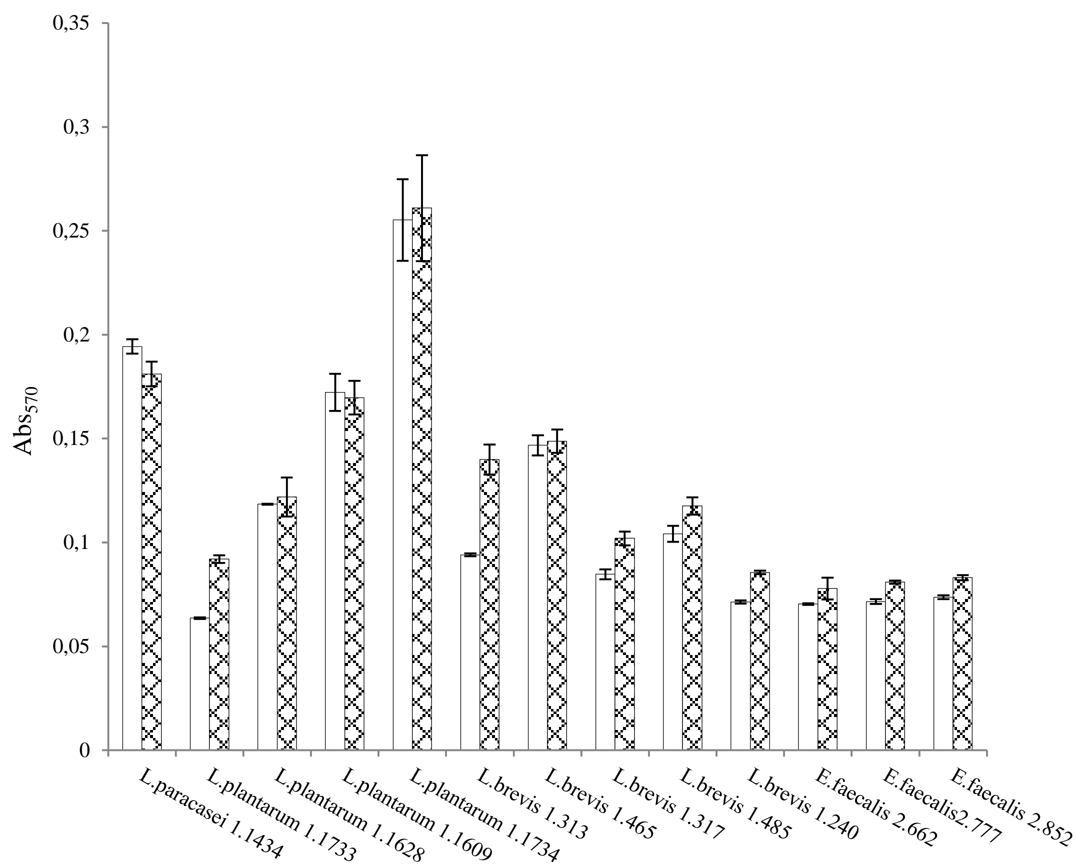


Fig. 20 Adhesion of ten lactobacilli and three enterococci to two concentrations of fibronectin. Statistics are average values ± standard deviations. □1 µg/well; ⊠10 µg/well

3.2 Characterization of phosphotransferase knock out mutants

3.2.1 *Pts* mutants construction

As the level of mannose/fructose/sorbose phosphotransferase systems in *E. faecalis* OG1RF was up to 7-fold up-regulated when bacterium passed through the intestine of mouse in comparison to the bacterium residing in culture media. Mannose/fructose/sorbose phosphotransferase system is therefore speculated to involve in bacterial virulence. PTS knock out mutants lacking the EIIC and EIID components were constructed to find out the influence of mannose/fructose/sorbose phosphotransferase systems on sugar utilization, aggregation ability, stress tolerance and survival in macrophages.

By homologous recombination, 79.5% of *pts*1 (OG1RF_12401 and OG1RF_12402) and 80.4% of *pts*2 (OG1RF_11511 and OG1RF_11512) were sucessfully deleted. As shown in Fig. 3, genes encoding EIIC and EIID in grey frame were deleted from PTS system. Take one mannose/fructose/sorbose PTS (mfs-PTS) system in *E. faecalis* OG1RF as a whole, 49.3% of the complete mfs-PTS system (from OG1RF_12399 to OG1RF_12402) was eliminated in Δ*pts*1, and 52.7% of the complete mfs-PTS system (from OG1RF_11510 to OG1RF_11513) was eliminated in Δ*pts*2. Components EIIA and EIIB remained intact in PTS, in principle, only the function of EIIC and EIID would be affected. Unexpectedly, seveal nucleotides mutated and caused two amino acid mutations in the *pts*2 and *pts*1/2 mutant (App. Fig. 9). Considering the mutated amino acids are located in the EIIC component residue, which is the part to be deleted, we assumed no impact of the mutated amino acids would exert on our outcomes. Causation of these mutations could derive from the ultraviolet radiation when ligation product was recycled from the electrophoresis gel since the same patter of mutation was observed in *pts*2 and *pts*1/2 mutant, which used the same ligation product for homologous recombination. Fig. 21 illustrates the length of PCR products with different primers. Process of *pts* mutants construction is displayed in Tab. 31, Fig. 22-25, and App. Fig. 1-7.

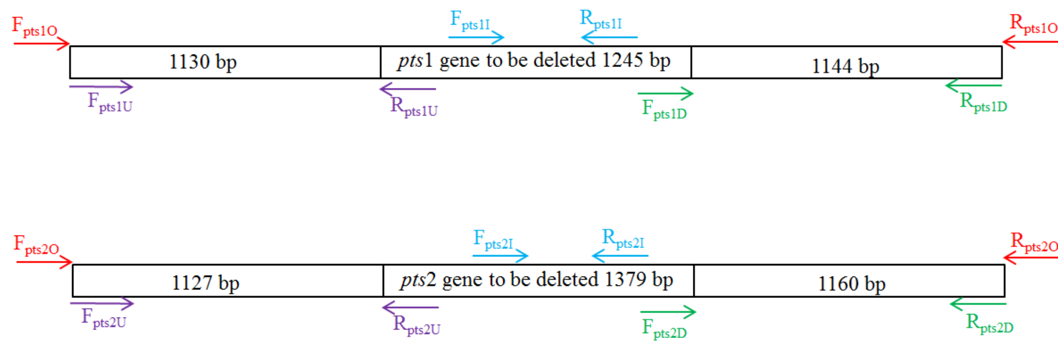


Fig. 21 Verification of successful deletion of *pts*1 and *pts*2 by PCR analyses. Binding sites of primers in the genome of *E. faecalis* OG1RF. Primers in the same color stand for primer pairs. F_{pts1U} - R_{pts1U} are used to amplify the upstream of *pts*1 gene; F_{pts1D} - R_{pts1D} are used to amplify the downstream of *pts*1gene; F_{pts1O} - R_{pts1O} and F_{pts1I} - R_{pts1I} are used to verify the mutants in outer and inner fragments. F_{pts2U} - R_{pts2U} are used to amplify the upstream of *pts*2 gene; F_{pts2D} - R_{pts2D} are used to amplify the downstream of *pts*2 gene; F_{pts2O} - R_{pts2O} and F_{pts2I} - R_{pts2I} are used to verify the mutants in outer and inner fragments.

Primer paris / Bacteria	F_{pts1O}-R_{pts1O}	F_{pts1I}-R_{pts1I}	F_{pts2O}-R_{pts2O}	F_{pts2I}-R_{pts2I}
E. faecalis OG1RF	Fig. 26 Lane B17 and Fig. 27 Lane A17 (3788 bp)	Fig. 26 Lane A17 and Fig. 27 Lane B17 (1058 bp)	Fig. 31 Lane A33, A34 and Fig. 32 Lane A11, A12 (3898 bp)	Fig. 31 Lane B33, B34 and Fig. 32 Lane B11, B12 (597 bp)
E. faecalis Δ*pts*1	Fig. 26 Lane B1-B16 and Fig. 27 Lane A1-A6 (2543 bp)	Fig. 26 Lane A1, A3, A4, A9, A11-A15 and Fig. 27 Lane B1-B6 (0 bp)	/	/
E. faecalis Δ*pts*2	/	/	Fig. 31 Lane A5, A6, A9, A10, A13 and Fig. 32 Lane A1-A5 (2519 bp)	Fig. 31 Lane B5, B6, B9, B10, B13, B14 and Fig. 32 Lane B1-B5 (0 bp)
E. faecalis Δ*pts*1/2	/	/	Fig. 31 Lane A15-A17, A19, A22, A24, A27, A30, A31 and Fig. 32 Lane A6-A10 (2519 bp)	Fig. 31 Lane B16, B19, B22, B27, B30 and Fig. 32 Lane B6-B10 (0 bp)

Tab. 31 View of key primer pairs, predicted fragment sizes, and corresponding figure numbers of PCR products in gel electrophoresis. Verification of successful deletion of *pts*1 and *pts*2 mutant. The presence / absence of PCR products of the predicted fragment sizes was demonstrated for all cases, rendering the deletion mutants correct. Sequences of the chomosomal areas are given in the App. Fig. 8-9.

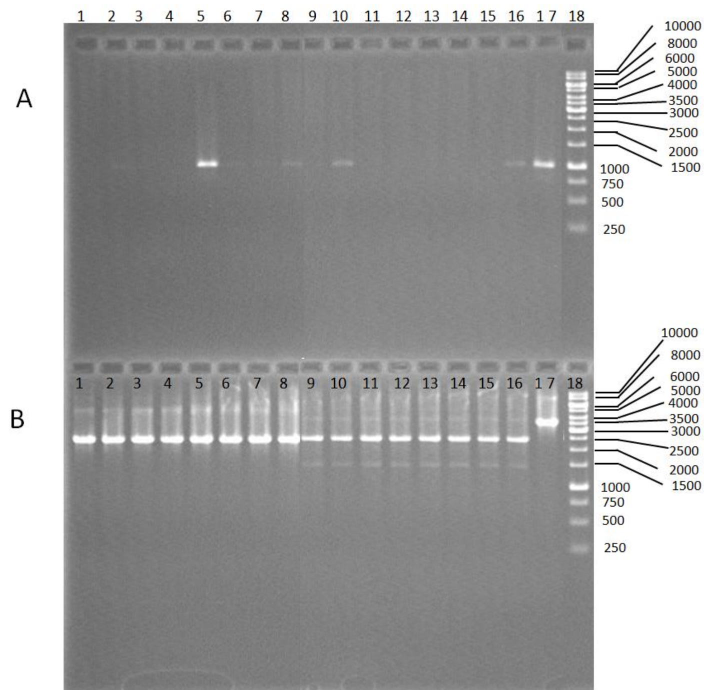


Fig. 22 Verification of DNA fragments for *pts*1 mutant construction. Gel electrophoresis of double-cross over colonies screening for *pts*1 mutant. A1-A16, colonies examined by F_{pts1I}-R_{pts1I} primers, gene length (0 bp) is as expected in bands A1, A3, A4, A9, A11, A15; B1- B16, colonies examined by F_{pts1O}-R_{pts1O} primers, gene length (2543 bp) is as expected in bands B1-B16; A17, positive control examined by F_{pts1I}-R_{pts1I} primer, gene length, 1058 bp; B17, positive control examined by F_{pts1O}-R_{pts1O} primers, gene length 3788 bp; A18 and B18, GeneRuler 1 kb DNA Ladder (ready-to-use, 250 to 10,000 bp)

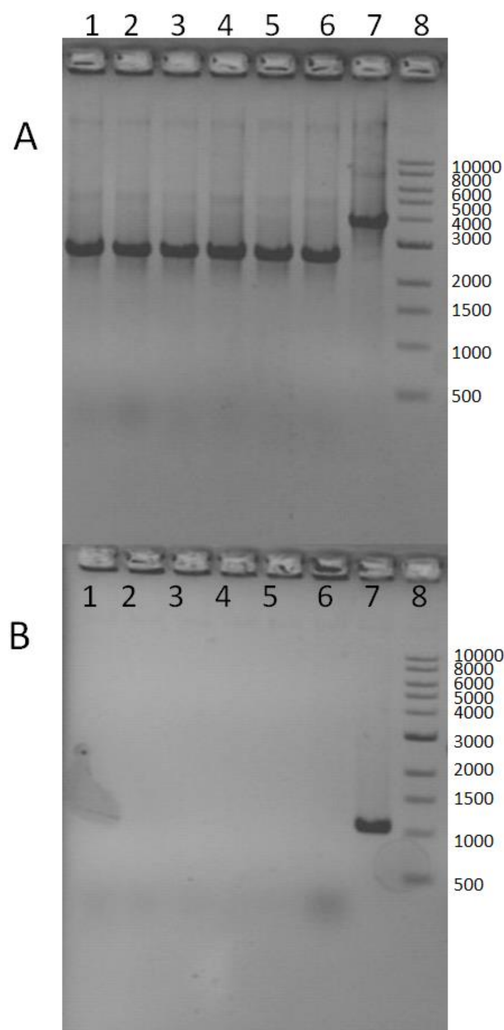


Fig. 23 Using reconstructed plasmid as template for verification of *pts*1 mutant construction. Gel electrophoresis of double-cross over colonies screening for *pts*1 mutant. A1-A6, colonies examined by F_{pts1O}-R_{pts1O} primers, gene length 2543 bp) is as expected in bands A1-A6; B1-B6, colonies examined by F_{pts1I}-R_{pts1I} primers, gene length (0 bp) is as expected in bands B1- B6; A7, positive control examined by F_{pts1O}-R_{pts1O} primers, gene length 3788 bp; B7, positive control examined by F_{pts1I}-R_{pts1I} primers, gene length 1058 b; A8 and B8, 1 kb DNA Ladder (Ready-to-load, 500 to 10,000 bp)

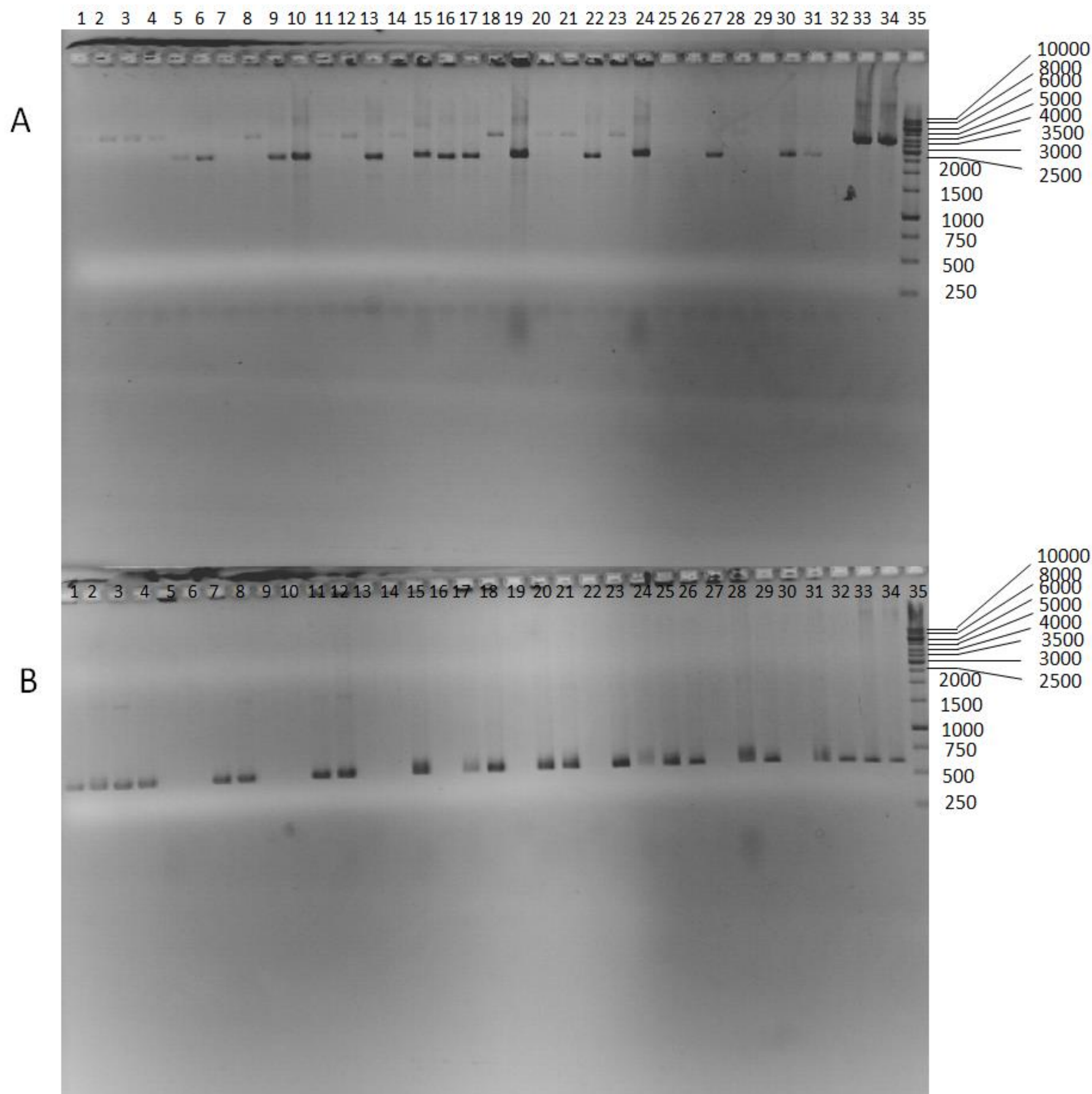


Fig. 24 Verification of DNA fragments for *pts*2 and *pts*1/2 mutants construction. Gel electrophoresis of double-cross over colonies screening for *pts*2 and *pts*1/2 mutants. A1-A14, *pts*2 colonies examined by F_{pts2O}-R_{pts2O} primers, gene length (2519 bp) is as expected in bands A5, A6, A9, A10, A13; A15-A32, *pts*1/2 colonies examined by F_{pts2O}-R_{pts2O} primers, gene length (2519 bp) is as expected in bands A15, A16, A17, A19, A22, A24, A27, A30, A31; A 33-A34, positive control examined by F_{pts2O}-R_{pts2O} primers (3898 bp); B1-B14, *pts*2 colonies examined by F_{pts2I}-R_{pts2I} primers, gene length (0 bp) is as expected in bands B5, B6, B9, B10, B13, B14; B15-B32, *pts*1/2 colonies examined by F_{pts2I}-R_{pts2I} primers, gene length (0 bp) is as expected in bands B16, B19, B22, B27, B30; B 33-B34, positive control examined by F_{pts2I}-R_{pts2I} primers (597 bp);A35 and B35, GeneRuler 1 kb DNA Ladder (ready-to-use, 250 to 10,000 bp)

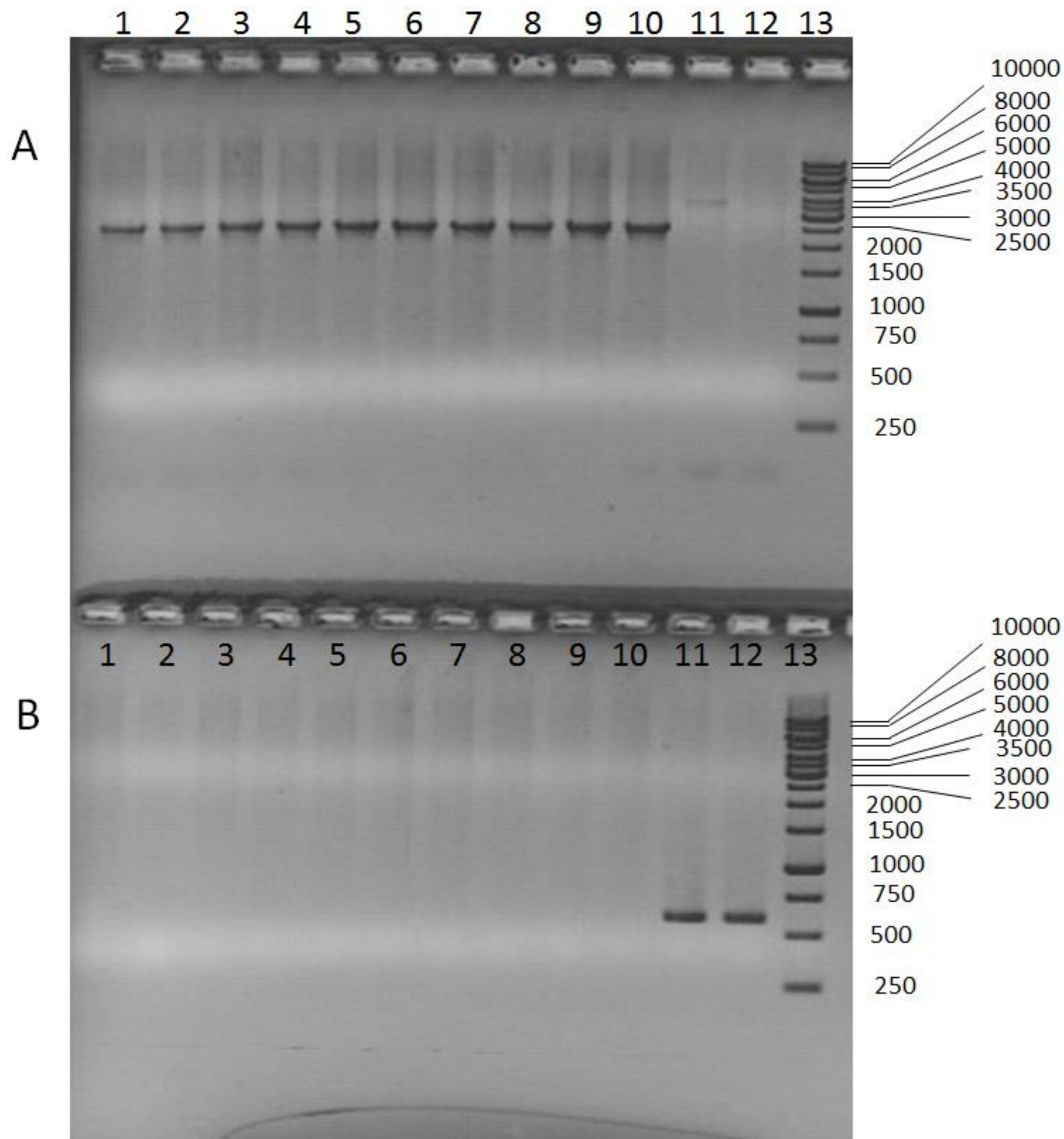


Fig. 25 Using reconstructed plasmid as template for verification of *pts*2 and *pts*1/2 mutants construction. Gel electrophoresis of double-cross over colonies screening for *pts*2 and *pts*1/2 mutants. A1-A5, *pts*2 colonies examined by F_{pts2O}-R_{pts2O} primers, gene length (2519 bp) is as expected in bands A1-A5; A6-A10, *pts*1/2 colonies examined by F_{pts2O}-R_{pts2O} primers, gene length (2519 bp) is as expected in bands A6- A10; B1-B5, *pts*2 colonies examined by F_{pts2I}-R_{pts2I} primers, gene length (0 bp) is as expected in bands B1-B5; B6-B10, *pts*1/2 colonies examined by F_{pts2I}-R_{pts2I} primers, gene length (0 bp) is as expected in bands B6-B10;A11-A12, positive control examined by F_{pts2O}-R_{pts2O} primers (3898 bp); B11-B12, positive control examined by F_{pts2I}-R_{pts2I} primers (597 bp); A13 and B13, GeneRuler 1 kb DNA Ladder (ready-to-use, 250 to 10,000 bp)

3.2.2 *In vitro* studies

3.2.2.1 Influence of PTS on sugar utilization

An API test of enterococci as shown in App. Fig. 26 did not display any significant difference in sugar utilization among *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, and Δ*pts*1/2. In an attempt to get a knowledge on how PTS affects non-preferential PTS-sugar utilization, *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, and Δ*pts*1/2 were grown in Davis and MRS-based broth media (containing galactose) supplemented with one putatively preferential sugar in each culture. Davis-based media is strictly chemically defined and could only support growth of enterococci when glucose was used as carbon source, indicating enteroccoci are not adaptive in Davis-based media (App. Fig. 18-25). MRS-based media supplemented with different PTS-sugars enable *E. faecalis* OG1RF and all *pts* mutants to grow to different degrees. Enterococcal growth in MRS-based media without sugar was set as background (Fig. 26). Sorbose was observed to support enterococcal growth in a background level, indicating the inability of enterococci to use sorbose as preferential carbon source for growth (Fig. 27). In MRS with glucose, fructose or mannose as the putative preferential carbon source and galactose as the non-preferential sugar, Δ*pts*1 displayed leading growth, followed by Δ*pts*1/2 and Δ*pts*2, whereas growth of *E. faecalis* OG1RF was repressed to the lowest level (Fig. 28-30). The discrepancy of growth in MRS-based media however disappeared when enterococci were grown in BHI (Fig. 35). In BHI medium, *E. faecalis* OG1RF recovered growth to a similar level as Δ*pts*1 and Δ*pts*1/2 while Δ*pts*2 displayed retardant growth. Growth of enterococci in MRS with other sugars and in modified BHI (low pH, hydrogen peroxide) was shown in App. Fig. 10-17. No diauxic growth was observed in MRS with two sugars as carbon sources (Fig. 31-34).

Sugars that require PTS, which consists of a series of protein components that together act as a phosphoryl transfer chain to accomplish sugar utilization is termed PTS-sugar. To evaluate if bacterial growth corelated with consumption of PTS-sugar, bacterial OD_{590} was recorded and sugar consumption was quantified (Fig. 38 and Tab. 32). *Pts*1 mutational strain with the highest OD_{590} value (0.8) consumed most glucose (11.69 U). OG1RF strain with the lowest OD_{590} value (0.59) consumed least glucose (5.84 U), which confirmed that absence of *pts*1, *pts*2 and *pts*1/2 in *E. faecalis* OG1RF led to improved utilization of non-preferential sugar (galactose) due to carbon catabolite repression.

Besides, redox potential and acidification of enterococci grew in BHI were examined by iCinac system (Brasca, Morandi, Lodi, & Tamburini, 2007). *E. faecalis* OG1RF displayed similar redox potential and acidification to that of all *pts* mutants (Fig. 36-37).

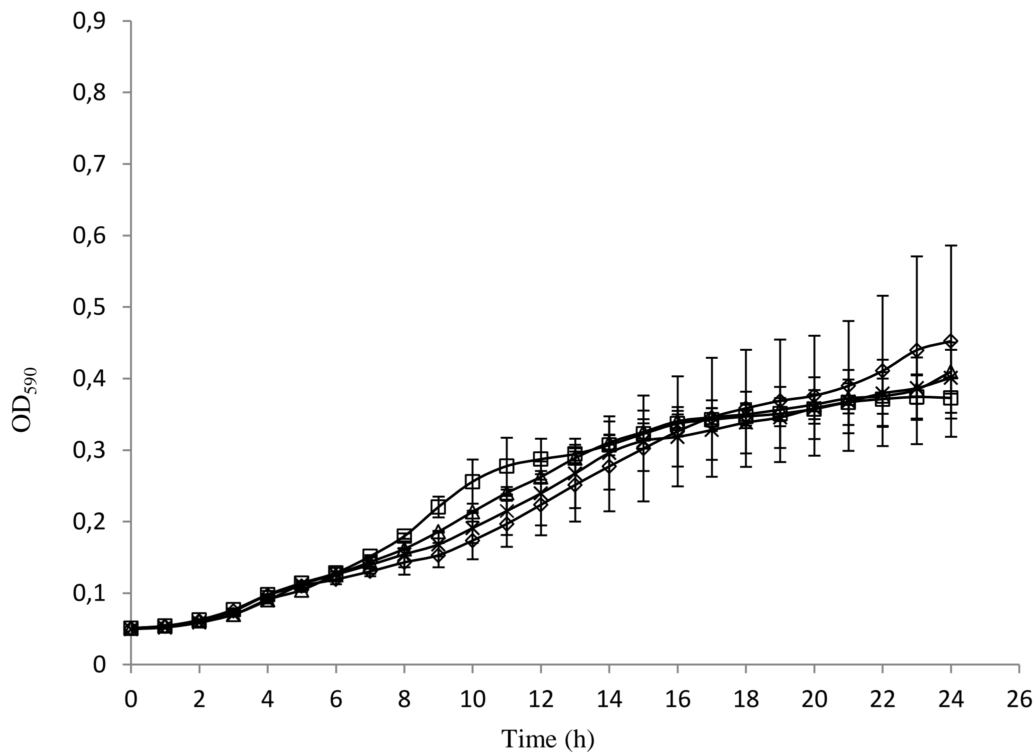


Fig. 26 Growth of enterococci in MRS without sugar. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —✕—*E. faecalis pts*1/2 mutant

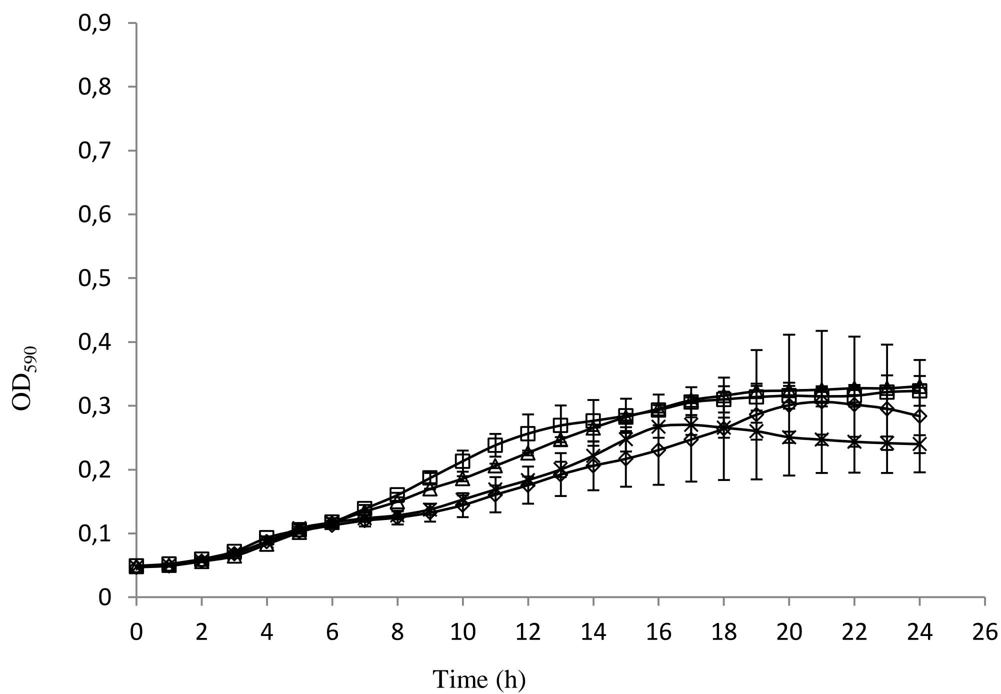


Fig. 27 Growth of enterococci in MRS with sorbose. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —✕—*E. faecalis pts*1/2 mutant

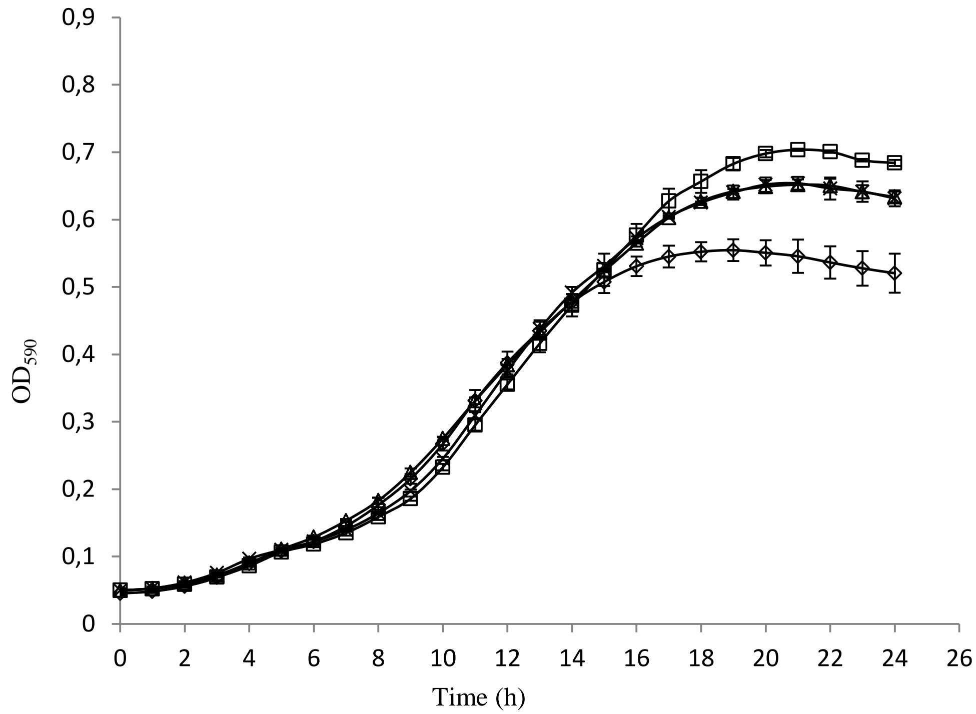


Fig. 28 Growth of enterococci in MRS with fructose. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —×—*E. faecalis pts*1/2 mutant

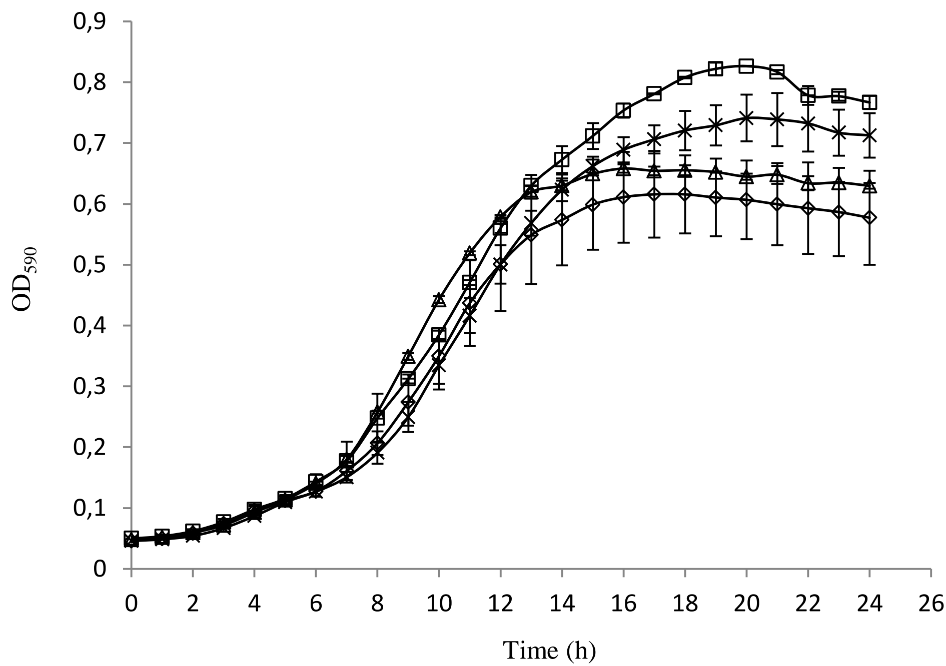


Fig. 29 Growth of enterococci in MRS with glucose. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —×—*E. faecalis pts*1/2 mutant

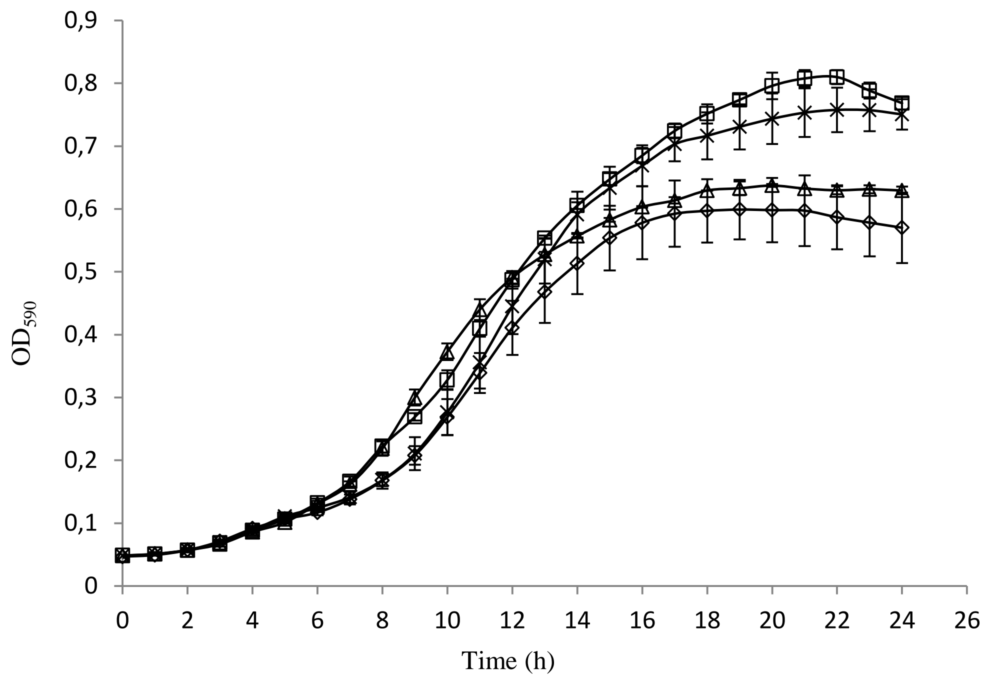


Fig. 30 Growth of enterococci in MRS with mannose. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —✕—*E. faecalis pts*1/2 mutant

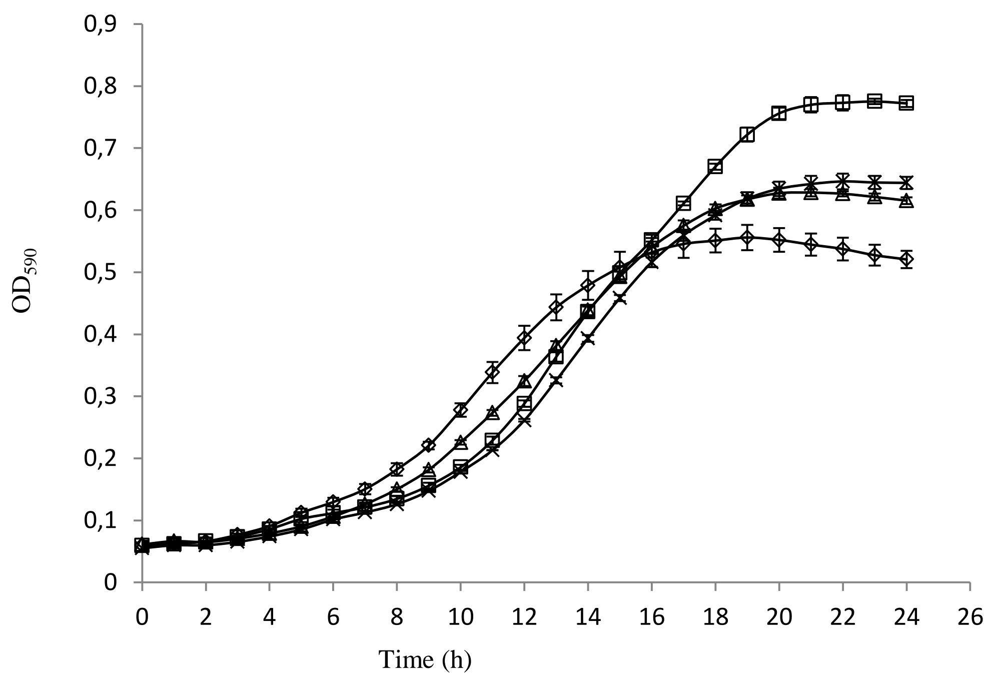


Fig. 31 Growth of enterococci in MRS with 0.005% glucose and 0.5% mannose. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —✕—*E. faecalis pts*1/2 mutant

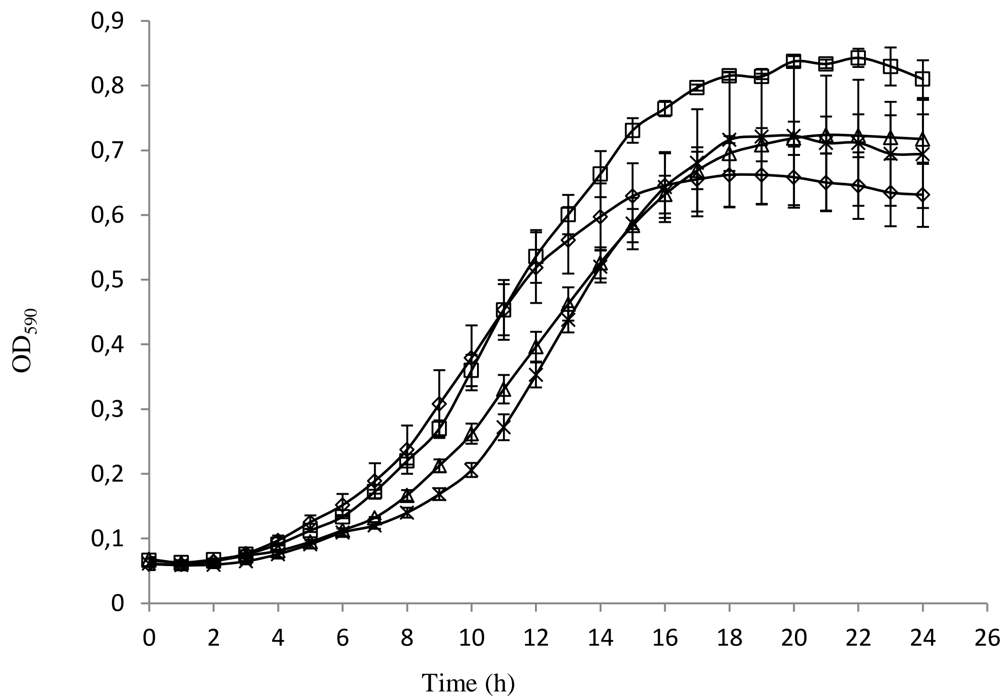


Fig. 32 Growth of enterococci in MRS with 0.05% glucose and 0.5% mannose. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —✕—*E. faecalis pts* 1/2 *mutant*

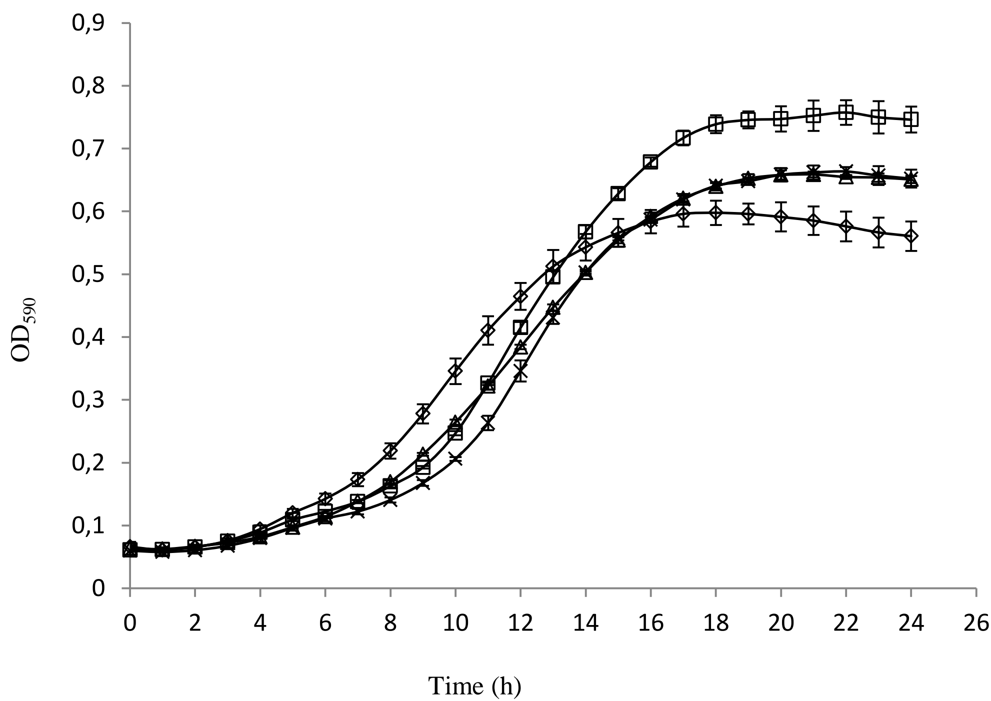


Fig. 33 Growth of enterococci in MRS with 0.15% glucose and 0.5% mannose. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —✕—*E. faecalis pts*1/2 mutant

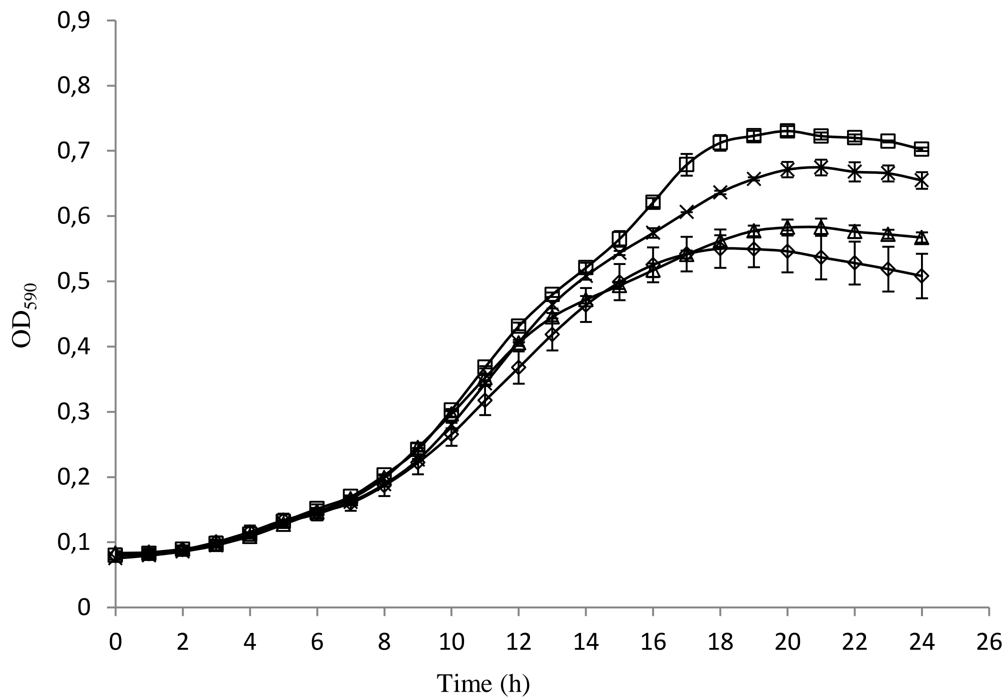


Fig. 34 Growth of enterococci in MRS with 0.005% glucose and 0.5% fructose. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —✕—*E. faecalis pts*1/2 mutant

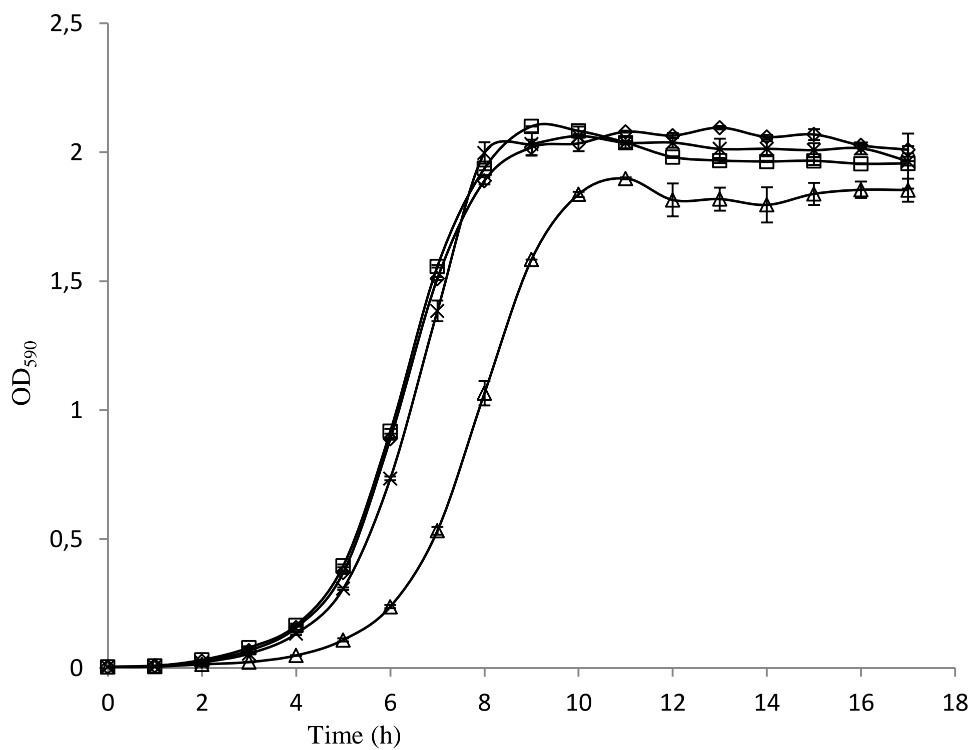


Fig. 35 Growth of enterococci in BHI (pH 7.23, aerobic). —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —✕—*E. faecalis pts*1/2 mutant

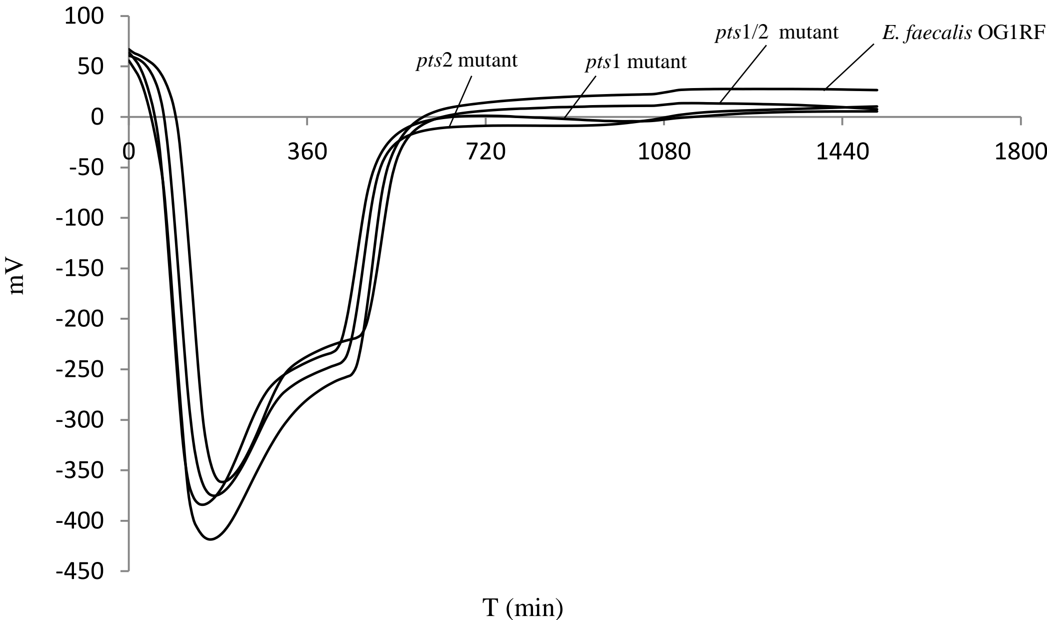


Fig. 36 Redox potential of BHI fermented by enterococci

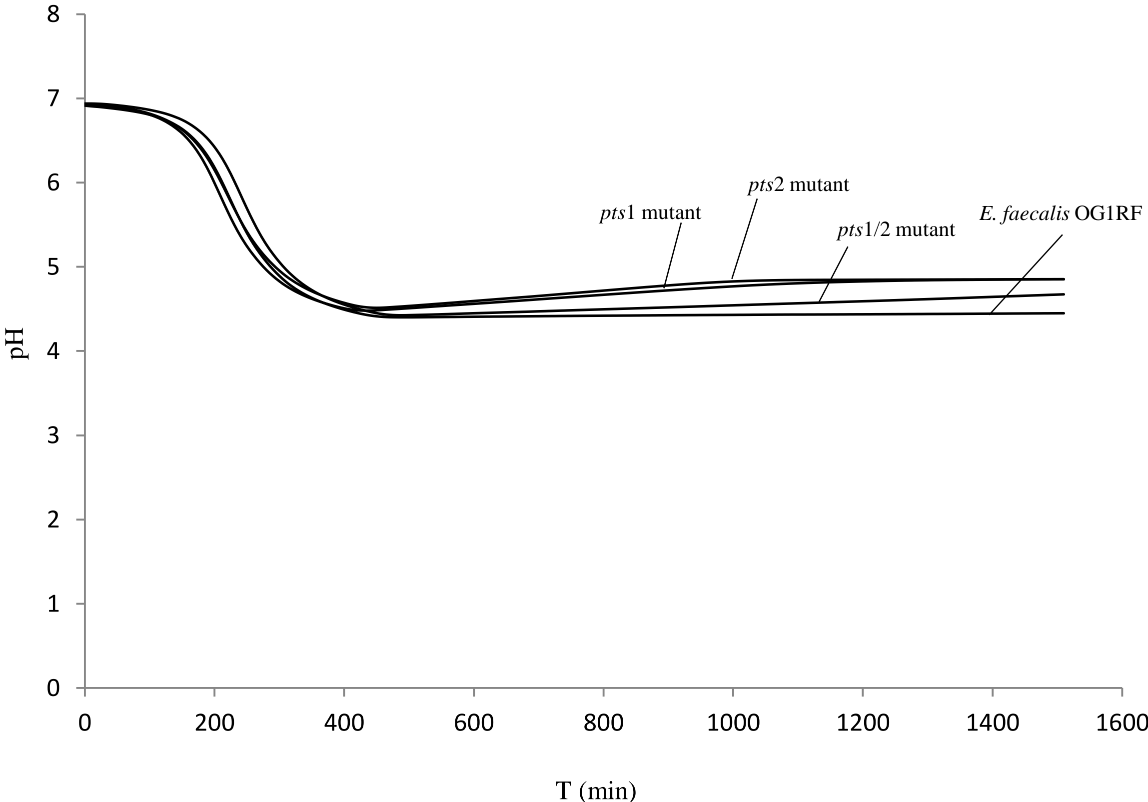


Fig. 37 pH of BHI fermented by enterococci

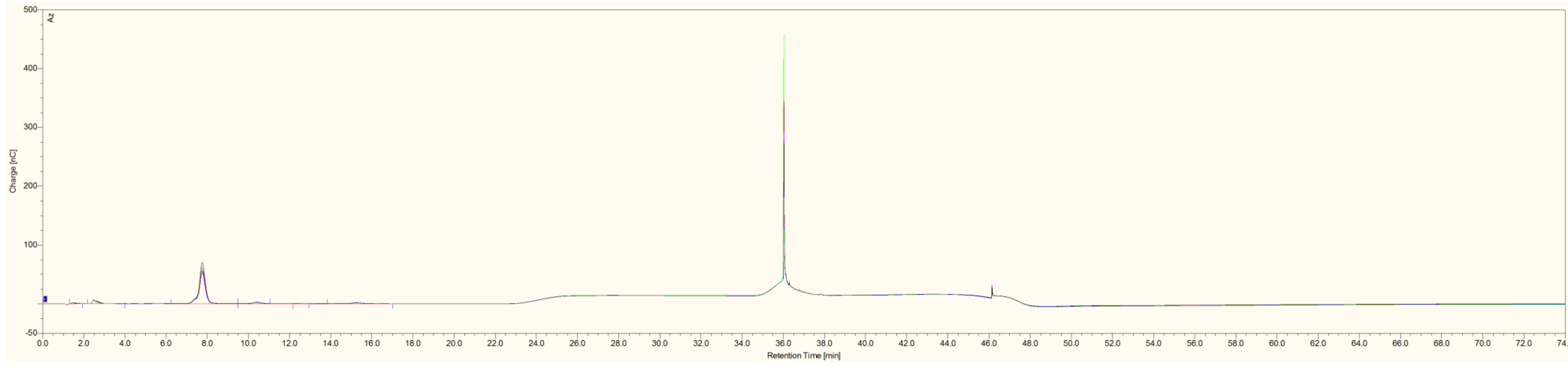


Fig. 38 Ion chromatography diagram of glucose consumption in MRS medium

Strains	Retention time	Consumed glucose	OD590
E.faecalis OG1RF	7,8	5,84	0.59
*pts*1 mutant	7,8	11,69	0.8
*pts*2 mutant	7,8	9,51	0.72
*pts*1/2 mutant	7,8	9,68	0.75

Tab. 32 Glucose consumption in MRS medium

3.2.2.2 Influence of PTS on oxidative stress tolerance

The influence of *pts* genes on oxidation tolerance was detected by growth behavior and stress killing assay. *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, and Δ*pts*1/2 all displayed repressed growth in BHI with 0.5 mM hydrogen peroxide (App. Fig. 12).

As for bacterial tolerance to 25 mM hydrogen peroxide (Fig. 39), at time point 15 min, survival of *E. faecalis* OG1RF, Δ*pts*1 and Δ*pts*1/2 all dropped to around 10% while Δ*pts*2 had a survival rate of merely 3%. At time point 30 min, survival rate of *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, and Δ*pts*1/2 decreased to 5%, 2%, 0.08% and 2%, respectively. This model displayed a stepwise and fierce killing of *E. faecalis* by hydrogen peroxide. *E. faecalis* OG1RF had comparatively stronger ability to survive while Δ*pts*2 was most susceptible to hydrogen peroxide.

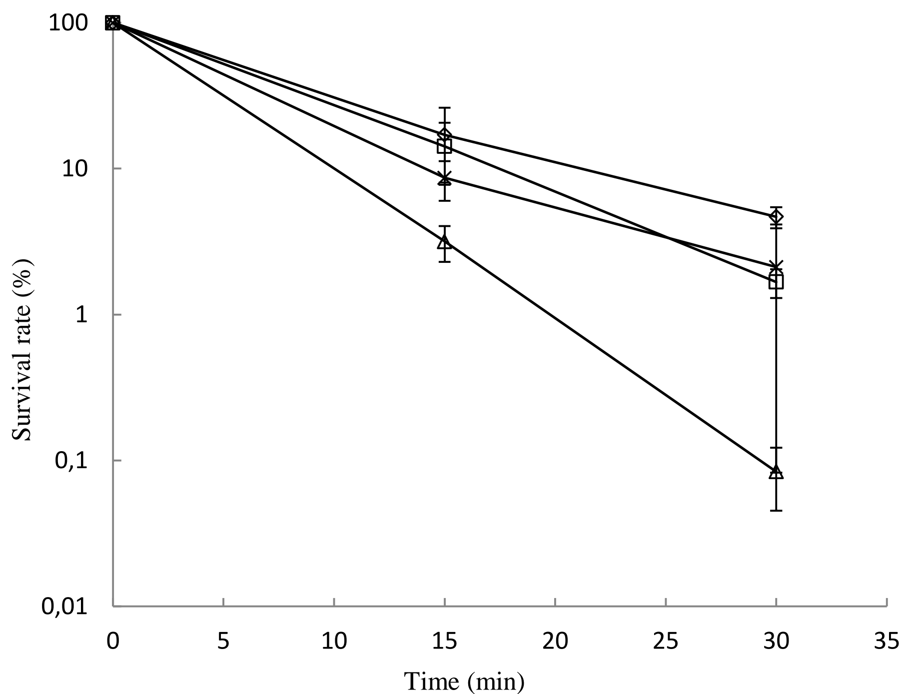


Fig. 39 Survival rate of enterococci in hydrogen peroxide (25 mM). —◇— *E. faecalis* OG1RF; —□— *E. faecalis pts*1 mutant; —△— *E. faecalis pts*2 mutant; —✕— *E. faecalis pts*1/2 mutant

3.2.2.3 Influence of PTS on acid tolerance

The influence of *pts* genes on acid tolerance was detected along their growth behavior and in a stress killing assay. *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, and Δ*pts*1/2 all displayed repressed growth in pH 5.5 BHI (App. Fig. 11).

As shown in Fig. 40, survival of *E. faecalis* OG1RF was subtly affected after incubation in pH 4.1 buffer for 30 min (95% survival rate), while survival rates of Δ*pts*1, Δ*pts*2, and Δ*pts*1/2 were 84%, 79%, 70%. At time point 60 min, *E. feacalis* OG1RF kept the highest survival rate (70%), followed by Δ*pts*1/2 (65%), Δ*pts*1 (64%) and Δ*pts*2 (57%). *E. faecalis* OG1RF displayed comparatively stronger ability to resist acid than *pts* mutants.

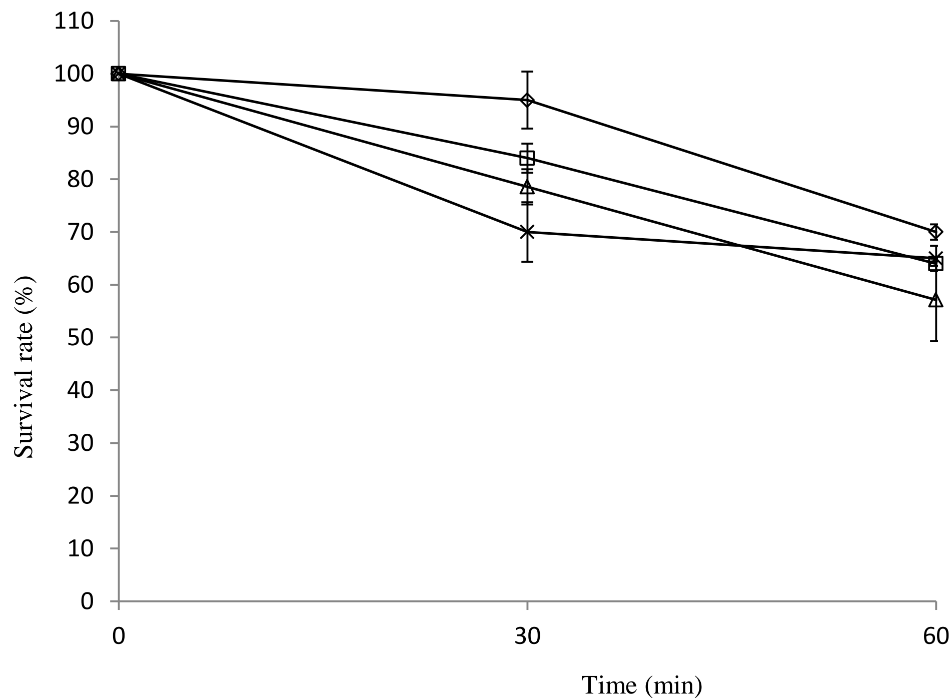


Fig. 40 Survival rate of enterococci in acid (pH 4.1). —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —✕—*E. faecalis pts*1/2 mutant

3.2.2.4 Influence of PTS on bacterial resistance to pediocin

Resistance of *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, and Δ*pts*1/2 to pediocin was demonstrated in two parallel groups. In the group without treatment of catalase, where substantial hydrogen peroxide produced by *Pediococcus* presented, *E. faecalis* OG1RF and all *pts* mutants displayed fairly the same susceptibility to pediocin with an inhibition zone of 20 mm (Tab. 33). To eliminate the interference of hydrogen peroxide, BHI medium in the second group was treated with 1 mg/mL catalase. A significant schrink of inhibition zones was observed (Fig. 41). Relative to *E. faecalis* OG1RF (14 mm), the inhibition zones of Δ*pts*1 and Δ*pts*1/2 were equally reduced (11 mm), followed by Δ*pts*2 with a less reduction (12 mm). In this context, deletion of *pts*1 or *pts*1/2 genes accounts for 20% loss of pediocin receptors whereas *pts*2 genes represents 15%. Loss of bacteriocin receptor in the mutants resulted in enhanced resistance to pediocin.

Strains / Treatment	*E. faecalis* OG1RF	*E. faecalis* OG1RF *pts*1 mutant	*E. faecalis* OG1RF *pts*2 mutant	*E. faecalis* OG1RF *pts*1/2 mutant
Non-treatment	20	20	20	20
Treated with catalase	14	11	12	11

Tab. 33 Diameter of inhibition zone (mm)

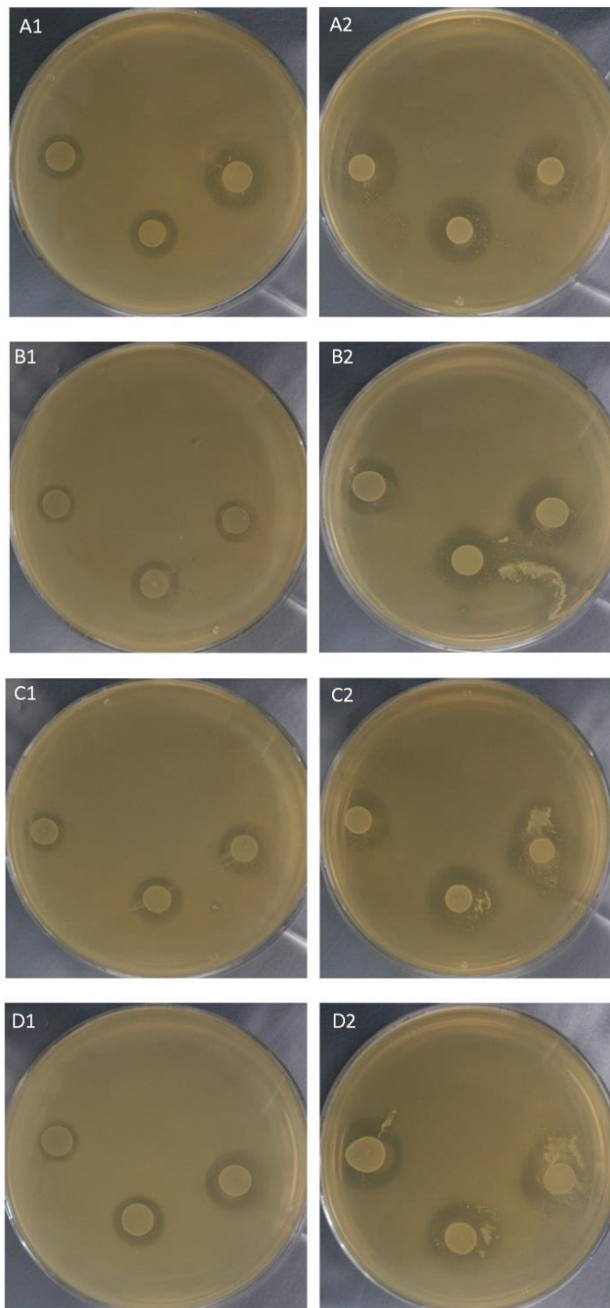


Fig. 41 Inhibition zone induced by pediocin (mm). Group1, Treatment by catalase; Group 2, Non-treatment by catalase; A, *E. faecalis* OG1RF; B, *pts*1 mutant; C, *pts*2 mutant; D, *pts*1/2 mutant

3.2.2.5 Behavior in yeast agglutination assay

Enterococci in MRS with different sugars were pre-cultured to induce mannose-sensitive enterococci. Since different sugars exerted no influence on the sensitivity of enterococci to mannose (data not shown), yeast cultured in MRS with glucose as carbon source was applied to agglutination assay. *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, and Δ*pts*1/2 were found to exhibit no much differences in generating yeast aggregation by microscopy and OD_{590} measurements. All (except Δ*pts*1/2, which enhanced yeast aggregation) resulted in little and similar effects on yeast agglutination (App. Fig, 27-29).

3.2.3 *In vivo* studies

3.2.3.1 Influence of PTS on SOD activation in nematode

C. elegans as an animal model uses oxidative stress response enzyme (superoxide dismutase, SOD) to protect itself from reactive oxygen species (ROS) intrigued by invading pathogens. Activity of SOD in *C. elegans* is therefore an indicator of self-defence against pathogen. *C.elegans* with SOD-GFP reacted differently when fed with *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, and Δ*pts*1/2. SOD-GFP in nematode was monitored by Leica DMRE fluorescence microscope (fluorescence images were taken at 2 s exposure time, gain 5×, magnification 5× and gamma 1.29. Transmitted light images were taken at 2 ms exposure time, gain 5×, magnification 5 × and gamma 1.29). Nematodes in similar size and age were picked and observed. Fluorescence were observed along the distribution of intestine (Fig. 42). Combination of imagines taken with both light sources were processed with photoshop online to have a better view. Intensity of fluorescence was analysed with ImageJ. Nematode fed with *E. faecalis* OG1RF stimulated the strongest fluorescence intensity (204.451 U), followed by *pts*1/2 (203.864 U) and *pts*2 mutants (189.569 U), while *pts*1 mutant displayed the lowest fluorescence intensity (167.544 U). In practice, the intensity of fluorescence could not exactly stand for the virulence of enterococci because the nematodes varied in size (even we chose nematodes in similar size) and consumed different amount of enterococci, which could cause different fluorescence intensities. The order of intrigued fluorescence intensity simply implied the tendency of virulence.

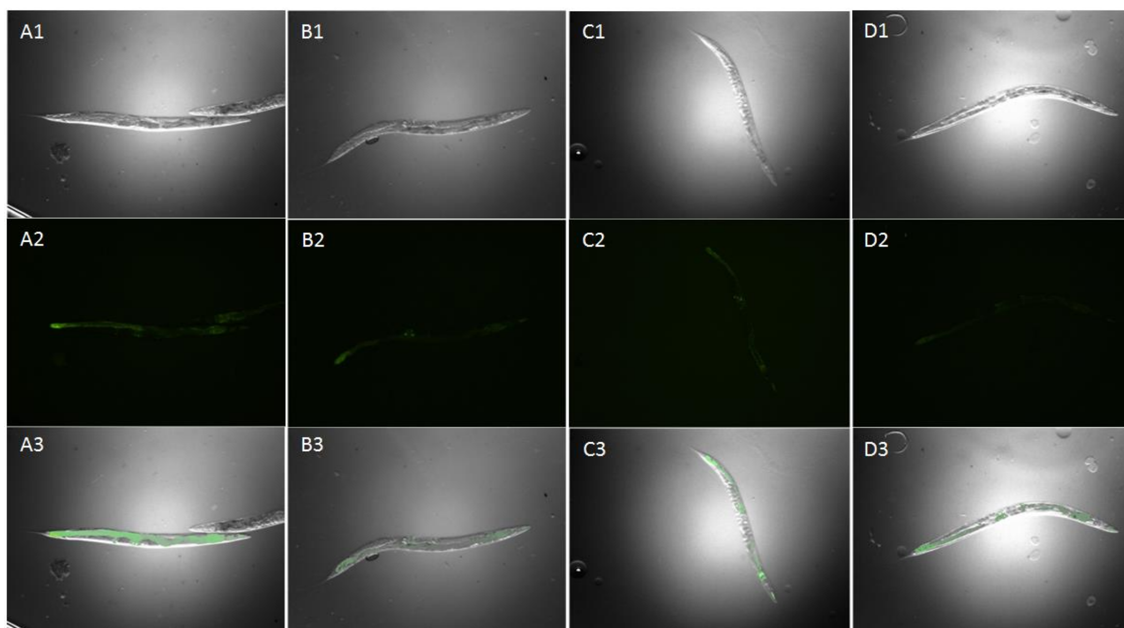


Fig. 42 Fluorescence in nematode induced by enterococci. Nematode with fluorescence reporter in the upstream of SOD (superoxide dismutase) fed with *E. faecalis* OG1RF and *pts* mutants were observed to investigate effects of *pts* genes on activation of SOD. Group1, bright light; Group 2, Fluorescence; Group 3, Combination of bright light and fluorescence; A, *E. faecalis* OG1RF; B, *pts*1 mutant; C, *pts*2 mutant; D, *pts*1/2 mutant

3.2.3.2 Influence of PTS on bacterial survival in different types of macrophage

Survival ability in macrophage is an important indicator of bacterial persistence and virulence. The mannose receptor was reported to promote the phagocytosis and endocytosis of host (Gordon, 2003). Therefore, survival of *E. faecalis* OG1RF and *pts* mutants in macrophages was investigated.

Each well of macrophage J774A.1 (10^6) was infected with 2×10^7 *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, or Δ*pts*1/2 (MOI=20). As shown in Fig. 44, the invasion ability of *E. faecalis* OG1RF and *pts* mutants varied. Ten macrophages J774A.1 could engulf 14 *E. faecalis* OG1RF, meanwhile, only 2 *pts*1 mutant, 6 *pts*2 mutant and 3 *pts*1/2 mutant were able to enter the same amount of J774A.1. *E. faecalis* OG1RF had the strongest capacity to intrude macrophage while the mutants showed inferior ability. Survival of enterococci in macrophage is another criterion to evaluate bacterial virulence (Fig. 43). 81% *E. faecalis* OG1RF, 23% *pts*1 mutant, 16% *pts*2 mutant, and 20% *pts*1/2 mutant were viable after engulfed by J774A.1 for 48 h. A prolonged incubation led to a decrease in survival rate. Around 42% *E. faecalis* OG1RF, 17% *pts*1 mutant, 12% *pts*2 mutant and 10% *pts*1/2 mutant survived in J774A.1 up to 72 h. *E. faecalis* OG1RF displayed much stronger survival ability in macrophage J774A.1.

BMM as a freshly developed macrophage was infected with *E. faecalis* OG1RF or *pts* mutants (MOI=20) under the same condition. Ten BMMs could engulf 25 *E. faecalis* OG1RF, 9 *pts*1 mutant, 29 *pts*2 mutant and 8 *pts*1/2 mutant, respectively (Fig. 46). Survival rates of *E. faecalis* OG1RF, *pts*1 mutant, *pts*2 mutant and *pts*1/2 mutant in BMM were: 25%, 6%, 20% and 5% after incubation for 48 h. Prolonged incubation (at time point 72 h) further decreased survival rates of *E. faecalis* OG1RF, *pts*1 mutant, *pts*2 mutant and *pts*1/2 mutant to: 4%, 0.9%, 5% and 1%, respectively (Fig. 45). BMM displayed better phagocytosis and killing ability owing to its primary and native capacity whereas J774A.1 as a continuous subculture in laboratory could generate function impairs (Wang et al., 2013).

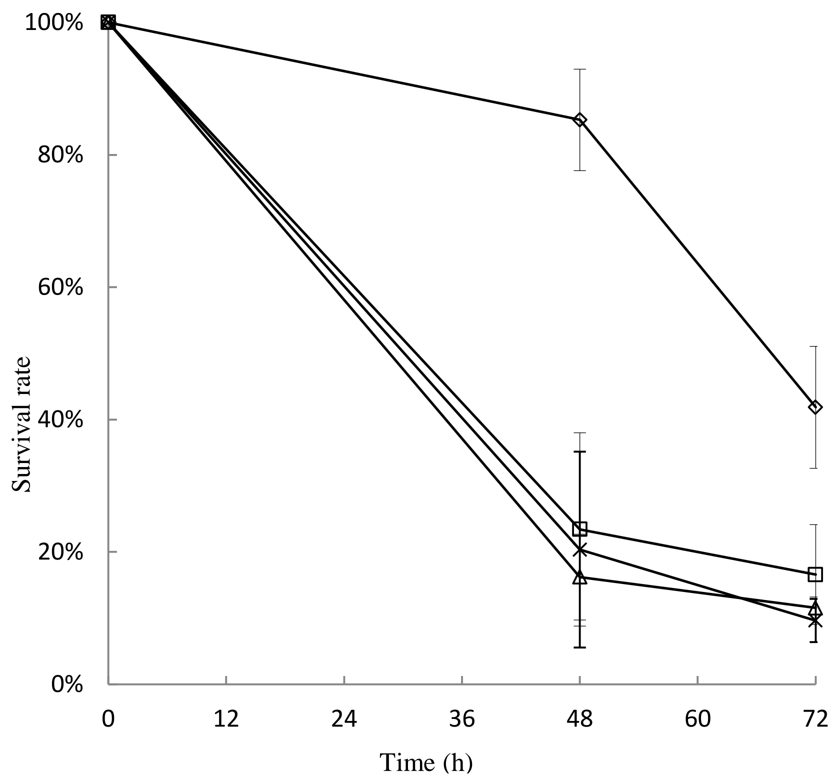


Fig. 43 Survival rate of enterococci inmacrophage J774A.1. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —×—*E. faecalis pts*1/2 mutant

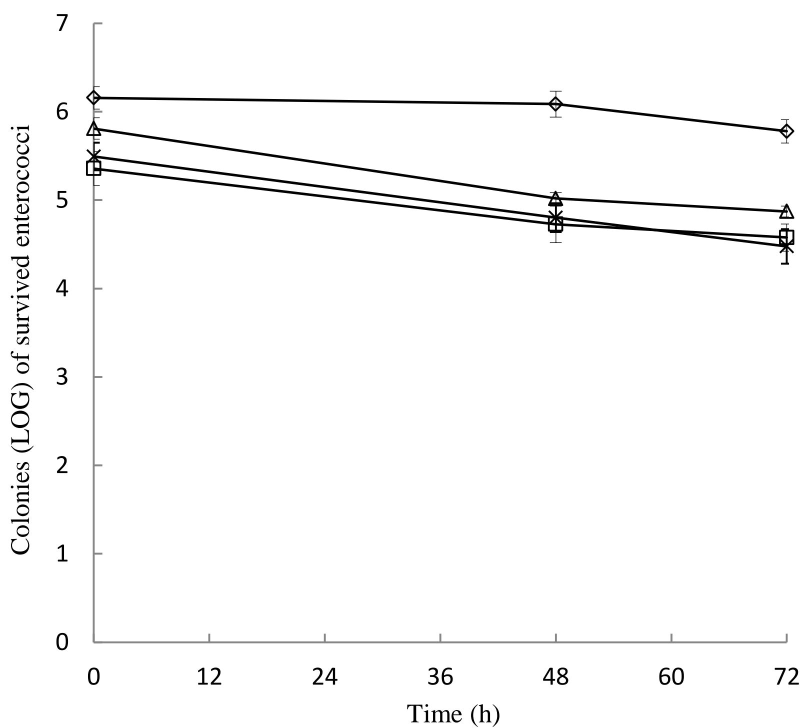


Fig. 44 Survival CFU of enterococci in macrophage J774A.1. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —×—*E. faecalis pts*1/2 mutant

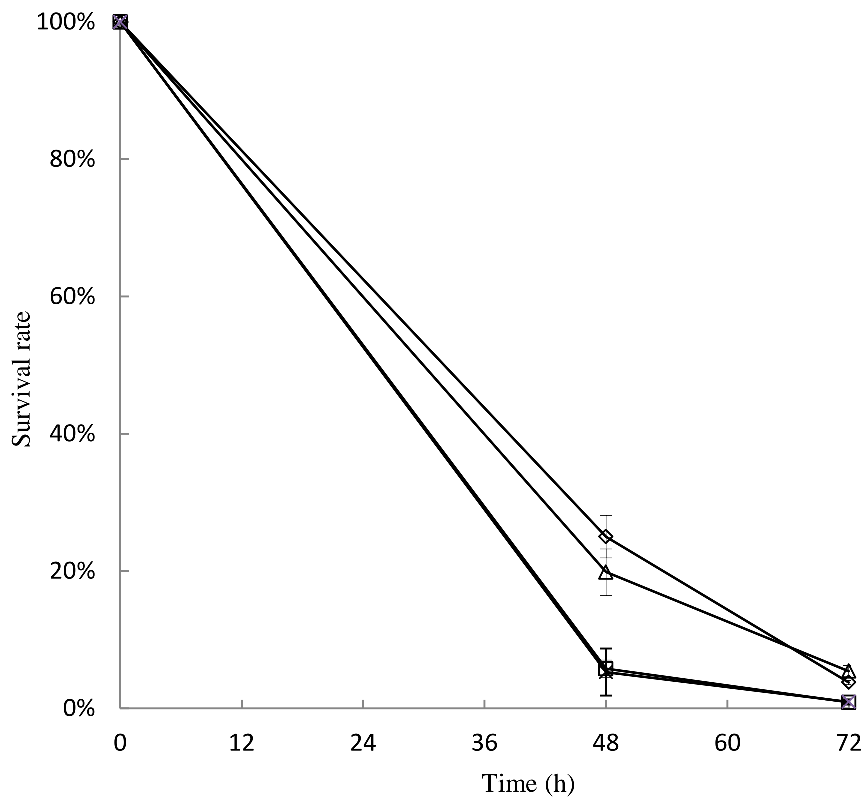


Fig. 45 Survival rate of enterococci in macrophage BMM. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —✕—*E. faecalis pts*1/2 mutant

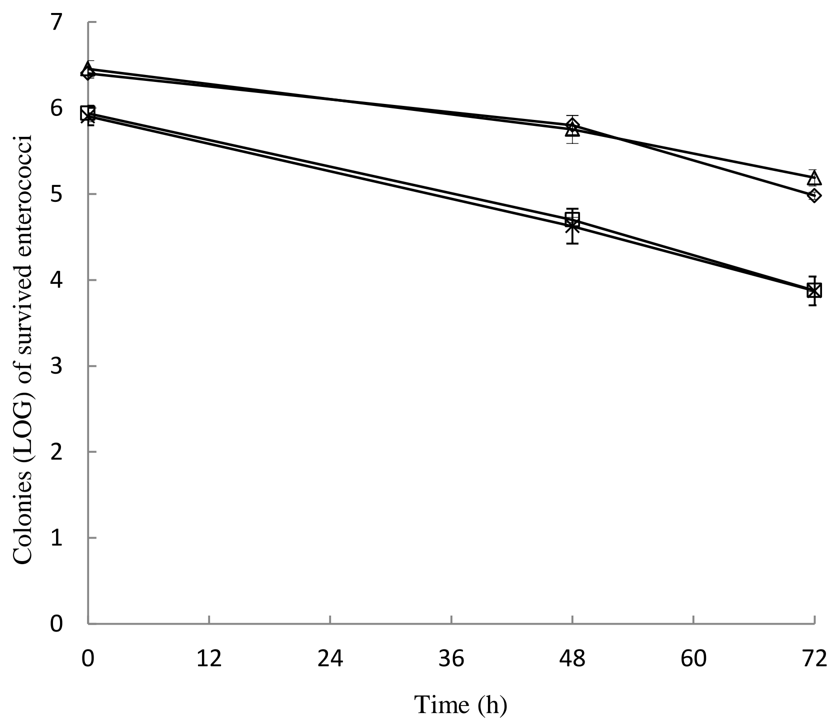


Fig. 46 Survival CFU of enterococci in macrophage BMM. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —✕—*E. faecalis pts*1/2 mutant

3.2.4 Relative quantification of antioxidant and energy-related genes by qRT-PCR

Transcription of antioxidant and energy-related genes in *E. faecalis* OG1RF and all *pts* mutants were quantified by RNA extraction, reverse transcription and qPCR. Amplification efficiency was examined for each pair of primer (in the range of 90%-98%, App. Fig. 30-41). As displayed in Tab. 35, comparing to that of wild type strain, expression of *hpr*K (master regulator of carbon metabolism in gram-positive bacteria) was 4.8 fold decreased in *pts*2 mutant while no change was identified in *pts*1 or *pts*1/2 mutant. This tallied with growth behaviors, in which *pts*2 mutant showed worst growth while OG1RF, *pts*1 and *pts*1/2 displayed equal growth behavior in BHI (pH 7.23, aerobic). As to antioxidative gene transcription, five antioxidant genes (*gor*, *kat*A, *lys*R, *hyp*R, *rex*) were 3~6 fold downregulated in *pts*2 mutant, which perfectly explained the mostly attenuated tolerance of *pts*2 mutant to hydrogen peroxide in molecular level. Besides, two antioxidant genes (*tpx, lys*) in *pts*1 and *pts*1/2 mutants were 2~3-fold increased. CcpA (catabolite control protein A) as catabolite repressor in CCR subjected no detectable influence to the absence of *pts* genes. Transcription of antioxidant and energy-related genes in hydrogen peroxide-treated *E. faecalis* OG1RF and all *pts* mutants was shown in Tab. 34 and discussed in chapter 4.5.

Genes	Gene annotation	Fold change* in expression in mutants (b, c, d) relative to OG1RF (a)			
		A (wild type)	b (Δ⁻*pts*1)	c (Δ⁻*pts*2)	d (Δ⁻*pts*1/2)
gor	Glutathione reductase	1	-2,77	ND	ND
katA	Heme-dependent catalase (peroxide reductase)	1	-3,00	ND	ND
npr	NADH peroxidase	1	ND	ND	ND
ahpC	Alkyl peroxide reductase	1	ND	ND	ND
tpx	Thiol peroxidase	1	-3,56	2,08	ND
lysR	Lysine family transcriptional, regulator for ahpC	1	-4,29	ND	ND
hypR	Hydrogen peroxide regulator	1	-3,61	ND	ND
rex	Redox-sensing transcriptional repressor	1	ND	ND	ND
ccpA	Catabolite control protein A, regulator of inducible genes	1	ND	ND	ND
hprK	Hpr kinase, master regulator of carbon metabolism in gram-positive bacteria	1	-3,34	2,17	ND

Tab. 34 Fold differences ($2^{-\Delta\Delta Ct}$) of 10 oxidative genes (mutant/wild type) in *E. faecalis* treated with 2.4 mM H_2O_2. Transcriptional level of wild type was set to 1.
*Twofold cutoff ($P \leq 0.05$)

Genes	Gene annotation	Fold change* in expression in mutants (b, c, d) relative to OG1RF (a)			
		A (wild type)	b (Δ*pts*1)	c (Δ *pts*2)	d (Δ*pts*1/2)
gor	Glutathione reductase	1	ND	-3,3	ND
katA	Heme-dependent catalase (peroxide reductase)	1	ND	-6,3	ND
npr	NADH peroxidase	1	ND	ND	ND
ahpC	Alkyl peroxide reductase	1	ND	ND	ND
tpx	Thiol peroxidase	1	3,5	ND	2,1
lysR	Lysine family transcriptional, regulator for ahpC	1	3,3	-4,1	2,3
hypR	Hydrogen peroxide regulator	1	ND	-6,0	ND
rex	Redox-sensing transcriptional repressor	1	ND	-3,2	ND
ccpA	Catabolite control protein A, regulator of inducible genes	1	ND	ND	ND
hprK	Hpr kinase, master regulator of carbon metabolism in gram-positive bacteria	1	ND	-4,8	ND

Tab. 35 Fold differences ($2^{-\Delta\Delta Ct}$) of 10 oxidative genes (mutant/wild type) in non-treated *E. faecalis*. Transcriptional level of wild type was set to 1.
*Twofold cutoff ($P \leq 0.05$)

4 Discussion

It is known that people with preexisting maladjustments like intestinal dysfuntion, immunodeficiency or open wounds apt to develop diseases more easily comparing with the healthy ones. It is yet unkonwn how the dysfunctional intestine facilitates the invasion of bacteria, and which factors in bacteria and which receptors in host cell are invovled in bacterial infection. Bacterial adhesion and translocation as the primary indicators of bacterial colonization and infection were studied in both intact and dysfunctional intestinal monolayers to compare the discrepancy. Moreover, several host matrixes such as actin, mucin and fibronectin as nominated receptors in host were evaluated for the ability to mediate bacterial adhesion, and actin-binding proteins in enterococci were isolated. In this thesis, EDTA-treated Ptk6 monolayers (dysfunctional intestine) were found to facilitate enterococcal translocation in comparison to the intact Ptk6 monolayer. Actin as a component in host cells is proposed to be an important receptor for enteroccal adhesion and translocation. Moonlighting proteins are highlighted among the obtained adhesins (actin-binding proteins) in enterococci.

Bacteria make adjustments to accommodate themselves to inferior qualifications by regulation of gene expression. The transcription level of mfs-PTS in enterococci passing the mouse intestine was found to be 7-fold upregulated comparing with the PTS in enterococci cultured in medium in former study. The modulation of gene expression by PTS led to the postulation that PTS may be a factor involved in confrontation to gross circumstances. In this study *pts* mutants were constructed to probe the impact of the mannose/fructose/sorbose phosphotransferase system on the morphological and physiological behavior of *E. faecalis* OG1RF. Several representative bacterial pathogenic traits such as aggregation ability, tolerance to hydrogen peroxide and acid, resistance to bacteriocin and survival in macrophages were characterized in *E. faecalis* OG1RF and *pts* mutants. It was found that absence of *pts* (encoding mannose/fructose/sorbose, EIICD) genes in *E. faecalis* OG1RF attenuated tolerance to hydrogen peroxide and acid, induced less signal of SOD in *C. elegans*, and decreased survival in macrophages.

This study suggests that *E. faecalis* OG1RF utilizes several moonlighting proteins to adhere to actin in the host and possibly uses this as a mechanism to enhance translocation. Hereby it apparently takes advantage of a dysfunctional intestinal barrier, which greatly promotes bacterial translocation but not adhesion. During infection, *E. faecalis* OG1RF adaptes to rough enviroment like low pH and oxidation, meanwhile, it orients itself on surviving in

macrophages. These adaptions appear to be supported in the presence of mfs-PTS, which enhances the chance of bacterial survival and thus infectivity.

4.1 Dysfunctional intestinal layer aggravates infection

Bacteria are allured to the human organism because the body provides a fitting niche (warmth, moisture, nutrients) for colonization and propagation. Technically speaking, the location such as gastrointestinal tract, skin, and upper respiratory tract are outside the body since they could directly contact outside, which means, the colonization of bacteria in these parts could hardly generate systematic destruction owing to the protection of barrier (mucus, ciliated epithelia, and secretions containing antibacterial substances like lysozyme) (Baron, 1996). However, diseases break out every year as a result of enterococcal infection, the reasons behind and underlying mechanisms between bacteria and host intestine await to be unveiled. Intestine has two contrary but complementary functions: 1, Gateway of nutrients. Intestine lining digests and absorbs carbohydrate, fats, vitamin, and other nutrients by a series of enzymes and transport proteins (Phan, 2001; Wood & Trayhurn, 2003). Nutrients recognized by intestine are allowed into blood, or adjacent lymphatics and glands; 2, Entrance guard of adverse compounds, which is often referred as barrier function. To accomplish the blockup of unfavorable substances, the epithelial layer constitutes a vast superficial area and physical insulation to granules and macromolecules. The phenomena that an increase in permeability of large compounds is not necessarily associated with a concomitant increase in permeability of all small compounds implies that the intestine functions in a sophisticated and dynamic way other than merely a physical barrier (Hollander, 1992). Increased intestinal permeability is interrelated with diseases like hepatocirrhosis, immunodeficiency, atopic eczema, celiac sprue and other bacterial associated infections (Earth, 2014; Hollander, 1992; Inagaki-Ohara et al., 2006; Peterson & Artis, 2014; Pike, Heddle, & Atherton, 1985). It is a bacterial maneuver to induce intestinal permeability and open up tight junctions and adherens junctions (namely barriers) to invade human organisms. As shown in Fig. 47, tight junctions are composed of teraspan transmembrane protein and single-span transmembrane protien, of which teraspan transmembrane protein determines the capacity and the selectivity of the paracellular diffusion pathway. Teraspan transmembrane protein includes occludin, tricellulin and claudins (Balda & Matter, 2008). Adherens junctions contain cadherin and catenin, and are morphologically associated with actin filaments (Meng & Takeichi, 2009). *S. pneumoniae* and *Haemophilus influenzae* induce disruption of tight junction to facilitate translocation by reducing claudin 7 (Clarke, Francella, Huegel, & Weiser, 2011). *Listeria* adhesion protein

(Lap) promotes bacterial paracellular translocation through epithelial cell junctions while infecting gastrointestine (Kim & Bhunia, 2013). *S. pyrogenic* is able to utilize SpeB (Streptococcal pyrogenic exotoxin B) to cleave junctional proteins like occludin and E-cadherin and translocate across the epithelial barrier (Sumitomo, Nakata, Higashino, Terao, & Kawabata, 2013). Enteropathogenic *E. coli* accomplished invasion by using EspF protein as weapon to break occludin of tight junctions in HeLa and T84 cell layer (Mcnamara et al., 2001). In this thesis, a simulated open barrier was created by treatment of EDTA buffer, which chelated Ca^{2+}, and inhibited Ca^{2+}-dependent E-cadherin in epidermal tissue thus formed destroyed adherens junctions. A comprehensive survey of effects of open barrier on bacterial adhesion and translocation was performed with three enterococcal strains and one *E. coli* strain. No difference of adhesion rates was found between open barrier and close barrier for any of the bacteria. This may be so because that bacteria could efficiently bind to the surface of eucaryote cells without being affected by the disruption of epidermal tissue. However, translocation was 10 to 10,000-fold enhanced for all tested bacteria when barrier was critically disrupted (treated by EDTA buffer for 20 min). This translocation assay is stable based on the result that *E. coli* Top10 served as a control for the study, and had a translocation rate of 10^3 out of 10^6, which is approximately in accordance with the study of Cruz (Cruz et al. 1994), who reported that 10^3 out of 10^7 *E. coli* DH5α translocated across a Caco-2 cell monolayer under similar conditions. The increased translocation rates in open barrier varied greatly among different bacterial strains. *E. faecalis* V583 came first with an elevation of around 10,000 fold, followed by *E. faecalis* OG1RF with an elevation of 100 fold and lastly was *E. faecalis* Symbioflor® with an elevation of 10 fold. This provides a hint that an open barrier (dysfunctional intestine) offers an identical platform for attachment but also a more effortless routeway for translocation of pathogen (*E. faecalis* V583) comparing with probiotic (*E. faecalis* Symbioflor®). It is reasonable to speculate that a dysfunctional intestine attracts as many bacteria as healthy intestine but allows more pathogen to stride across intestinal barrier and spread into other organs. To keep the integrality of intestine is therefore to prevent pathogen from translocating across intestinal barrier, thereby, further infections in blood, glands and other tissues are restrained.

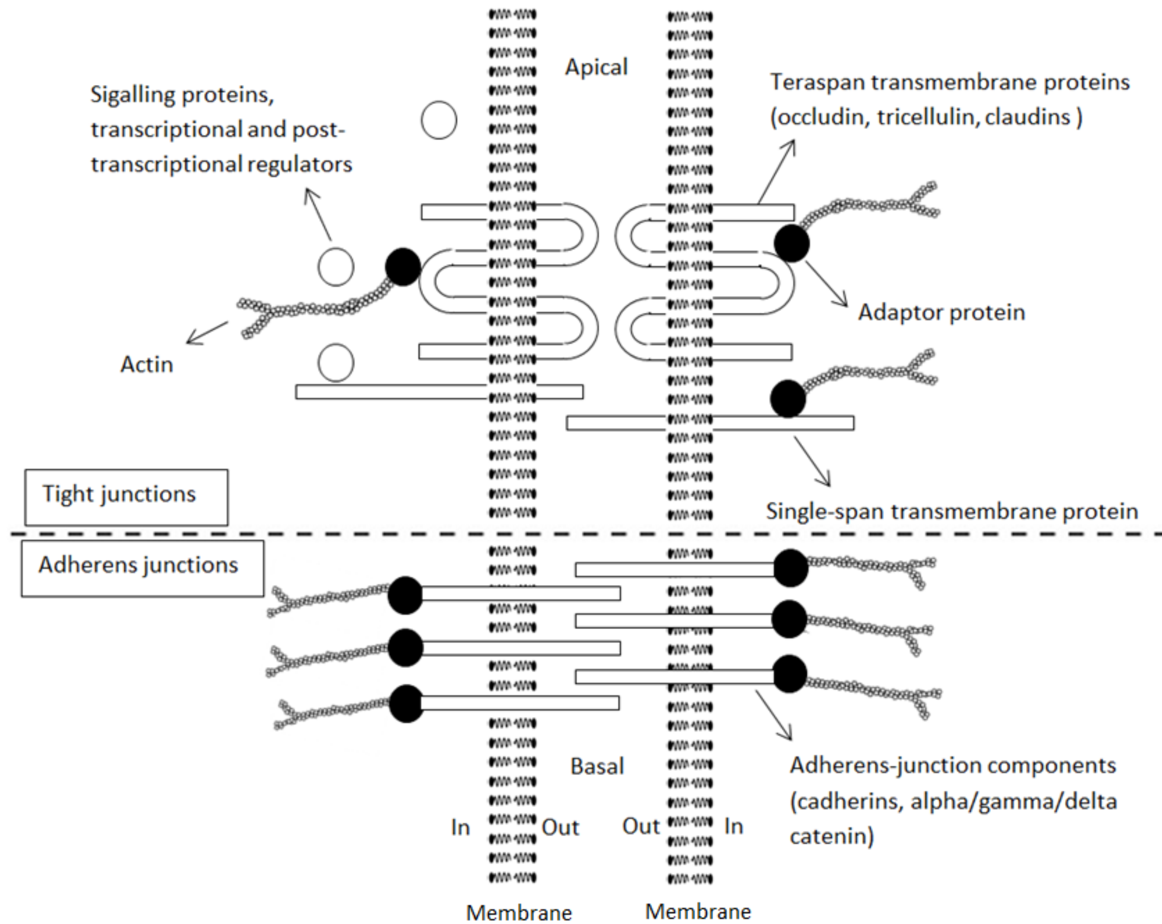


Fig. 47 Composition of tight junctions and adherens junctions between two adjacent epitheial cells. Tight junctions are the most apical component of the epithelial junctional complex while adherens junctions are more basal. Tight junctions contain Teraspan transmembrane protein (occludin, tricellulin, claudins) and single-span transmembrane protein. Sigaling proteins, transcriptional and post-transcriptional regulators are in the cytoplasm to complete the full function. Adherens junctions are constituted by cadherins and alpha/gamma/delta cateninn. Both tight junctions and adherens junctions are interrelated with actin by adaptor protein and are crucial for integrity of intestinal layer.

4.2 Actin as mediator for enterococcal paracellular migration

It is comprehensively agreed upon that a pathogen could expand its infectious territory if it manages to overcome the block of epithelial layer and diffuses into other organs, yet the brace for bacterial adhesion and translocation is under debate. Bacteria accomplish invasion by secreting adhesins which are invovled in anchoring the receptors in epithelial cells (Jepson, Schlecht, & Carla, 2000). Actin as the principal structure of eukaryotic cell skeleton has been suggested to participate the activity of bactrial invasion. In this thesis, the hypothesis that actin acts as the mediator of *E. faecalis* OG1RF to achieve paracellular migration has been proposed, being followed by the observation that the majority of bacteria stick to the actin of epithelial periphery in a series of experiments in this study.

Whether bacteria conquer the intestinal barrier by transcellular or paracellular migration, depends on intricate determinants. As an example, *Campylobacter* (*C.) jejuni*, which can be considered as commensal in chicken, is able to cross the intestinal barrier and enter underlying tissues, bloodstream and even other organs by both paracellular and transcellular migration. Factors with regard to bacteria (protein biosynthesis) and host (temperature, pH) determine the manner of *C. jejuni* migration. In other words, *C. jejuni* switches ways of migration complying with diversifications of ambience (Backert, Boehm, Wessler, & Tegtmeyer, 2013). Not all bacteria are able to utilize either way of migration of their own choices. Some commensal bacteria like *E. coli* choose endocytosis in preference to paracellular migration while infecting obstructive mouse distal small intestine and gluten-sensitive mice (Silva et al., 2012; Wu, Kuo, & Yu, 2011). The current thesis introduced laser confocal scanning microscope (LCSM) to observe the dynamic translocation of enterococci across a Ptk6 confluent monolayer. Actin as the primary component of the cellular skeleton dyed with phalloidine enabled a clear visual sense of the cytoskeleton. As each sheet of LCSM is equivalent to a cell slide, bacteria passing Ptk6 monolayer intracellularly or extracellularly could be captured by LCSM during translocation. *Enterococcus* and *E. coli* were observed to entangle with periphery actin, endocellular bacteria were rarely seen. A close up view made actin-tangling bacteria conspicuous (Fig. 11). Moreover, to statistically ascertain the outcomes in LCSM obeservation, cytochalasin D was adopted to control the density of actin, and corresponding bacterial translocation rates were recorded. In an intact Ptk6 monolayer, the order of bacterial translocation was: *E. faecalis* Symbioflor® > *E. faecalis* V583 > *E. faecalis* OG1RF. Cytochalasin D treatment at disruptive level increased *E. faecalis* Symbioflor® translocation only by 10 fold while *E. faecalis* V583 translocated at a 10,000-fold increased level starting from lower translocation than strain Symbioflor® in intact

monolayers. This suggests that *E. faecalis* Symbioflor® can modulate actin aggregation by itself to a higher extent than V583 to pass the barrier better in an intact mucosal barrier. As *E. faecalis* Symbioflor® lacks a major pathogenicity island in its genome, this strain may be tolerated in the host despite an increased translocation through a dysfunctional intestine (Fritzenwanker et al., 2013). The latter strain (V583), in turn, can take advantage of an impaired barrier and translocate to a very high extent in that situation. Moreover, it expresses a large array of known virulence factors (Lindenstrauss et al., 2011) and may have more and different ways to overcome the intestinal barrier in a compromised situation, followed by severe infections. OG1RF has the lowest translocation rate and may be considered as the "true commensal" in this context. We can´t exclude the possibility that enterococci and *E. coli* translocate the intestinal barrier by transcellular migration, but we infer that paracellular migration is the dominant route for enterococci and *E. coli* to go through Ptk6 barrier. Moreover, the pilus backbone in group B *Streptococcus* was suggested to contribute to paracellular translocation through epithelial cells (Pezzicoli et al., 2008). Other bacteria like *E. coli* C-25 and *L. monocytogenes* make use of paracellular migration to step across the mice ileum and Caco-2 epithelial barrier, whether the mechnism is also related to actin is yet unknown (Kim & Bhunia, 2013; Nadler et al., 2000).

4.3 Actin-binding proteins in enterococci and lactobacilli

Up to this study, three actin-binding proteins have been discovered in three different species of bacteria. Phosphoglycerate kinase (PGK) in group B *Streptococcus* has been reported to utilize amino acids 126–134 (KKESKNDEE) as binding site to bind actin in HeLa cells (Boone, Burnham, & Tyrrell, 2011; Boone & Tyrrell, 2012). SPA (staphylococcal protein A) in *S. aureus* targets β-actin in renal epithelial cells to achieve internalization in host cells (Jung, Kim, 2001). *S. enterica* manages to span two adjacent actin monomers in a filament by using SipA protein to directly bind to F-actin (Mitra, Zhou, & Gala, 2000). Due to its capacity to anchor host cells, actin-binding protein is considered as virulence factor in pathogen. *S. agalactiae* was also observed to recruit cytoskeletal actin at the site of adherent chains of streptococci, and actin was speculated to be involved in bacterial dissemination (Tyrrell, Kennedy, Shokoples, & Sherburne, 2002), yet the actin-binding protein in *S. agalactiae* awaits to be discovered. Since enterococci utillize periphery actin as vehicle to traverse the intestinal epithelial barrier. It is consequently of interest for us to find out the path-breaker, i.e., the actin-binding elements in enterococci. The current thesis endeavored to isolate actin-binding proteins in enterococci based on the inspection of enterococci-actin

interaction during bacterial infection in Ptk6 cells. A preliminary experiment was set up to verify the ability of enterococci to bind actin. Actin immobilized in 96-well plates was able to attract generally more enterococci than BSA as determined by OD_{570} measurement. Subsequently, this study addresses novel actin-binding proteins being isolated from *E. faecalis*. These identified adhesins were originally described to play a role in the central carbon metabolism. As the actin-binding function is second to the primary carbon metabolism function in these proteins, this second functionality is called “moonlighting”. Colloquially, moonlighting protein is a term introduced to describe the ability of a protein or peptide, which has more than one biological action (Henderson and Martin 2011). Moonlighting protein as an evolutionary tactic of tackling protean internal and external environments enables bacteria to make adaptions thereby develop survival mechanism. Invasion-mediating proteins usually reside on the bacterial surface and act as natural “ports” to receive outer material and hand them over intracellularly. Thus, the majority of proteins in supernant are expected to be secreted proteins, from which actin-binding proteins should be easier to obtain. However, no such proteins were found in this study. Moonlighting proteins discovered in this study are pyruvate formate lyase, enolase, glyceraldehyde-3-phosphate dehydrogenase, and GroEL from cell debris of *E. faecalis* OG1RF, Symbioflor®, and V583. The former three proteins are involved in the downstream of glycolytic pathway, and GroEL is a molecular chaperone. Indeed, the metabolic enzymes of the glycolytic pathway and molecular chaperone were previously suggested to exhibit moonlighting functions of adhesion and modulation of leukocyte activity in other bacteria. Surface enolase from *S. mutans* was demonstrated to bind to human plasminogen and salivary mucin MG2 (Ge et al. 2004). The major surface protein in group A *Streptococcus* is glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activities (Pancholi and Fischetti 1992). GroEL protein in *L. johnsonii* La1 and other lactic acid bacteria was speculated to play a role in gastrointestinal homeostasis due to its ability to bind to components of the gastrointestinal mucosa (Bergonzelli, Granato, Pridmore, et al., 2006). The identification of novel proteins in *E. faecalis* enhances our knowledge of the diversity of bacterial origins of moonlighting proteins, suggests additional targets to be studied in the mechanisms of enterococcal approaches to their host, and suggests new functions for proteins of the central metabolic pathways.

In addition to enterococci, ten lactobacilli (probiotic) were applied to find out if actin is a receptor that is specific for enterococci. To our knowledge, no paper on probiotic utilizing actin as mediator to colonize host has been published so far. In the 96-well plate assay, *L. paracasei* TMW 1.1434, *L. plantarum* TMW 1.1734 and *L. paracasei* TMW 1.1434 of the ten

lactobacilli were found to display strong adhesion to actin. Thereafter, actin-binding adhesins in lactobacilli were isolated and characterized. The newly discovered actin-binding proteins in lactobacilli are: pyruvate kinase (PK), glucose-6-phosphate isomerase (PGI) and phosphoglycerate kinase (PGK) from *L. paracasei* TMW 1.1434; PK, chaperonin GroEL and EF-Tu from *L. plantarum* TMW 1.1734 and GroEL from *L. brevis* TMW 1.485. Regardless of the bacterial species, the outcome that PK, PGI, PGK, EF-Tu and GroEL pop up as actin-binding proteins is not unexpected due to the fact that these five proteins from pathogens as well as lactobacilli were previously reported to be host-targeting in many other studies. For example, chemically purified EF-Tu was testified to bind actin in metastatic rat mammary adenocarcinoma (Edmonds et al., 1996). GroEL and EF-Tu from *L. johnsonii* NCC533 La1 were successively found to bind to components of the gastrointestinal mucosa by Bergonzelli and his coworkers (Bergonzelli, Granato, Pridmore, et al., 2006; Granato, Bergonzelli, Pridmore, Marvin, Rouvet, & Corthésy-Theulaz, 2004). The adhesion to actin interprets GroEL and EF-Tu as multiaspect adherence factors. In the year 1999 Jeffery introduced the term “moonlighting proteins” to represent such multifunctional proteins (Jeffery, 1999). Since that time, more and more moonlighting proteins have been discovered. PK, PGI, and PGK were previously defined as moonlighting proteins as a result of the ability to bind to plasminogen (Brian & Andrew, 2011). In the light of the multiple functions of moonlighting proteins, these proteins do not exclusively bind to just one substance, but are able to anchor to several targets. The cell surface protein PGK from *L. lactis* was described as capable of binding mannan of yeast (Katakura et al., 2010), PGK from oral streptococci could bind to plasminogen (Kinnby, Booth, & Svensa, 2008), and PGK from group B *Streptococcus* binds to cellular actin, which is in accordance with our present study. This leads to the deduction that PGK may play a role in host intimacy and internalization of bacteria (Burnham, Shokoples, & Tyrrell, 2005). PGI is a major human/mammalian moonlighting protein with funtion of cell signaling and it plays a role in malignancy as autocrine motility factor (Yanagawa, Funasaka, Tsutsumi, Watanabe, & Raz, 2004). Based on this a hypothesis was developed that bacterial PGIs may have arisen from eukaryotic gene transfer (Katz, 1996). The recombinant crystallized PGI from *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*) has been shown to have both autocrine motility factor and neuroleukin activity (Medical, Hospital, & Science, 1999), suggesting that this bacterium could utilize its PGI to elicit signals once it colonized humans. In this thesis, we report that PK, PGI, and PGK from *L. paracasei* TMW 1.1434, and PK from *L. plantarum* TMW 1.1734 could bind to actin, therefore confirmed the multifunctionality of PK, PGI, and PGK.

It was reported that the succesful capture of PGK in bacteria by actin in the host owed to a specific amino acid motif. Site directed mutagenesis suggests that a consensual sequence (KKESKNDEE) between PGK in group B *Streptococcus* and actin (or plasminogen) in the host is involved in binding (Boone & Tyrrell, 2012). PGK from ten lactobacilli in our study were sequenced and the hypothetical PGK-actin binding domains were characterized. One amino acid difference between *L. paracasei* TMW 1.1434 and the other nine lactobacilli was identified. The existence of lysine (K) at the position of amino acid 127 in *L. paracasei* TMW 1.1434 and GBS, which displayed strong actin adhesion ability may explain the reason why the other nine lactobacilli, which had arginine (R) at the same position did not adhere to actin so strongly. Lysine probably dominates the adhesion ability in this structrue. Therefore, lysine in the consensus sequence of the binding domain is suggested to play important role in actin-binding activities.

The possibility to immobilize receptors like actin, mucin and fibronectin enables bacterial substrates to bind specifically and adhesins in bacteria are likely to be isolated, which helps to concrete the adhesin-receptor model when bacteria infect host.

4.4 Influence of PTS on sugar utilization

PTS was reported to influence bacterial sugar metabolic pathways, biofilm formation, initial adherence of cells to substrate, which are important factors of bacterial colonization and persistence (Houot, Chang, Absalon, & Watnick, 2010; Iyer & Hancock, 2012). The principal and obvious function of PTS as a major carbohydrate transport system is to enable effective sugar uptake resulting in activated sugar mono- and dimers. EIIC and EIID in the chain of enzyme II are key membrane proteins that buid up the connection between sugar and phosphorylation in carbohydrates metabolism, thereofore, gene encoding EIICD was deleted to investigate its role in sugar utilization and other effects on bacterial virulence. Besides, disruption of PTS could generate a different content of intermediate phosphoryl transporter. As the critical intermediate phosphoryl transporter of glycometabolism, the phosphorylated status of HPr determines miscellaneous regulations and controls. In a comprehensive view of PTS, the intermediate status (phosphorylation status) of all PTS catalyzing enzymes codetermine the aftermath of many regulation routes. We discuss only gram positive bacteria in this context. The unique character of gram positive bacteria is the two statuses of phosphorylation in HPr: P~Ser-HPr and P~His-HPr (Fig. 2). Both P~Ser-HPr and P~His-HPr are phosphoryl intermediates, whose contents of phosphoryl are complemented, i.e., more P~Ser-HPr will lead to less P~His-HPr and vice versa. In a medium with rapidly consumed

sugar (glucose), P~Ser-HPr accounts for 95% of phosphoryl groups while P~His-HPr accounts for 5%. Even though P~His-HPr takes up only a minor proportion, it is the key for sugar metabolism. P~Ser-HPr however does not play an direct role in sugar utilization but is associated with many regulations. Disruption of PTSs may modify content of P~Ser-HPr and cause:

(1) Release of carbon catabolite repression (CCR). Cre (catabolite responsive element) is the valve of sugar utilization, which could be inactivated by the P~Ser-HPr:CcpA complex. When bacteria grow in rapidly consumed sugars like glucose and fructose, abundant P~Ser-HPr is produced in the process of sugar metabolism. As the coopressor of CcpA (catabolite control protein A), the great quantity of P~Ser-HPr significantly increased the amount of P~Ser-HPr:CcpA complex, which binds to cre and turns off sugar utilization, this is termed CCR. Nevertheless, it is worth noting that this phenomena (CCR) does not occur in complex media, which also includes the intestinal tract. Deletion of IIC (Man) or IID (Man) was reported to create substantial P~His-HPr but rare P~Ser-HPr (Yebra et al., 2006), therefore, *pts* (encoding mannose/fructose/sorbose, EIICD) mutants are expected to decrease quantity of P~Ser-HPr and switch on sugar utilization, finally release from CCR in nutrient-limited medium but not in rich medium.

(2) Release of inducer exclusion. Mechanism of inducer exclusion: the plenty of P~Ser-HPr in glucose-containing medium results in low quantities of P~His-HPr, which bring EIs and EIIs to intensive competition for phosphoryl groups. As a result, less P~EIs and P~EIIs distract the system from utilizing non-preferential sugars such as glycerol, lactose and galactose. Moreover, the unphosphorylated form of enzyme IIAs can inhibit the permease of non-PTS sugars by direct interaction. Therefore, *pts* (encoding mannose/fructose/sorbose, EIICD) mutants are expected to decrease quantity of P~Ser-HPr and increase P~EIs and P~EIIs, finally release from inducer exclusion.

(3) Change of bacterial virulence. P~Ser-HPr was deduced to positively regulate virulence by binding to virulence operator *cre*, which locates upstream from the virulence promoter (*mga*) in streptococci (Fig. 2). Therefore, *pts* (encoding mannose/fructose/sorbose, EIICD) mutants are expected to decrease quantity of P~Ser-HPr and attenuate bacterial virulence. Other regulatory functions of P~Ser-HPr present in: glycerol kinase acitivation, inducer expulsion and antitermination.

In an effort to define the role of PTS in CCR, a chemically defined medium (Davis medium) was applied for the cultivation of *E. faecalis* OG1RF and all *pts* mutants. Enterococci residing in this medium could only utilize glucose as carbon source while other commonly used sugars

like fructose and mannose were not able to support a thriving propagation (App. Fig. 18-25). However, MRS with glucose, mannose or fructose as the preferential carbon source enabled the flourishing growth of *E. faecalis* OG1RF and all *pts* mutants, owing to the more diverse nutrients that are important for bacterial growth. As shown in Fig. 26-30, the preferential sugars for enterococi are mannose, glucose, and fructose. Sorbose however like other non-preferential carbon sources such as galactose, sorbitol, gluconate did not boost bacterial growth (App. Fig. 13-17), which was also reflected in the API test (App. Fig. 26), where little sorbose could be utilized. Release of CCR in *pts* mutants was observed in MRS with glucose, fructose or mannose as the preferential carbon source and galactose as the non-preferential carbon source. Glucose and fructose are the two common sugars that induce the glucose repression effect (namely CCR), whereas class A (glucose, mannitol) and class B (glycerol) substrates are also able to generate CCR (Postma & Lengeler, 1985). It is obvious that the *pts*1, *pts*2 and *pts*1/2 mutants displayed more vigorous growth than wild type due to release of CCR. The effect of CCR release could stem from two mechnisms: 1, Allosterism of transporters or cytoplasmic enzymes. The allosteric structure is not able to activate and promote synthesis of inducer and thus fails to utilize relevant sugars. 2, Inhibition of transcriptinal regulators. The activity of *cre*, which is in the promoter region of genes involved in carbon metabolism could be inhibited by the P~Ser-HPr:CcpA complex. As indicated above, removal of EIIC (Man) or EIID (Man) greatly reduces P~Ser-HPr, therefore less P~Ser-HPr:CcpA complexes are formed, which results in more activated cre sites and more sugar utilizations are thus switched on. Although the release of CCR due to disruption of PTS is common for different bacteria (Galinier et al., 1998; Görke & Stülke, 2008; Kremling et al., 2001; Veyrat, Monedero, & Perez-martinez, 1994; Viana et al., 2000), CCR disappears when nutrient-limited medium is switched to rich medium. The influence of growth conditions on CCR in *pts* mutants were seen in a few cases. For example, the CCR generated by deletion of *pts*H1 in *B. subtilis* disappeared when grown in complex medium but resumed in minimal medium (Deutscher, Reizer, Fischer, & Galinier, 1994; Zalieckas, Jr, & Fisher, 1999). To verify the influence of complex medium on CCR, *E. faecalis* OG1RF and all *pts* mutants were cultivated in BHI medium. As expected, *E. faecalis* OG1RF and all *pts* mutants displayed identical growth potential (except for the *pts*2 mutant, whose growth was slightly repressed), no obvious CCR effect was seen in wild type strain. CCR was suggested to be an adaptive mechanism that ensures an optimal bacterial metabolic network activity for growth, meaning, bacteria manage to make good use of limited carbon sources upon growth in a nutrient-restricted environment, but abandon this principle when the environment

supplies enough nutrients such as LB medium (Zalieckas et al., 1999; Zhou et al., 2013). This principle is applicable to *E. faecalis*, which lost CCR when nutrient-limited medium was switched to rich medium. The animal intestine is considered to be nutrient-rich, therefore removal of *pts* genes is assumed not to affect bacterial growth in the host (Fabich et al., 2011). The mechanism how *pts* mutants in different media modulate CCR is veiled but was suggested to be related to the different nitrogen sources present in the media (Faires et al., 1999).

Another phenomenon due to the CCR effect in bacterial growth is diauxic growth or biphasic growth. The privileged utilization of a small amount of preferential sugar delays the comsuption of less preferential sugar, and thus leads to diauxic growth. In the first growth phase, glucose or other preferential sugars cause a flourishment of bacterial growth till the sugar is exausted. Before the second growth phase begins, bacteria usually need an adjustment period to get ready for the use of the less preferential sugar, this adjustment period terms lag time. The second prosperity of bacterial growth resumes by consuming less preferential sugar. It was reported that deletion of EIIAB (Man) in *S. salivarius* led to disappearance of diauxic growth, indicating the disruption of PTS released bacterial growth from CCR. With this mechanism, bacteria could directly use less preferential sugars without inhibition by a preferential sugar from the start. Our study however observed no diauxic growth in wild type or in any of the *pts* mutants when using glucose and fructose (or mannose) combination as carbon source (Fig. 31-34), illuminating that *E. faecalis* OG1RF had equal preference for glucose, fructose and mannose. This is consistent with that glucose and fructose were simultaneously metabolized by *E. faecalis* RKY1 when these two sugars were applied to one medium (Yun & Ryu, 2001). Moreover, this also explains why glucose, mannose, and fructose in MRS promote the growth of *E. faecalis* OG1RF and all *pts* mutants in a similar pattern (Abranches, Candella, Wen, Baker, & Burne, 2006; Kok, 2003).

Bisides the effect of CCR release, removal of PTS enzyme II components could bring many other aftereffects. Elimination of EIID (Man) in *E. faecalis* V583 or EIIAB (Man) in *S. mutans* resulted in differential expression of many genes, indicating these *pts* genes are involved in global carbon catabolite control (Jacqueline et al., 2003; Opsata et al., 2010). To be more specific, deletion of EIID (Man) in *E. faecalis* V583 led to repressed metabolism while using serine, galactose, lactose, glycerol or sucrose as carbon source, caused differential expression of 184 genes, most of which are involved in energy metabolism, transport, binding and signal transduction. This demonstrates that EIID (Man) could comprehensively influence glucolysis to a universal extent. It is worthwhile to notice that deletion of EIID (Man) could

defect the operon of EII (Man) and lead to decreased transcription of the mannose PTS operon, indicating that a fully functional PTS is necessary for operon´s integrality (Opsata, Nes, & Holo, 2010).

The profiles of sugar consumption were analysed by ion chromatography. It has shown that utilization of glucose is compatible with the CCR effect in bacterial culture (Zhou, 2011). Consumption of glucose positively proportionated to bacterial OD values.

Moreover, oxidation-reduction potential (Eh) and acid production as indicators of bacterial fermentation displayed no significant differences between wild type and all *pts* mutants during fermentation in BHI (Fig. 36-37), i.e., loss of *pts* genes does not affect bacterial fermentaion ability.

4.5 Influence of PTS on acid and oxidative stress tolerance

Bacteria accommodate themselves to a certain range of pH by regulating expression of related genes to enable survival and propagation. The ability of bacteria to resist low pH are broadly studied since low pH is used in fermented food as means to prevent growth of potential pathogens. Observations assist the point of view that energy systems react to low pH discrepantly due to bacterial species. How energy-related genes respond to low pH is not universally agreed on. Significant repression of the energy-related gene HPr in *L. lactis* was detected by two-dimentional electrophoresis when bacteria were exposed to pH 4.5 or 5.5 (adjusted with hydrochloric acid) for about 25 min (Frees, Vogensen, & Ingmer, 2003). A counterexample was found in *E. coli*, which had an increased expression of energy-related genes (*man*X and *pts*H) by the inducement of propionic acid (pH 6.0) (Blankenhorn, Phillips, & Slonczewski, 1999). Generally, these envidenes above manifest that energy-related genes respond to low pH inconsistently. *E. faecalis* OG1RF and all *pts* mutants displayed repressed growth in pH 5.5 BHI comparing with those in normal BHI (aerobic, pH 7.23) as indicated in chapter 3.2.2.3. In acid killing assay, OG1RF displayed stronger tolerance to acid than any of its *pts* mutants when enterococci were exposed to pH 4.1 buffer. This indicates that *pts* genes in enterococci enhanced bacterial tolerance to low pH.

Bacteria evolved to protect themselves from oxidative stress in mal-conditions by many mechansims such as global regulators *oxy*R and *per*R, hydrogen peroxide scavengers catalase, *spx*A1 and *spx*B, and NADH peroxidase (L. Chen, Ge, Wang, Patel, & Xu, 2012; Pericone, Park, Imlay, & Weiser, 2003). *E. faecalis* has more than one aptness to tolerate oxidation by producing Npr (NADH peroxidase), Spx, heme-dependent KatA, etc. In this study, growth of

OG1RF and all *pts* mutants were found to be repressed by hydrogen peroxide (0.5 mM), and *pts*2 mutant was most vulnerable to hydrogen peroxide. The underlying mechanism is illustrated by determination of the differential gene transcription via qRT-PCR discussed below. A higher concentration of hydrogen peroxide (25 mM) caused an almost linear killing of *E. faecalis* OG1RF and all *pts* mutants. The incubation in hydrogen peroxide for 15 and 30 min led to drasticly decreased survival rates for OG1RF and *pts* mutants. Of all the enterococci survived in hydrogen peroxide, OG1RF maintained better survival ability than any of the *pts* mutants at different intervals. Deletion of *pts*1 and *pts*1/2 genes decreased the survival rate by 2~3 fold while deletion of *pts*2 gene decreased survival rate by 62 fold, indicating *pts*2 gene significantly enhances the tolerance of *E. faecalis* OG1RF to oxidative stress.

Since absence of *pts* genes in *E. faecalis* OG1RF led to attenuated resistance to hydrogen peroxide, it is therefore speculated that *pts* genes are involved in regulation of antioxidant genes. Related studies by Nicolas, *et al* showed that the deletion of *hyp*R (antioxidant gene) generated a survival reduction by 200 fold in 20 mM hydrogen peroxide in comparison with wild type *E. faecalis* JH2-2 (Verneuil et al., 2004). The inactivation of catalase katA (heme-dependent) was found to down-regulate survival of *E. faecalis* OG1RF in 30 mM hydrogen peroxide (for 15 min) by 30% (Baureder et al., 2012). Moreover, it was reported that disruption of the *spx* gene reduced tolerance of *E. faecalis* OG1RF to hydrogen peroxide, and resulted in down-regulation of several antioxidant genes including *ahpC*, *gor*, *hypR*, *katA*, *npr*, and *tpx* (Kajfasz et al., 2012). This implies that one gene lost could generate a “butterfly effect”, i.e., the absence of one gene would affect the transcription or expression of many other genes, which are regulated by this one gene. To confirm the role of *pts* genes in regulation of antioxidant and energy-related genes, quantitative real time PCR was applied to quantify the level of gene transcription. Quantitative RT-PCR was executed with RNA samples under two different conditions: 1, RNA was isolated from *E. faecalis* OG1RF and *pts* mutants subjecting to 2.4 mM hydrogen peroxide for 30 min after exponential growth in BHI. 2, RNA was isolated from *E. faecalis* OG1RF and *pts* mutants cultivated in BHI till the exponential phase. In case 1, as displayed in Tab. 34, the transcriptional level of antioxidant genes in *E. faecalis* OG1RF and *pts* mutants has no rule to follow considering its relevance to hydrogen peroxide-tolerant ability. That is to say, the susceptibility of any of the *pts* mutants to hydrogen peroxide did not corelate to the transcription of genes suspected to play a role in tolerance to oxidative stress when RNA samples were obtained from hydrogen peroxide-treated enterococci. Such “chaotic” transcription changes were also seen in a similar study by

Giard, who found that inactivation of *ers* (antioxidant gene) in *E. faecalis* JH2-2 caused reduced tolerence to hydrogen peroxide but disordered transcriptional levels of antioxidant genes in qRT-PCR assays (Giard et al., 2006). This may take into account the fact that cultivation in hydrogen peroxide for 30 min could have activated different mechnisms for bacteria to regulate gene expression or other potential antioxidant gene regulators were activated. In case 2, as shown in Tab. 35, based on the premise that no significant detectable difference was traced in internal controls (23S rRNA and *gyr*A genes), elimination of *pts*1 and *pts*1/2 led to increased transcription of two hydrogen peroxide stress response genes *tpx* and *lys*R (+2~3 fold), which is in line with former observed better growth potential of *pts*1 and *pts*1/2 mutants than *E. faecalis* OG1RF in BHI containing 0.5 mM hydrogen peroxide (App. Fig. 12). Moreover, the down-regulated transcription of five hydrogen peroxide stress response genes *gor*, *kat*A, *lys*R, *hyp*R, and *rex* (-3~6 fold) in *pts*2 mutant made an explanation for repressed growth of *pts*2 mutant in low concentration of hydrogen peroxide (0.5 mM) and lowest survival rate at high concentration of hydrogen peroxide (25 mM). Besides, energy-related genes like *hpr*K and *rex* were 4.8 and 3.2 fold down-regulated in *pts*2 mutant, which is probably the reason why *pts*2 displayed repressed growth in BHI under all conditions (aerobic, anaerobic, with hydrogen peroxide, without hydrogen peroxide). *Rex* is redox-sensing repressor, which regulates transcription of respiratory genes in response to the intra cellular NADH/NAD+ redox poise (Gyan, Shiohira, Sato, Takeuchi, & Sato, 2006; McLaughlin et al., 2010; Royer & Lukosi, 2007). This assay illuminates that *pts* genes participate regulation of energy-related and antioxidant genes, which in turn influence bacterial metabolism and tolerance to oxidation.

4.6 Effects of PTS on yeast agglutination

Yeast is commonly used as a model for evaluation of bacterial adhesion capacity owing to its abundant content of mannose, which is one component of bacterial receptors in epithelial cells. It is natural to hypothesize that bacteria with ability to bind intestinal epithelial cells is likely to possess the capacity to generate yeast agglutination. The disruption of mannose/fructose/sorbose phosphotransferase system in enterococci is speculated to alter its ability to assemble yeast according to the principle that an incomplete mannose/fructose/sorbose phosphotransferase system attenuates bacterial ability to receive and transfer mannose, thereby, disruption of PTS leads to inferior ability to aggregate yeast. However, no such assumed phenomena was observed in the yeast agglutination assay by visual observation or by statistical study. The probable reasons are interpreted as: 1,

enterococci prefer other receptors to mannose when anchoring to epithelial cells. Mannose is not the only target for enterococci to bind, other molecules may play a more important role than mannose. Actin as a receptor in epithelia for example is important for enterococcal adhesion. Consequently, the disruption of mannose/fructose/sorbose phosphotransferase system may not associate with the bacterial ability to agglomerate yeast. 2, the disruption of selected mannose/fructose/sorbose PTS is too puny to cause an inability to hinder PTS assembly, and a truncated PTS is assembled, which still enables adhesion. 3, there are eight mannose/fructose/sorbose PTS in enterococci as indicated by gene annotation, and all display different gene elements. This could imply that the eight mannose/fructose/sorbose PTS are hierarchical in regulating sugar phosphorylation and transport or mannose specific adhesion. When the mannose/fructose/sorbose PTS eliminated in this study happens to be the dispensable ones, then it is reasonable that the all *pts* mutants displayed similar yeast-agglutination ability like the wild type. 4, it is worth noting that, although aggregation substance is frequently nominated as one of the important virulence for host-adhesion, enterococci however attach more importance to adhesins other than aggregation substances in infection (Jett et al., 1994). Hence, it may not be *pts* itself, which is involved in mannose specific binding. A vigorous adhesion to mannose base group normally requires MSA (mannose specific adhesin), which was found in *L. plantarum* WCFS1 (protein encoded by lp_1229) and in *S. aureus* (protein SasA). No homologous sequence to such genes was found in enterococci, illuminating why also *E. faecalis* OG1RF is incapable of generating notable yeast agglutination.

4.7 Influence of PTS on bacterial resistance to pediocin

Mannose PTS has been reported to be involved in sensitivity to mesentericin Y105 and has been suggested to be receptor for class II bacteriocins (Dalet, Cenatiempo, & Cossart, 2001). Enterococci as the second bacteria after *Listeria*, which possess abundant *pts* in the genome, displayed in a similar manner resistance to mesentericin Y105 when IID unit in mannose phosphotransferase system was eliminated (Héchard, Pelletier, Cenatiempo, & Frère, 2001). Pediocin produced by *Pediococcus acidiactici* as a class IIa bacteriocin was known as inhibitor of several bacteria such as *L. monocytogenes*, *E. coli,* and sublethal injuried (gentle heating, exposure to lactic acid or EDTA) *Salmonella* (*S.*) *typhimurium* (Rodríguez, Martínez, & Kok, 2002; Technics & Box, 1988). The removal of EIID (Man) in *E. faecalis* V583 generated resistance to pediocin while the wild type is sensitive to this bacteriocin (Opsata et al., 2010). Our investigation illustrates that deletion of *pts* (encoding

mannose/fructose/sorbose, EIICD) genes in *E. faecalis* OG1RF enhanced bacterial ability to resist bacteriocin to different degree. However, the absence of *pts* genes did not enable mutants to completely conquer the inhibition of pediocin (Tab. 33; Fig. 41). This is likely due to the fact that there are 8 mfs-PTSs, which could simultaneously act on the reception of pediocin in *E. faecalis* OG1RF, therefore, one or two missing *pts* genes did not necessarily induce full pediocin resistance.

4.8 Influence of PTS on bacterial survival in different types of macrophages

A stunt the human system protecting itself from infection by pathogen is phagocytosis by macrophages. Macrophages have versatile weapons such as antimicrobial peptides, lysozymes, acidification, ROS and nitrite to kill pathogens. Moreover, severe limitation of nutrients limits survival of pathogens inside the macrophage (Slauch, 2011). Only 10% *E. faecalis* JH2-2 survived in macrophage J774A.1 after incubation for 48 h (Verneuil et al., 2004). Survival rates of *E. faecalis* V583 and *E. faecalis* OG1X in murine peritoneal macrophages were 16% and 10% after incubation for 48 h (Gentry-weeks, Karkhoff-schweizer, Pikis, Estay, & Keith, 1999; Michaux et al., 2012). Survival of bacteria in macrophages is related to the species of bacteria and types of macrophage (Bokil et al., 2011). The current thesis has shown that 81% *E. faecalis* OG1RF could survive in J774A.1 macrophages after 48-h postinfection. Further incubation (till 72 h) in macrophages displayed continuous killing effects on *E. faecalis* OG1RF, which led to a 42% survival rate. The data of *E. faecalis* OG1RF surviving in J774A.1 for 48 h is approximately in accordance with Kajfasz´s study, which had a survival rate of around 70% (Kajfasz et al., 2012). As to the mutants, the survival rates of Δ*pts*1, Δ*pts*2, and Δ*pts*1/2 at time point 48 h were 23%, 16% and 20%, and at time point 72 h were 17%, 12% and 10%, which were significantly decreased comparing to the wild type at the same time point and implies that the elimination of *pts* had severely attenuated the survival ability of *E. faecalis* OG1RF in J774A.1 macrophages. As *in vitro* study, *pts* was shown to enhance tolerance to acid and hydrogen peroxide, which are arguments support the outcome that *pts* genes prevent *E. faecalis* from being killed by acid and other toxic substances produced by macrophage, thereby survive better in macrophage.

Some researchers argue that the J774A.1 cell line lacks many of the characteristics of native macrophages isolated from animals. Monocytes originate from progenitor cells in the bone marrow undergo three divisions (monoblasts, promonocytes, and monocytes) before they are released into the circulation (Cohn, 1978; Gordon & Taylor, 2005). Monocytes eventually differentiate into macrophages as they leave the blood and enter the tissue (Manuscript &

Isolation, 2008). Therefore, the present study prepared primary BMMs (bone marrow-derived macrophages) from murine bone marrow to mimic the dynamic biochemical activities of authentic macrophage. Survival of *E. faecalis* OG1RF, Δ*pts*1, Δ*pts*2, and Δ*pts*1/2 in BMM were 25%, 6%, 20% and 5%, respectively after 48-h incubation. Further incubation (till 72 h) led the survival rates to 4%, 0.9%, 5% and 1%. Here we observed different effects of *pts* genes on bacterial phygocytosis and survival in macrophage BMM and J774A.1. These two macrophages exhibit different features of bacterial killing: 1, BMM showed a stronger (2-fold) ability to engulf and eliminate endocellular *E. faecalis* OG1RF and *pts* mutants than J774A.1 at the same time point; 2, absence of the *pts*2 gene made similar contributions as *pts*1 and *pts*1/2 genes to encterococcal survival in J774A.1. But in BMM, deletion of *pts*2 gene had no influence (similar to wild type) on enterococcal survival while *pts*1 and *pts*1/2 mutants displayed a much worse survival ability. These discrepancies could stem from the different mechanisms of two macrophages to cope with bacteria. Even though BMM is a proximal macrophage in contrast to J774A.1, the application of latter macrophage in experimental protocols is much more often reported than that of BMM, which requires more sophisticated and longer cultivating time (one week for preparation). It was found that around 80%-90% group B *Streptococcus* serotype I survived in BMM after a postinfection for 60 min (Cheng et al., 2001). This suggests that the factors involved in bacterial survival in macrophages include type of macrophage, species of bacteria, MOI (multiplicity of infection), time of initial infection. In conclusion, the loss of *pts* (encoding mannose/fructose/sorbose, EIICD) genes in *E. faecalis* OG1RF attenuated bacterial survival in macrophages to different extents.

4.9 Interlinks between PTS regulation and virulence in bacteria

It is commonly approved that disruption of EI in PTS led to attenuated bacterial virulence. Omissive EI down-regulated expression of virulence genes *hly* and *actA* to 4 fold when *L. monocytogenes* AML73 was grown in minimal medium supplimented with 10 mM glucose (Aké, Joyet, Deutscher, & Milohanic, 2011). Disruption of EI in *L. monocytogenes* inhibited the activity of virulence regulator PrfA (Herro et al., 2005). In an *in vivo* study conducted by Kok *et al*, removal of EI in *S. typhimurium* lowered its survival rate in mice, indicating an attenuated virulence was generated (Kok, 2003). However, several lines of envidence suggest that the aftereffects brought by inactivation of EII in PTS varied in different bacterial species, the mechanism is not yet systematicly and explicitly clarified. There are three outcomes and viewpoints: 1, disruption of EII (Man) upgrades bacterial virulence. The mechanism probably lies in the activation of the virulence genes by P~EI and P~EII. According to Yebra´s study,

deletion of EIID (Man) transformed the majority of HPr into P~His-HPr and therefore led to more P~EI and P~EII thus activated bacterial virulence (Yebra et al., 2006). A study of PTS by Ake *et al* is compliant with the above-mentioned illustration: omissive EIIAB (Man) and EIIC (Man) enchanced expression of two prfA-controlled virulence genes (*hly*, *actA*) up to 12 fold when *L. monocytogenes* AML73 was grown in minimal medium supplemented with 10 mM glucose (Aké et al., 2011). Disrupted *manN* gene (coding the EIID component of mannose class PTS) in *S. suis* induced vast synthesis of suilysin, the hyperactive hemolysin generatd hyperhemolytic phenotype. Complementation of EIID in the mutants banished over-produced suilysin (Lun & Willson, 2005). 2, disruption of EII (Man) would on the contrary attenuate bacterial virulence. P~Ser-HPr as co-activator of virulence regulator (*mga*) in streptococci is suggest to enhance bacterial virulence. Disruption of *pts* (encoding mannose/fructose/sorbose, EIICD) genes is to reduce P~Ser-HPr and attenuate bacterial virulence. Elimination of EIIAB (Man) in *S. mutans* resulted in reduced presence of the virulence factor fructan hydrolase, meanwhile, impaired the formation of biofilms (Abranches et al., 2006). This is the theory that supports the outcomes in this thesis. 3, disruption of EII (Man) regulated the expression of different virulence genes in one bacterial strain differently. Deletion of EIIAB (Man) in *S. mutans* attenuated the expression of the virulence gene *gtf*BC by three fold, whereas enhanced the expression of virulence gene *ftf* by two fold (Jacqueline et al., 2003). The distinguishing regulation of virulence genes by PTS could stem from several aspects: 1, difference of culture medium. Type of sugar, concentration of sugar, and pH in medium have influence on expression of bacterial virulence genes (Aké et al., 2011; Ferrando et al., 2014; Iii et al., 2008). 2, difference of bacterial species. Bacteria vary in virulence as pathogens. The interlink between virulence genes and PTS is diversiform. 3, definition of real virulence genes. Virulence, in a narrow sense of the word, refers to the critical factors that contribute to the causation of lesion or disease. Some researchers tend to classify growth-promoting facotrs into bacterial virulence, consequently, the regulation of glycolytic enzyme by PTS is referred to as virulence regulation. 4, different models for virulence evaluation. Modification of bacterial virulence by PTS could be assessed at the molecular level (gene transcription) and by animal experiments (survival in animal or animal killing). Outcomes of studies *in vitro* and *in vivo* are not always consistent. 5, confusion of virulence regulator and virulence gene. Researchers are inclined to describe up-regulated virulence regulator as up-regulated virulence genes. Actually, the increased expression of virulence regulator does not necessarily stimulate activity of virulence genes under some conditions. Due to the intricate and global regulation of PTS, it is not fair to make an arbitrary conclusion, claiming that PTS

has a general positive or negative influence on the virulence of different bacteria. This study demonstrates that *pts* (encoding mannose/fructose/sorbse, EIICD) gene in *E. faecalis* OG1RF promotes sugar utilization and positively regulates bacterial virulence in vitro and *in vivo* assay, interpreting the observation that *pts* (encoding mannose/fructose/sorbse, EIICD) was up-regulated when *E. faecalis* OG1RF passed through murine intestine in former study.

4.10 Influence of PTS on SOD activation in nematodes

Caenorhabditis (*C.*) *elegans* has been developed as an inexpensive and easily compassable animal model for screening gram-positive pathogenic bacteria to assess bacterial virulence (Garsin et al., 2001). The intruding-defending interaction between pathogen and host is mutual and intricate. *C. elegans* generates oxidative stress response enzymes (like superoxide dismutase, SOD) to protect itself from reactive oxidative species (ROS) stimulated by pathogens when bacteria enter host. Signs of oxidative stress occur in the site of the host-pathogen interface (normally in the intestine), suggesting that ROS release is localized to this tissue (Chávez, Mohri-Shiomi, Maadani, Vega, & Garsin, 2007). *C. elegans* utilizes SOD to degrade self-produced or pathogen-produced superoxide to hydrogen peroxide and finally to water and oxygen. Activity of SOD in *C. elegans* is therefore an indicator of self-defence against pathogens. Superoxide dismutase (sod-2) coupled with a fluorescent reporter (GFP) was introduced in *C. elegans* to investigate the effects of sod-2 on longevity (Van Raamsdonk & Hekimi, 2009). It is interesting for us to find out if *E. faecalis* OG1RF and all *pts* mutants as antigens would activate sod-2 in *C. elegans* to different extents, consequently generate inequable fluorescence intensity in worms. The degressive order of fluorescence intensity in *C. elegans* generated by *E. faecalis* OG1RF and *pts* mutants was: OG1RF (204.451 U) >*pts*1/2 mutant (203.864 U) >*pts*2 mutant (189.569 U) >*pts*1 mutant (167.544 U). The fluorescence intensity stands for sod::gfp signals. Sod::gfp signals derive from self-defence of *C. elegans* by producing SOD against ROS, which was generated by administrated enterococci. The excitated fluorescence intensity suggests that *E. faecalis* OG1RF bears more virulence properties than *pts* mutants. As a whole, this is in accordance with our former study that the *pts* mutants displayed attentuated virulence comparing with *E. faecalis* OG1RF. Strictly speaking, *C. elegans* as an animal model could not fully replace classic animal model like mouse due to biomorphic and phylogenesis differences. A mutational strain testified to be virulence-attenuated in *C. elegans* killing assay could be virulence-identical in mouse killing assay (Maadani, Fox, Mylonakis, & Garsin, 2007). More work on mouse model being infected by *E. faecalis* OG1RF and *pts* mutants would be appealing to us in the future

experimental design to further confirm the role of *pts* (encoding mannose/fructose/sorbse, EIICD) genes in bacterial virulence.

5 Summary

E. faecalis OG1RF as a commensal in human intestine draws attention due to its pathogenicity in compromised conditions. A threat to public health as it is, the mechanism how *E. faecalis* OG1RF is introduced into host remains unclear. To be more specific, only few molecules in *E. faecalis* OG1RF and its host involved in adhesion and translocation were characterized. Following colonization in the intestine, *E. faecalis* OG1RF takes advantage of disordered conditions (trauma, maladjusted immune system) in the host to facilitate diffusion into adjacent blood stream or tissues. Yet how *E. faecalis* OG1RF makes use of the dysfunctional intestine as portal of invasion is not clearly defined. To persist under adverse conditions (low pH, hydrogen peroxide, endocytosis), *E. faecalis* OG1RF accommodates itself to the enviroments by regulating gene expression, thereby, genes that are expedient for persistence and survival will be activated and up-regulated. Since increased expression (~7 fold) of mannose/fructose/sorbse-PTS has been identified when enterococci passed through murine intestine in a former study, it was hypothesized that mannose/fructose/sorbse-PTS is able to upgrade the technique of enterococci to withstand aggressive niches. The role of mannose/fructose/sorbse-PTS in bacterial metabolism and virulence was investigated.

By laser scanning confocal microscope observation, the peripheral actin in epithelial cells Ptk6 was found to play an important role in mediating bacterial adhesion and translocation. It is therefore suggested that *E. faecalis* OG1RF spreads into adjacent organs by using paracellular migration (mainly relys on actin) to achieve infection. Furthermore, four putatively actin-binding adhesins were isolated from *E. faecalis* OG1RF, Symbioflor®, and V583, and characterized by LC–MS/MS. The four actin-binding adhesins are: pyruvate formate lyase, GroEL, enolase and glyceraldehyde-3-phosphate dehydrogenase. All these proteins belong to two major groups of moonlighting proteins, i.e., proteins, which display additional functions other than their described major biochemical catalysis. Both groups of moonlighting proteins were reported to be associated with epithelial cell binding. Besides, actin as bacterial receptor in the host also acts as the receptor for pyruvate kinase, glucose-6-phosphate isomerase, and phosphoglycerate kinase in *L. paracasei* TMW 1.1434; pyruvate kinase, chaperonin GroEL, and EF-Tu in *L. plantarum* TMW 1.1734, and chaperonin GroEL in *L. brevis* TMW 1.485.

Determination of adhesion and translocation of *E. faecalis* OG1RF in confluent Ptk6 epithelial monolayer (functional intestine) and in disrupted Ptk6 epithelial monolayers (dysfunctional intestine) in a two-chamber assay revealed that an open barrier (disrupted Ptk6

epithelial monolayer) facilitated bacterial translocation (10- to 10,000- fold enhanced) whereas the adhesion ability remained identical. This demonstrates that dysfunctional epithelial layers allow much more bacteria, especially those with enhanced virulence (*E. faecalis* V583, 10,000-fold enhanced) to cross the intestinal barrier and elevate the risk of bacterial infection.

By construction of three *pts* (encoding mannose/fructose/sorbose, EIICD) mutants, the role of *pts* genes playing in bacterial metabolism and virulence was identified. *E. faecalis* OG1RF lack of *pts* retrieved from CCR. Loss of *pts* genes deprived part of the receptors for class IIa bacteriocin therefore nurtured more tolerance to pediocin. More importantly, comparing with *E. faecalis* OG1RF, *pts* mutants displayed less tolerance to acid and hydrogen peroxide (half-lethal concentration), lower survival rate in macrophage J774A.1 and BMM (except for *pts*2 mutant in BMM), and weaker activation of superoxide dismutase in *C. elegans*. The evidences suggest that *pts* genes play roles not only in sugar metabolism but also in resistance to rough circumstances in niche. This thesis tends to attribute *pts* genes (encoding mannose/fructose/sorbse, EIICD) in *E. faecalis* OG1RF to virulence-regulating factor, together with other regulatory factors to make contributions to survival and persistence in host.

6 Zussamenfassung

E. faecalis OG1RF ist ein natürlich vorkommendes Bakterium der menschlichen Darmflora, welches ein hohes Gesundheitsrisiko für immungeschwächte Personen darstellen kann. Der Mechanismus, wie *E. faecalis* OG1RF einen Wirt infiziert, ist jedoch noch unklar. Derzeit sind nur wenige bakterielle und wirtsspezifische Moleküle charakterisiert, welche an der Adhäsion und Translokation beteiligt sind. Nach der Kolonisierung des Darms nutzt *E. faecalis* OG1RF die beeinträchtigten Funktionen des Wirts (Trauma, Immuninsuffizienz), um in die angrenzende Blutbahn oder in benachbarte Gewebe zu diffundieren. Auf welche mechanistische Art und Weise sich *E. faecalis* OG1RF die Dysfunktionalität des Darms zunutze macht, ist jedoch noch unklar. Durch Regulation der Genexpression kann sich *E. faecalis* OG1RF an widrige Lebensbedingungen (niedriger pH-Wert, hohe Wasserstoffperoxid Konzentration, Endozytose) anpassen. Studien haben gezeigt, dass die Expression von Mannose/Fructose/Sorbse-PTS codierende Gene um das Siebenfache erhöht ist, wenn Enterokokken durch den Darm einer Maus translozieren. Es wird angenommen, dass eine erhöhte Mannose/Fructose/Sorbse-PTS Konzentration die Anpassungsfähigkeit von Enterokokken an harsche Lebensbedingungen begünstigt. In dieser Arbeit wurde die Funktion von Mannose / Fructose / Sorbse-PTS in bakteriellen Stoffwechselwegen und deren Einfluss auf die Virulenz untersucht.

Mittels konfokaler Laser-Scanning-Mikroskopie wurde gezeigt, dass das Peripherie Aktin in Ptk6 Epithelzellen eine wichtige Rolle bei der Vermittlung der bakteriellen Adhäsion und Translokation spielt. Daher wird vermutet, dass *E. faecalis* OG1RF während der parazellulären Migration in benachbarte Organe, hauptsächlich auf das Aktin angewiesen ist, um eine Infektion hervorrufen zu können. Des Weiteren wurden vier mutmaßliche Aktin-bindende Adhäsine (Pyruvatformiatlyase, GroEL, Enolase und Glyceraldehyd-3-Phosphat-Dehydrogenase) von *E. faecalis* OG1RF, Symbioflor® und V583 identifiziert und mittels LC-MS/MS charakterisiert. Diese potenziellen Adhäsine lassen sich zwei spezifischen Hauptgruppen von "moonlighting proteins" zuordnen, welche neben katalytischen Eigenschaften noch sekundäre Funktionen übernehmen. Berichten zu Folge sind Vertreter dieser zwei Gruppen an der Bindung von Epithelzelle beteiligt. Zusätzlich wurden sowohl die Pyruvat-Kinase, die Glucose-6-phosphat-Isomerase und die Phosphoglyceratkinase von *L. paracasei* TMW 1.1434; die Pyruvatkinase, das Chaperonin GroEL und EF-Tu von *L. plantarum* TMW 1.1734; als auch das Chaperonin GroEL von *L. brevis* TMW 1.485 als Aktin-bindende Adhäsine identifiziert.

Die Bestimmung der Haft- und Translokations-Eigenschaften von *E. faecalis* OG1RF in einem Zwei-Kammer-Assay ergab, dass die Translokationsrate der Bakterien in gestörten Ptk6 Epithelzellen-Monoschichten bis zu 10-10.000-fach höher war, als bei der Verwendung von konfluenten Ptk6 Epithelzellen Monoschichten (funktioneller Darm). Hingegen wurde die bakterielle Haftfähigkeit, nicht von der Beschaffenheit der Epithelzellen Monoschicht beeinflusst. Dies zeigt, dass die Darmbarriere von dysfunktionellen Epithelschichten von einer weitaus höheren Anzahl an Bakterien passiert wird, insbesondere wenn diese eine erhöhte Virulenz aufweisen, was somit das Risiko einer bakteriellen Infektion steigert.

Durch die Generierung von drei *pts* Mutanten (Mannose / Fructose / Sorbose, EIICD Codierung) wurde die Rolle der *pts*-Gene in bakteriellen Stoffwechselwegen und deren Einfluss auf die Virulenz untersucht. *E. faecalis* OG1RF *pts* Mutanten wiesen einen inaktiven CCR-Mechanismus auf. Des Weiteren zeigten sie eine erhöhte Toleranz gegenüber Pediocin, was auf den Verlust von Rezeptoren für Klasse IIa Bakteriozine zurück zu führen ist. Darüber hinaus zeigten *pts* Mutanten eine geringere Toleranz gegenüber Säuren und Wasserstoffperoxid (halb letale Konzentration), geringere Überlebensraten in Makrophagen (J774A.1 und BMM) und induzierten eine verminderte Aktivierung der Superoxiddismutase in *C. elegans*. Daher ist davon auszugehen, dass *pts*-Gene nicht nur eine Rolle im Zuckerstoffwechsel spielen, sondern auch die Anpassungsfähigkeit an raue Lebensbedingungen beeinflussen. Diese Beobachtungen führen zu der Annahme, dass die *pts*-Gene (Mannose/Fructose/Sorbse, EIICD Codierung) von *E. faecalis* OG1RF als Virulenzfaktoren fungieren und somit einen Einfluss auf die Überlebensfähigkeit und die Persistenz im Wirt ausüben.

7 Reference

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8 Appendix

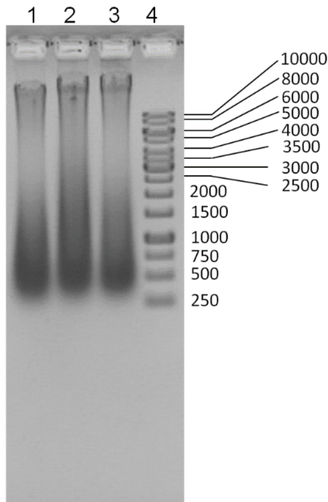

Fig. 1 Gel electrophoresis of genomic DNA extracted from *E. faecalis* OG1RF. Lane1-3, genome from *E. faecalis* OG1RF; Lane 4, GeneRuler 1 kb DNA Ladder (ready-to-use, 250 to 10,000 bp)

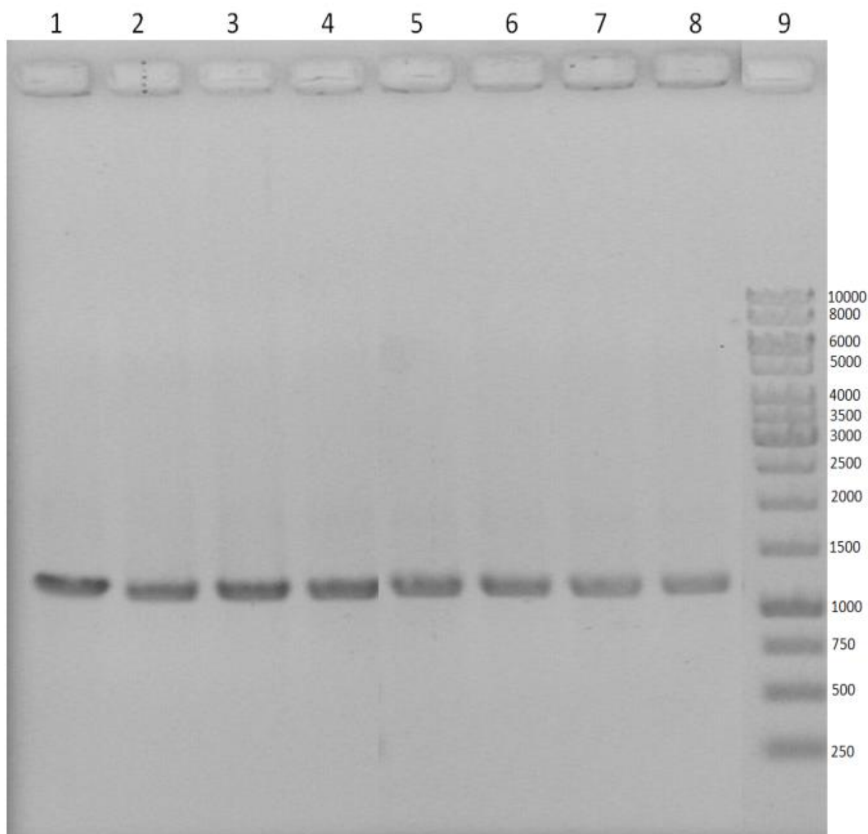

Fig. 2 Verification of DNA fragments for *pts*1 mutant construction. Gel electrophoresis of PCR amplificates with primers upstream and downstream of *pts*1 gene. Lane1-4, OG1RF_12403 (upstream amplified with primers F_{pts1U} and R_{pts1U}) in *E. faecalis* OG1RF, gene length (1130 bp) is as expected ; Lane 5-8, OG1RF_12400 (downstream amplified with primers F_{pts1D} and R_{pts1D}) in *E. faecalis* OG1RF, gene length (1144 bp) is as expected; Lane 9, GeneRuler 1 kb DNA Ladder (ready-to-use, 250 to 10,000 bp).

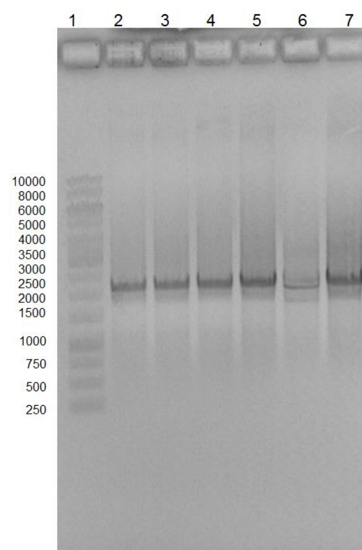

Fig. 3 Verification of DNA fragments for *pts*1 mutant construction. Gel electrophoresis of PCR amplificates of OG1RF_12403 and OG1RF_12400 ligation products. Lane1, GeneRuler 1 kb DNA Ladder (ready-to-use, 250 to 10,000 bp); Lane 2-7, OG1RF_12403 and OG1RF_12400 ligation product (amplified with primers F_{pts1U} and R_{pts1D}), gene length (2274 bp) is as expected.

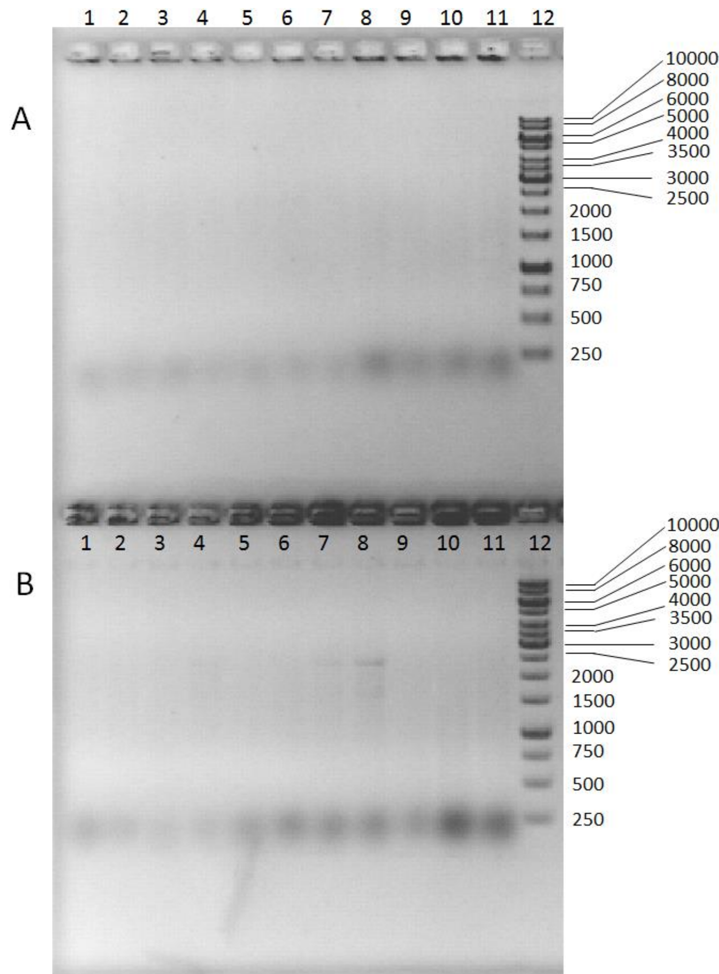

Fig. 4 Verification of DNA fragments for *pts*1 mutant construction. Gel electrophoresis of single-cross over colonies screening for *pts*1 mutant. A1-A11, colonies examined by F_{pts1O}-R_{pts1D} primers, no bands with length of 2543 bp was seen; B1-B11, colonies examined by F_{pts1U}-R_{pts1O} primers, gene length (2543 bp) is as expected in bands B7 and B8; A12 and B12, GeneRuler 1 kb DNA Ladder (ready-to-use, 250 to 10,000 bp)

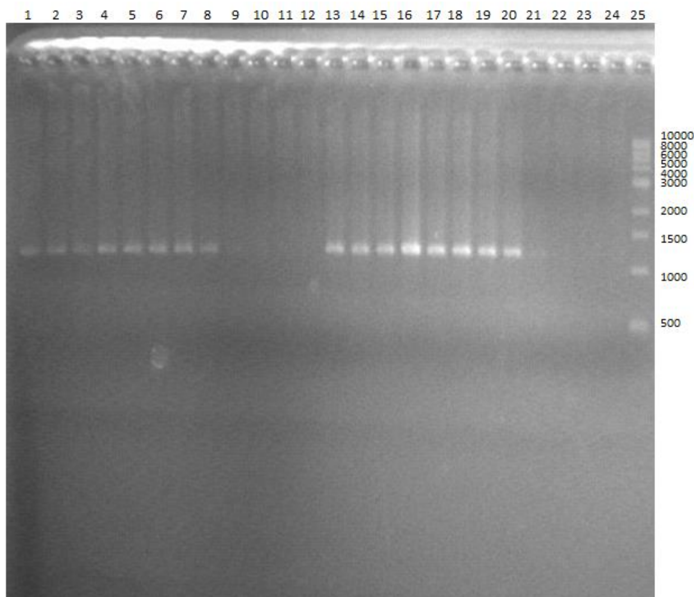

Fig. 5 Verification of DNA fragments for *pts*2 and *pts* 1/2 mutants construction. Gel electrophoresis of upstream and downstream of *pts*2 and *pts*1/2 genes. Lane1-12, OG1RF_11513 (upstream gene amplified with primers F_{pts2U} and R_{pts2U}) from *E. faecalis* OG1RF, gene length (1127 bp) is as expected in bands 1-8; Lane 13-24, OG1RF_11510 (downstream gene amplified with primers F_{pts2D} and R_{pts2D}) from *E. faecalis* OG1RF, gene length (1160 bp) is as expected in bands 13-20; Lane 25, 1 kb DNA Ladder (Ready-to-load, 500 to 10,000 bp)

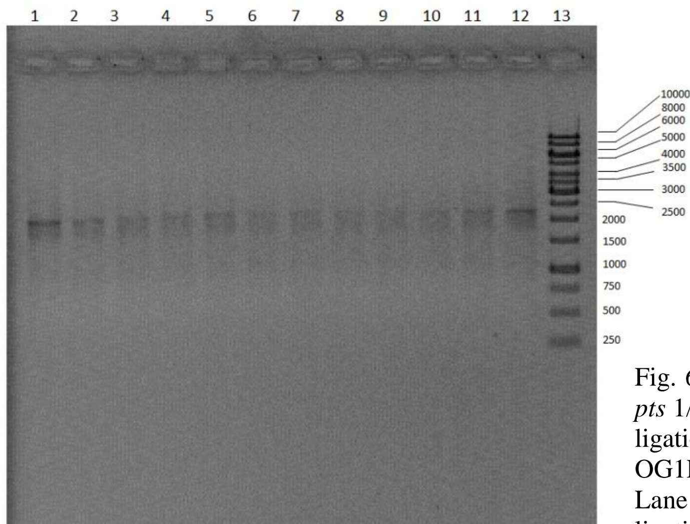

Fig. 6 Verification of DNA fragments for *pts*2 and *pts* 1/2 mutants construction. Gel electrophoresis of ligation product of OG1RF_11513 and OG1RF_11510 genes with primers F_{pts2U} - R_{pts2D} . Lane 1-12, OG1RF_11513 and OG1RF_11510 ligation product, gene length (2287 bp) is as expected in bands 1-12 ; Lane13, GeneRuler 1 kb DNA Ladder (ready-to-use, 250 to 10,000 bp)

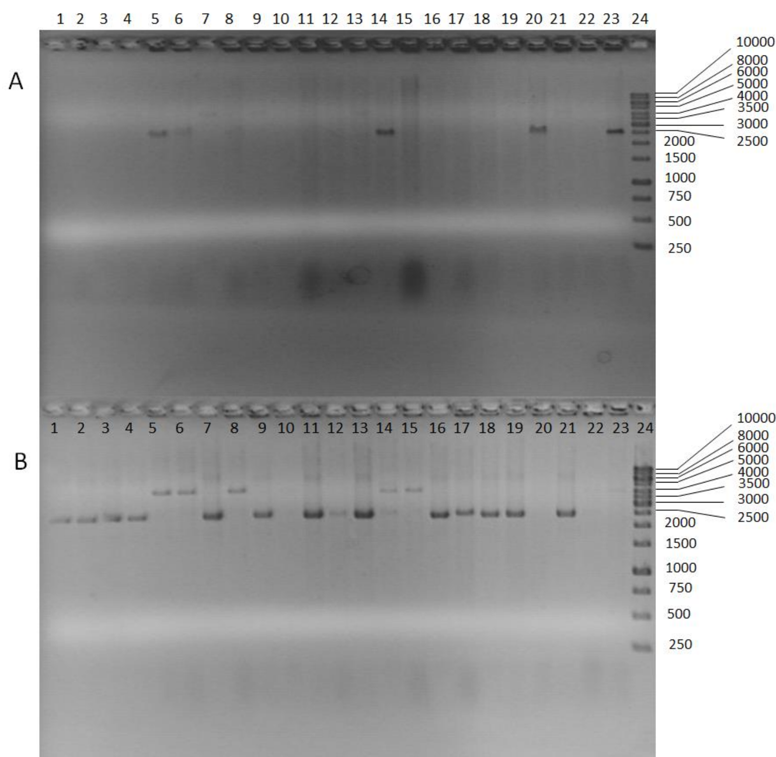

Fig. 7 Verification of DNA fragments for *pts*2 and *pts* 1/2 mutants construction. Gel electrophoresis of sing-cross over colonies screening for *pts*2 and *pts*1/2 mutants. A1-A19, *pts*2 colonies examined by F_{pts2O}-R_{pts2D} primers, gene length (2519 bp) is as expected in bands A5, A6, A8, A14; A20-A23, *pts*1/2 colonies examined by F_{pts2O}-R_{pts2D} primers, gene length (2519 bp) is as expected in bands A20, A23; B1-B19, *pts*2 colonies examined by F_{pts2U}-R_{pts2O} primers, gene length (2519 bp) is as expected in bands B1, B2, B3, B4, B7, B9, B11, B12, B13, B16, B17, B18, B19; B20-B23, *pts*1/2 colonies examined by F_{pts2U}-R_{pts2O} primers, gene length (2519 bp) is as expected in bands B21; A24 and B24, GeneRuler 1 kb DNA Ladder (ready-to-use, 250 to 10,000 bp)

Fig. 8 Sequence aligment between reconstructed *pts*1 mutant and OG1RF
Note: *pts*1 sequence, which has been deleted is not shown in the black frame. No amino acid mutated

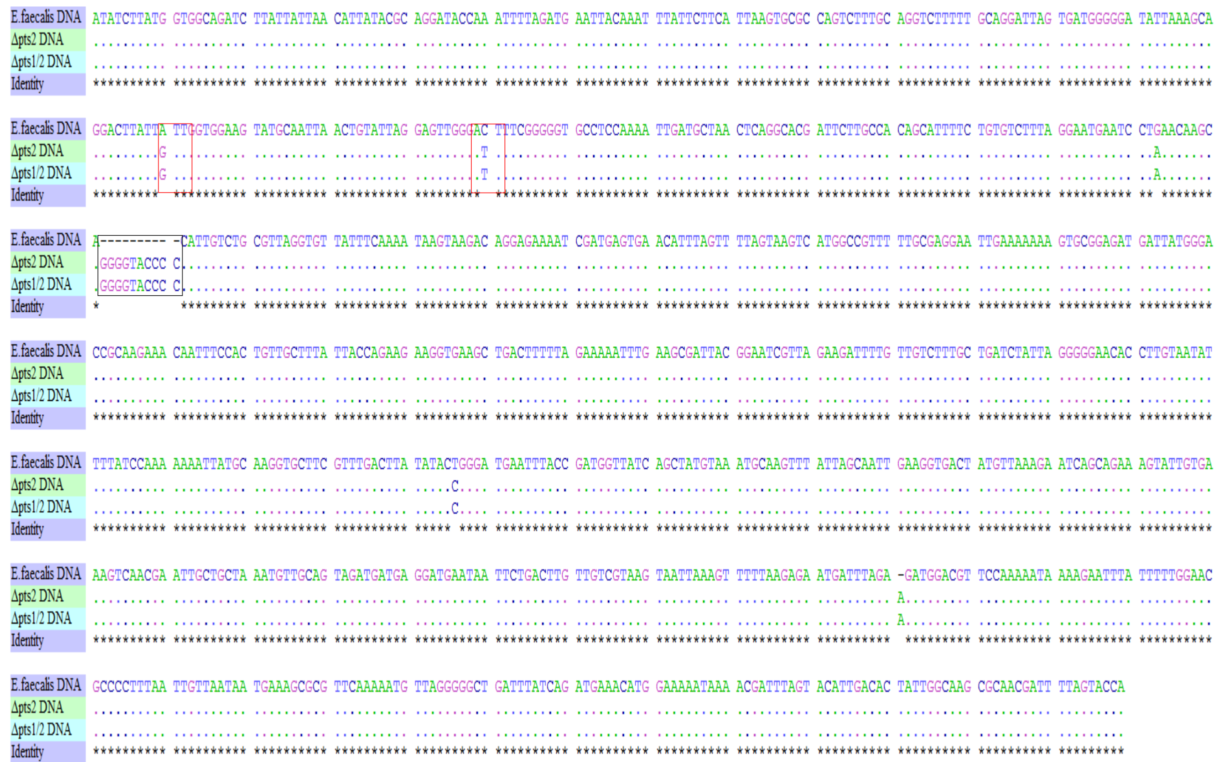

Fig. 9 Sequence aligment between reconstructed *pts*2, *pts*1/2 mutants and OG1RF
Note: *pts*2 sequence, which has been deleted is not shown in the black frame. Mutation of ATT into GTT caused amino acid mutated from Ile to Val; mutation of ACT into ATT caused amino acid mutated from Thr to Ile

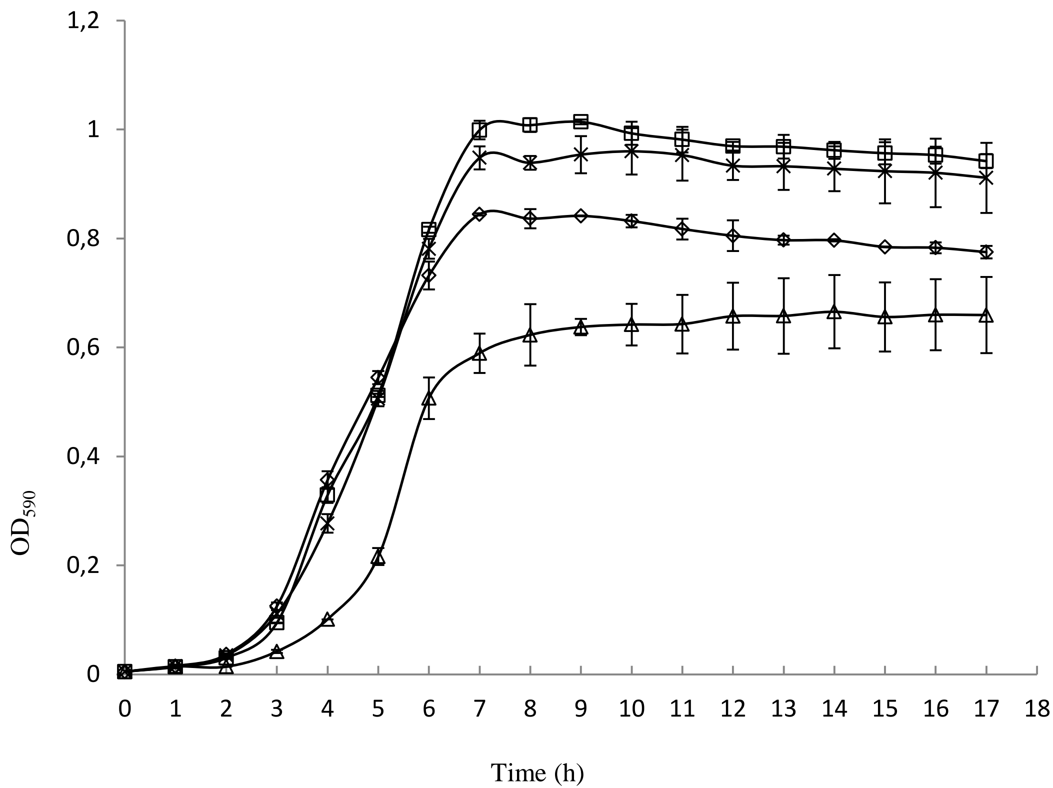

Fig. 10 Growth of enterococci in BHI (anaerobic). —◇— *E. faecalis* OG1RF; —□— *E. faecalis pts*1 mutant; —△— *E. faecalis pts*2 mutant; —✕— *E. faecalis pts*1/2 mutant

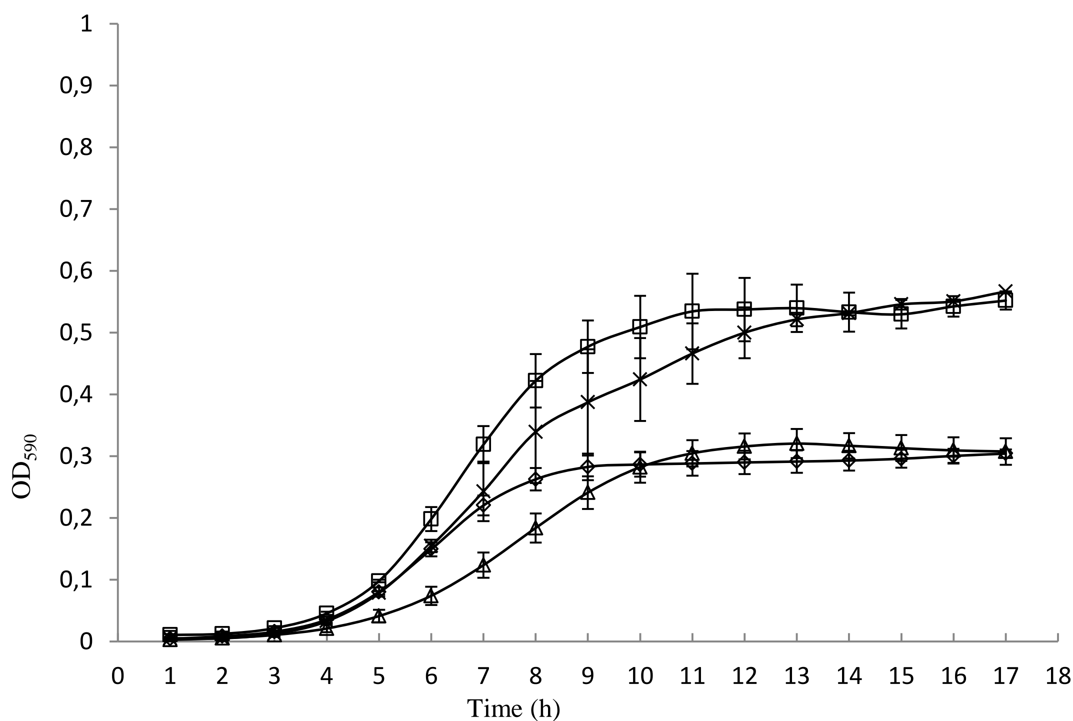

Fig. 11 Growth of enterococci in BHI (pH5.5). —◇— *E. faecalis* OG1RF; —□— *E. faecalis pts*1 mutant; —△— *E. faecalis pts*2 mutant; —✕— *E. faecalis pts*1/2 mutant

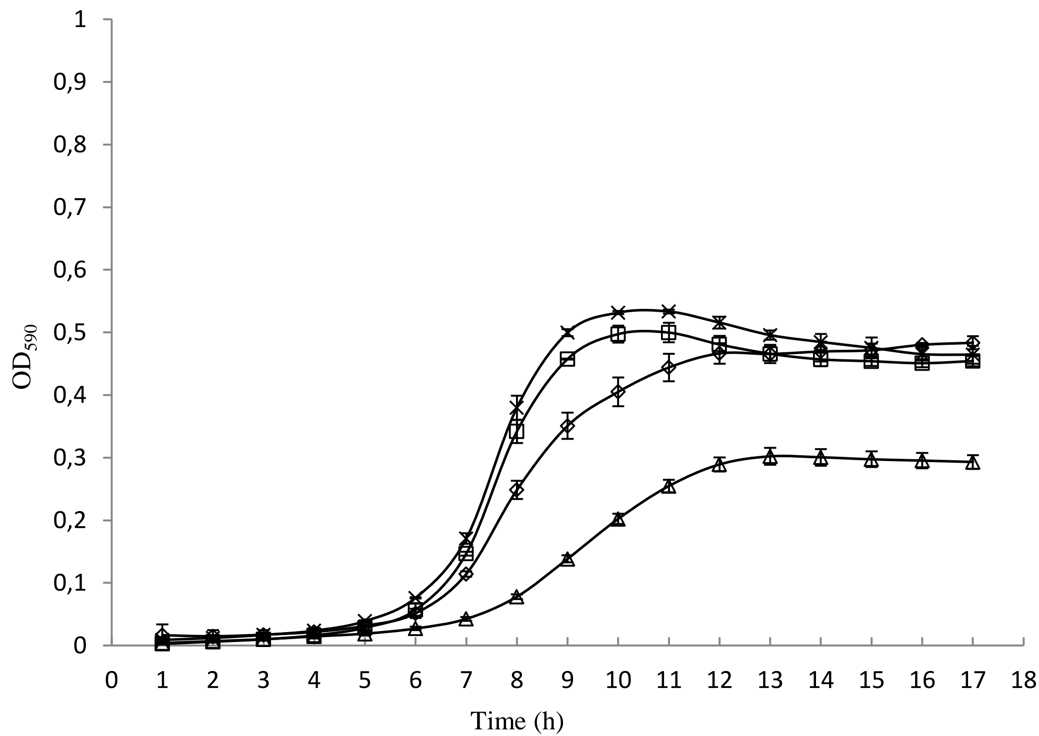

Fig. 12 Growth of enterococci in BHI (0.5 mM H2O2). ─◇─ *E. faecalis* OG1RF; ─□─ *E. faecalis pts*1 mutant; ─△─ *E. faecalis pts*2 mutant; ─✕─ *E. faecalis pts*1/2 mutant

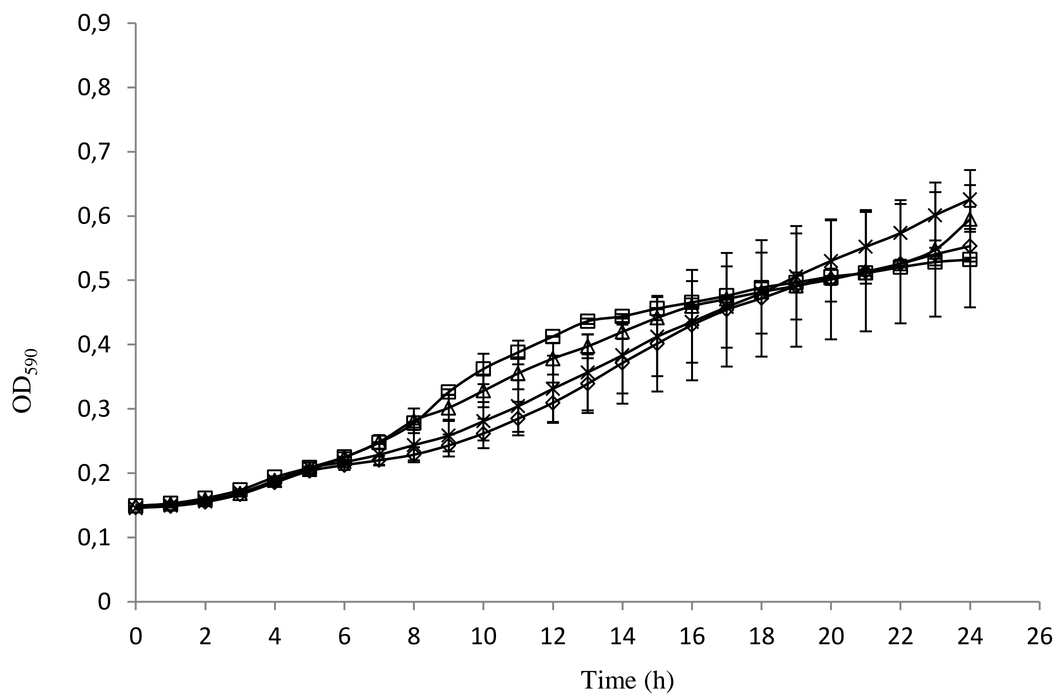

Fig. 13 Growth of enterococci in sucrose-MRS. ─◇─ *E. faecalis* OG1RF; ─□─ *E. faecalis pts*1 mutant; ─△─ *E. faecalis pts*2 mutant; ─✕─ *E. faecalis pts*1/2 mutant

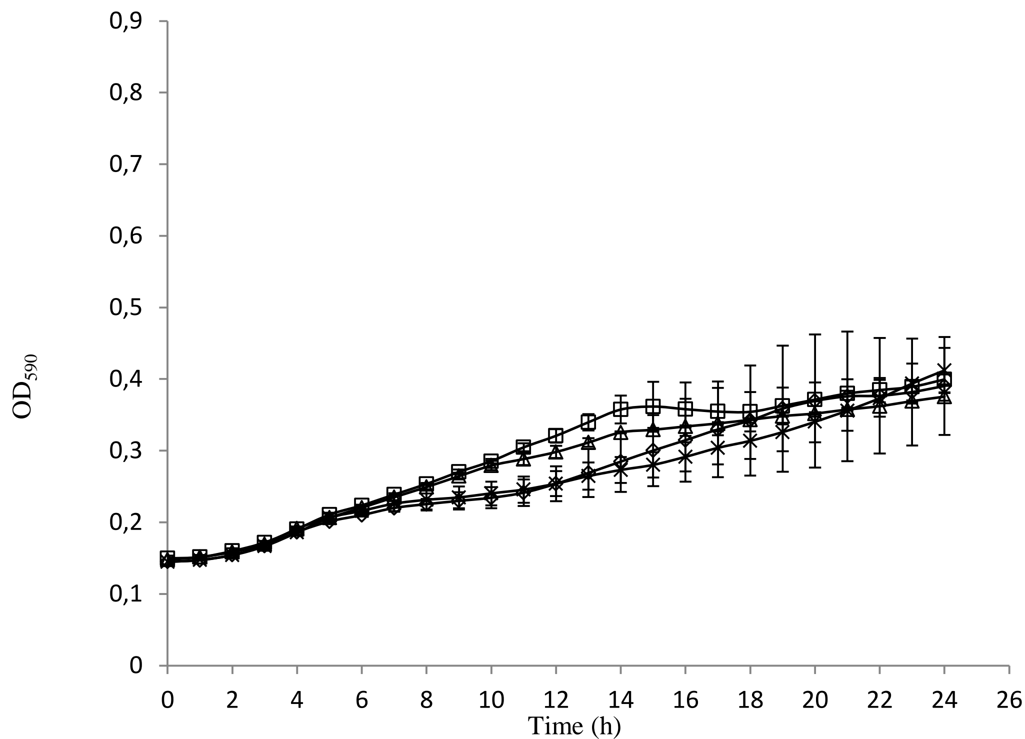

Fig. 14 Growth of enterococci in galactose-MRS. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —✕—*E. faecalis pts*1/2 mutant

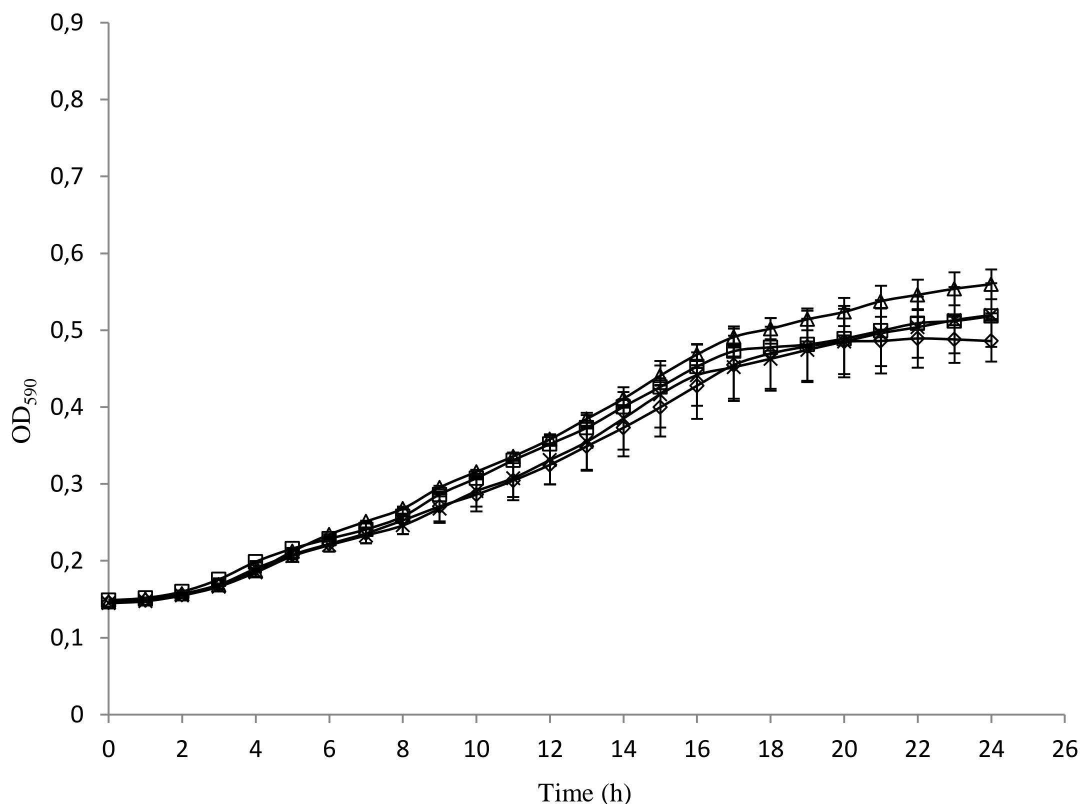

Fig. 15 Growth of enterococci in sorbitol-MRS. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —✕—*E. faecalis pts*1/2 mutant

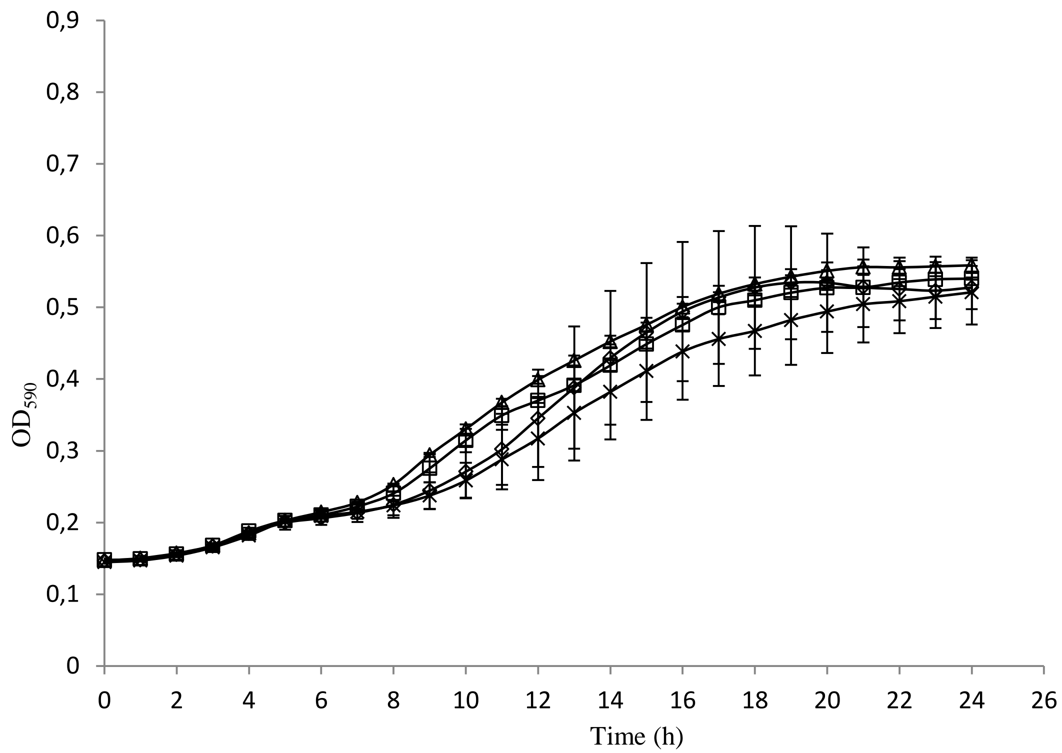

Fig. 16 Growth of enterococci in glycerol-MRS. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —×—*E. faecalis pts*1/2 mutant

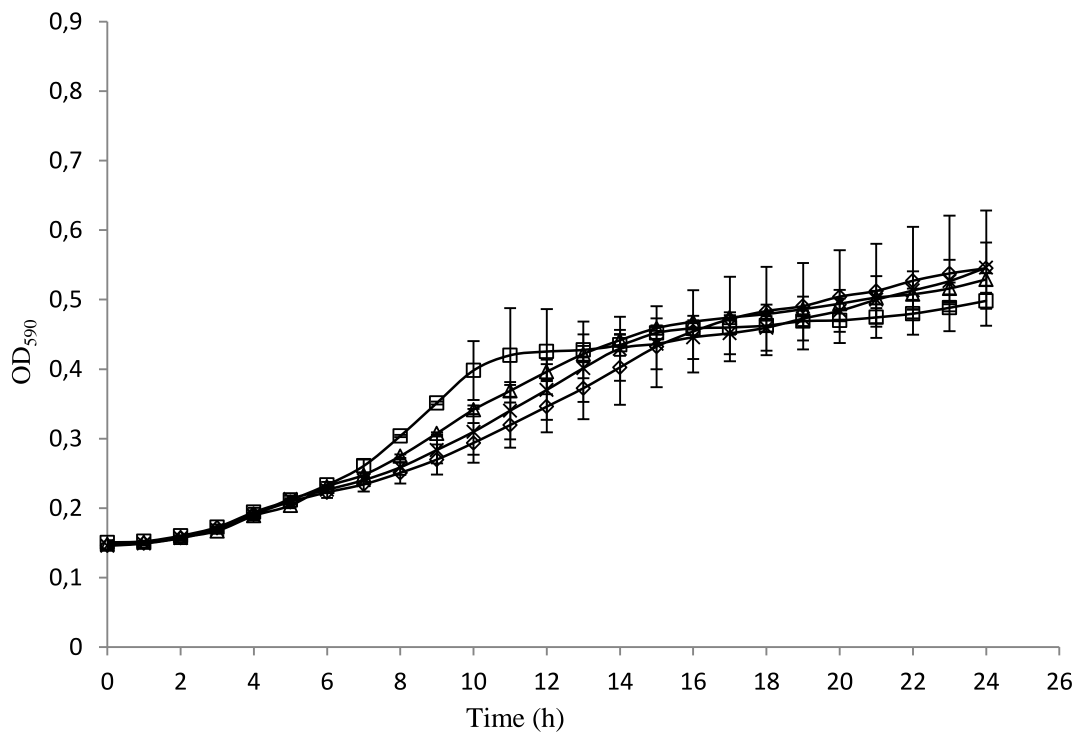

Fig. 17 Growth of enterococci in sodium gluconate-MRS. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —×—*E. faecalis pts*1/2 mutant

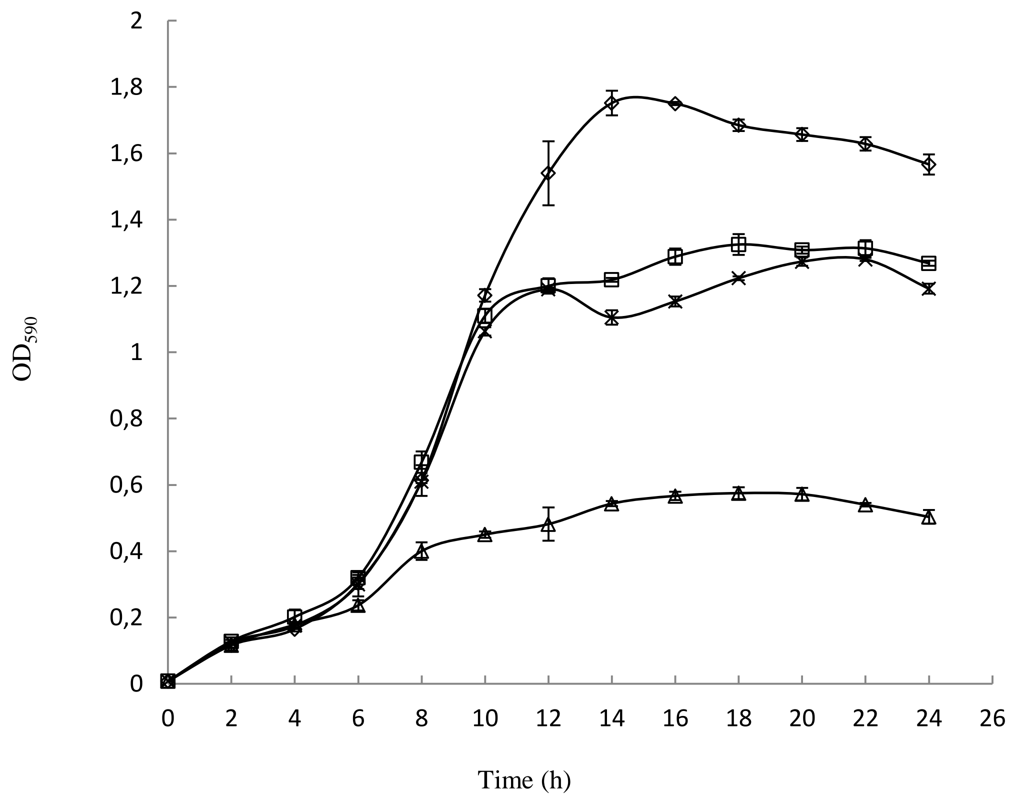

Fig. 18 Growth of enterococci in glucose-DAVIS. —◇— *E. faecalis* OG1RF; —□— *E. faecalis pts*1 mutant; —△— *E. faecalis pts*2 mutant; —×— *E. faecalis pts*1/2 mutant

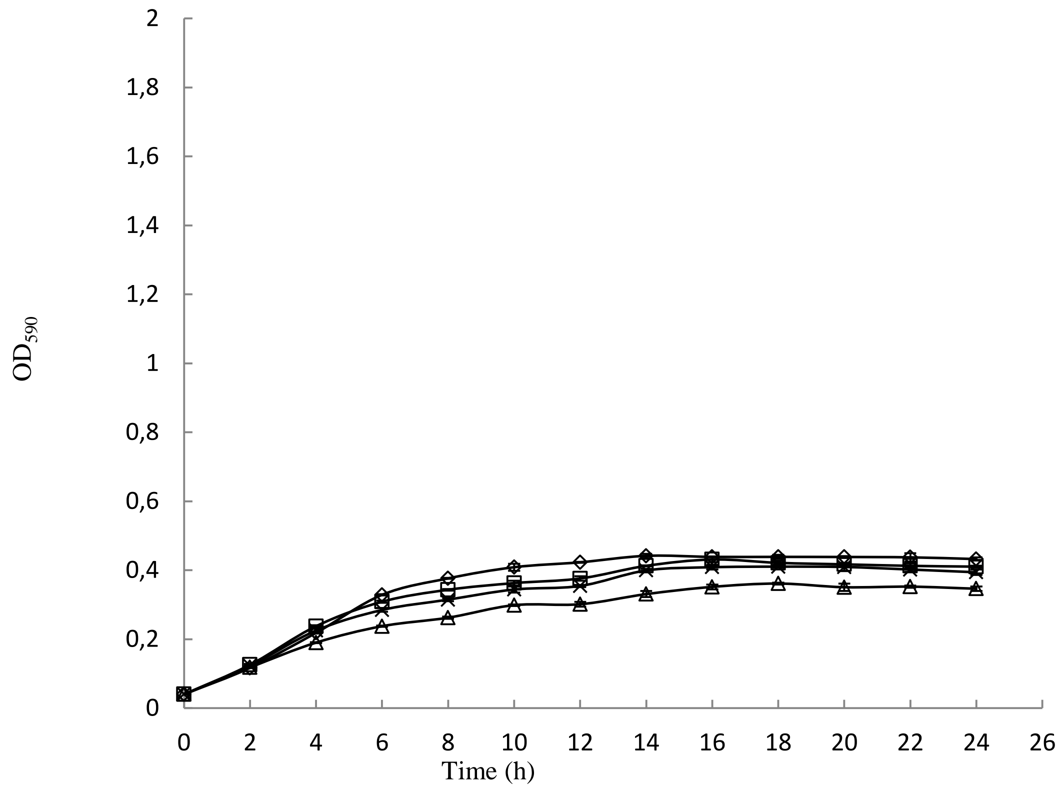

Fig. 19 Growth of enterococci in mannose-DAVIS. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —✕—*E. faecalis pts*1/2 mutant

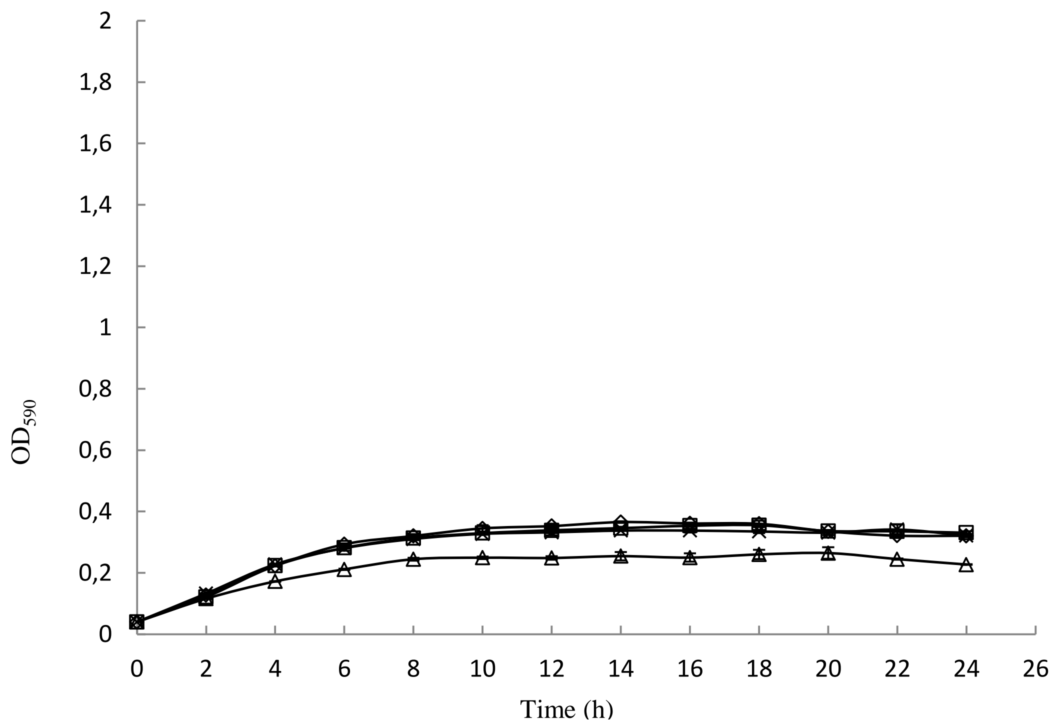

Fig. 20 Growth of enterococci in fructose-DAVIS. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —✕—*E. faecalis pts*1/2 mutant

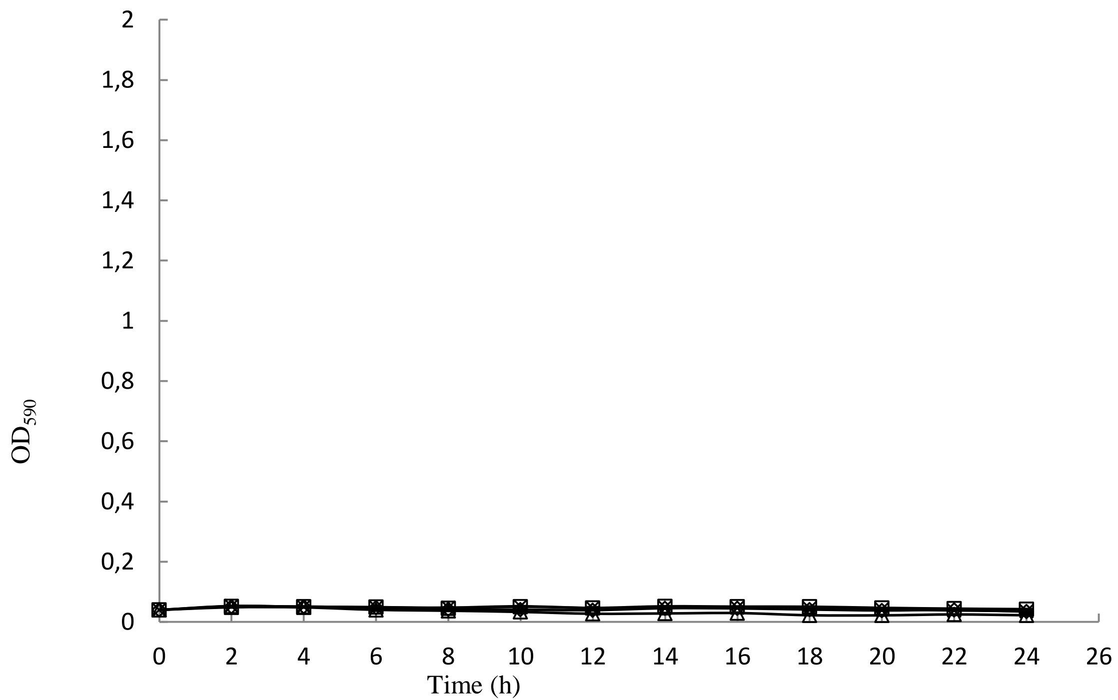

Fig. 21 Growth of enterococci in sorbose-DAVIS. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —×—*E. faecalis pts*1/2 mutant

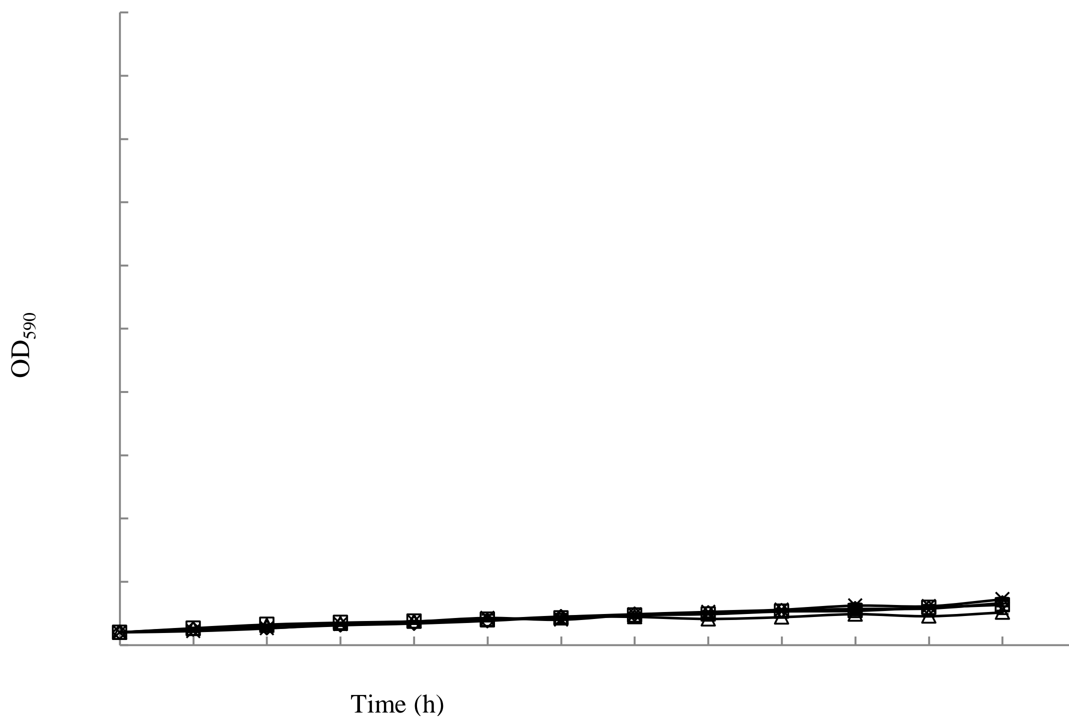

Fig. 22 Growth of enterococci in galactose-DAVIS. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —×—*E. faecalis pts*1/2 mutant

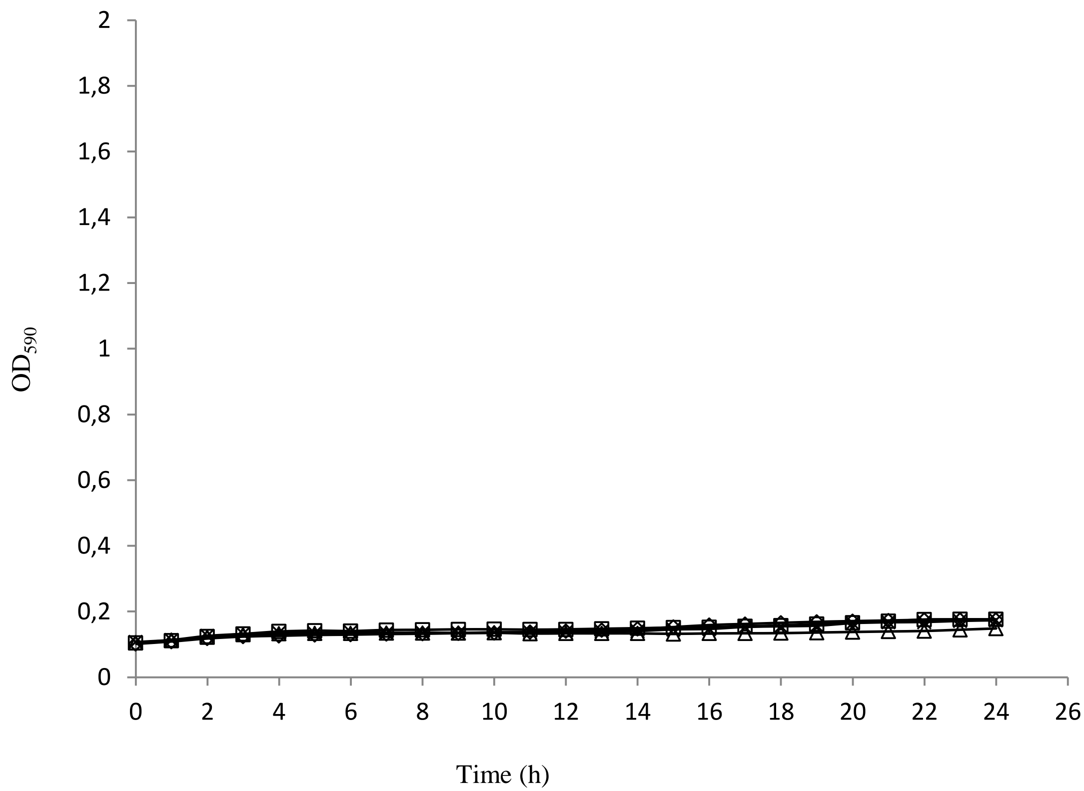

Fig. 23 Growth of enterococci in sorbitol-DAVIS. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —✕—*E. faecalis pts*1/2 mutant

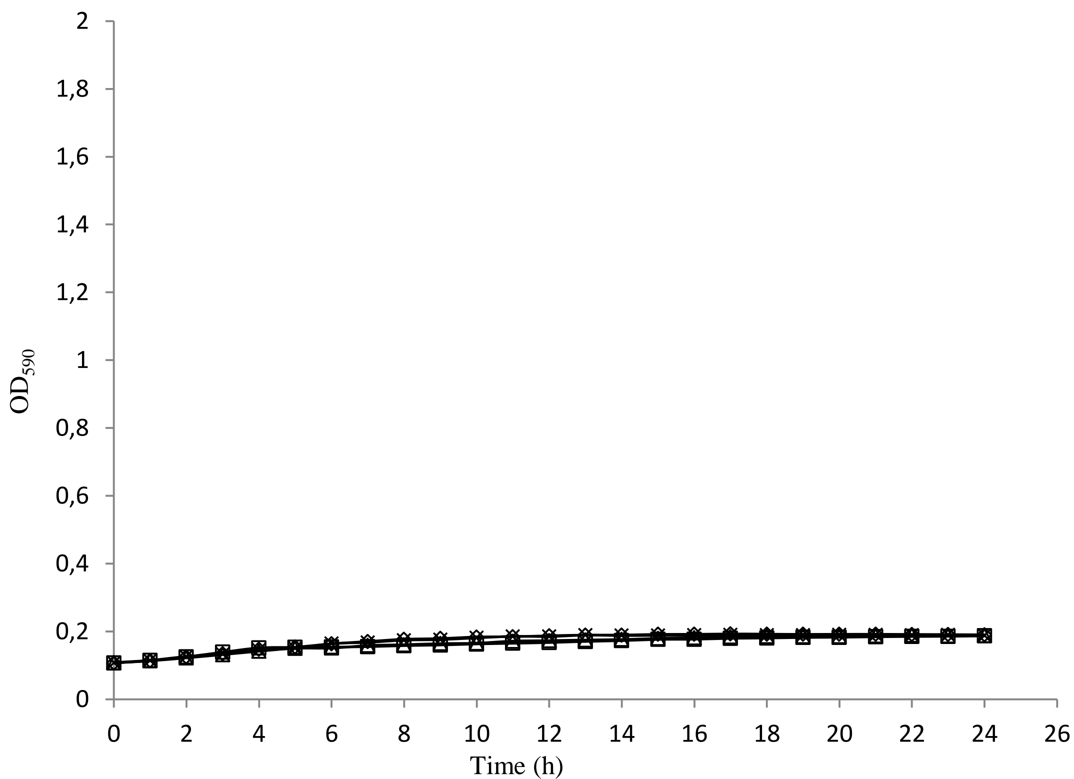

Fig. 24 Growth of enterococci in glycerol-DAVIS. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△—*E. faecalis pts*2 mutant; —✕—*E. faecalis pts*1/2 mutant

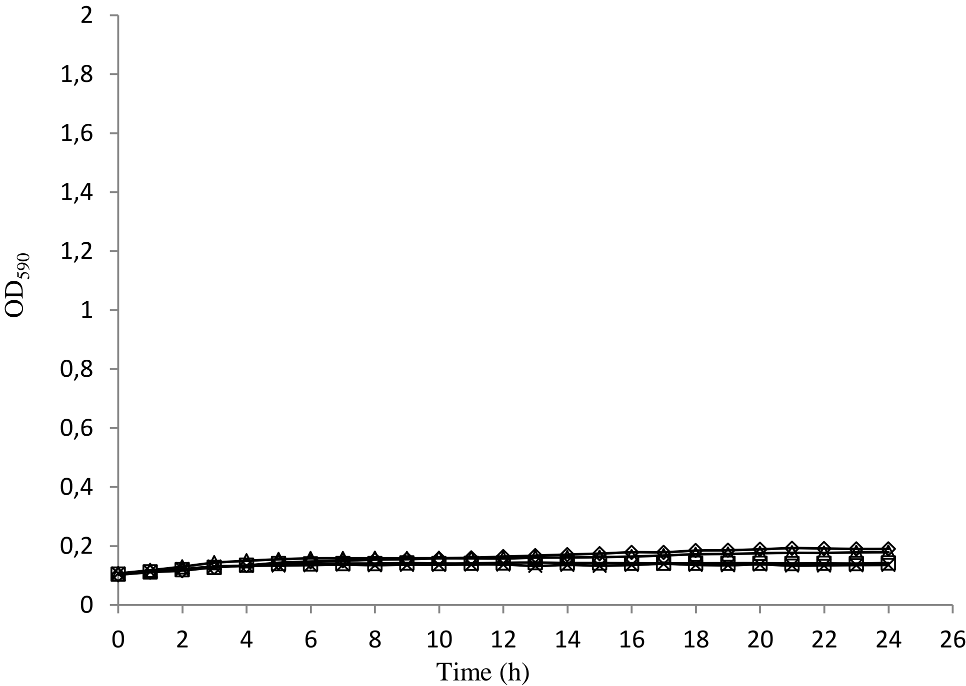

Fig. 25 Growth of enterococci in sodium gluconate-DAVIS. —◇—*E. faecalis* OG1RF; —□—*E. faecalis pts*1 mutant; —△— *E. faecalis pts*2 mutant; —×—*E. faecalis pts*1/2 mutant

E. faecalis OG1RF			
N.o	Name	24 h	48 h
0			
1	GLY	+	++
2	ERY		
3	DARA		
4	LARA		
5	RIB	++	+++
6	DXYL		
7	LXYL		
8	ADO		
9	MDX		
10	GAL	+	++
11	GLU	+++	++++
12	FRU	+++	++++
13	MNE	+++	++++
14	SBE		
15	RHA		±
16	DUL		
17	INO	±	+
18	MAN	±	+
19	SOR		±
20	MDM		
21	MDG		
22	NAG	+++	++++
23	AMY	±	+
24	ARB	++++	+++++
25	ESC		
26	SAL	+++	++++
27	CEL	+++	++++
28	MAL	+++	++++
29	LAC	+	++
30	MEL		
31	SAC	±	+
32	TRE	±	+
33	INU		
34	MLZ	±	+
35	RAF		
36	AMD		
37	GLYG		
38	XLT		
39	GEN	++	+++
40	TUR		
41	LYX		
42	TAG	++	+++
43	DFUC		
44	LFUC		
45	DARL		
46	LARL		
47	GNT		+
48	2KG		
49	5KG		

E. faecalis Δ *pts*1			
N.o	Name	24 h	48 h
0			
1	GLY	+	++
2	ERY		
3	DARA		
4	LARA		
5	RIB	++	+++
6	DXYL		
7	LXYL		
8	ADO		
9	MDX		
10	GAL	+	++
11	GLU	+++	++++
12	FRU	+++	++++
13	MNE	+++	++++
14	SBE		
15	RHA		±
16	DUL		
17	INO	±	+
18	MAN	±	+
19	SOR		+
20	MDM		
21	MDG		
22	NAG	+++	++++
23	AMY	±	+
24	ARB	++++	+++++
25	ESC		
26	SAL	+++	++++
27	CEL	+++	++++
28	MAL	+++	++++
29	LAC	+	++
30	MEL		
31	SAC	±	+
32	TRE	±	+
33	INU		
34	MLZ	±	+
35	RAF		
36	AMD		
37	GLYG		
38	XLT		
39	GEN	++	+++
40	TUR		
41	LYX		
42	TAG	++	+++
43	DFUC		
44	LFUC		
45	DARL		
46	LARL		
47	GNT		±
48	2KG		
49	5KG		

E. faecalis Δ *pts*2			
N.o	Name	24 h	48 h
0			
1	GLY	++	++
2	ERY		
3	DARA		
4	LARA		
5	RIB	++	+++
6	DXYL		
7	LXYL		
8	ADO		
9	MDX		
10	GAL	++	++
11	GLU	+++	++++
12	FRU	+++	++++
13	MNE	+++	++++
14	SBE		
15	RHA		±
16	DUL		
17	INO	±	+
18	MAN	±	+
19	SOR		±
20	MDM		
21	MDG		
22	NAG	+++	++++
23	AMY	±	+
24	ARB	++++	+++++
25	ESC		
26	SAL	+++	++++
27	CEL	+++	++++
28	MAL	+++	++++
29	LAC	+	++
30	MEL		
31	SAC	±	+
32	TRE	±	+
33	INU		
34	MLZ	±	+
35	RAF		
36	AMD		
37	GLYG		
38	XLT		
39	GEN	++	+++
40	TUR		
41	LYX		
42	TAG	++	+++
43	DFUC		
44	LFUC		
45	DARL		
46	LARL		
47	GNT		±
48	2KG		
49	5KG		

E. faecalis Δ *pts*1/2			
N.o	Name	24 h	48 h
0			
1	GLY	+	++
2	ERY		
3	DARA		
4	LARA		
5	RIB	++	+++
6	DXYL		
7	LXYL		
8	ADO		
9	MDX		
10	GAL	+	++
11	GLU	+++	++++
12	FRU	+++	++++
13	MNE	+++	++++
14	SBE		
15	RHA		±
16	DUL		
17	INO	±	+
18	MAN	±	+
19	SOR		±
20	MDM		
21	MDG		
22	NAG	+++	++++
23	AMY	±	+
24	ARB	++++	+++++
25	ESC		
26	SAL	+++	++++
27	CEL	+++	++++
28	MAL	+++	++++
29	LAC	+	++
30	MEL		
31	SAC	±	+
32	TRE	±	+
33	INU		
34	MLZ	±	+
35	RAF		
36	AMD		
37	GLYG		
38	XLT		
39	GEN	++	+++
40	TUR		
41	LYX		
42	TAG	++	+++
43	DFUC		
44	LFUC		
45	DARL		
46	LARL		
47	GNT		±
48	2KG		
49	5KG		

Fig. 26 API test of enterococci
At 24 h, *pts*2 mutant consumed glycerol (1) and D-galactose (10) slightly faster than other strains;
At 48 h, *pts*1 mutant consumed D-sorbitol (19) slightly faster than other strains, wild type strain consumed potassium gluconate (47) slightly faster than other strains

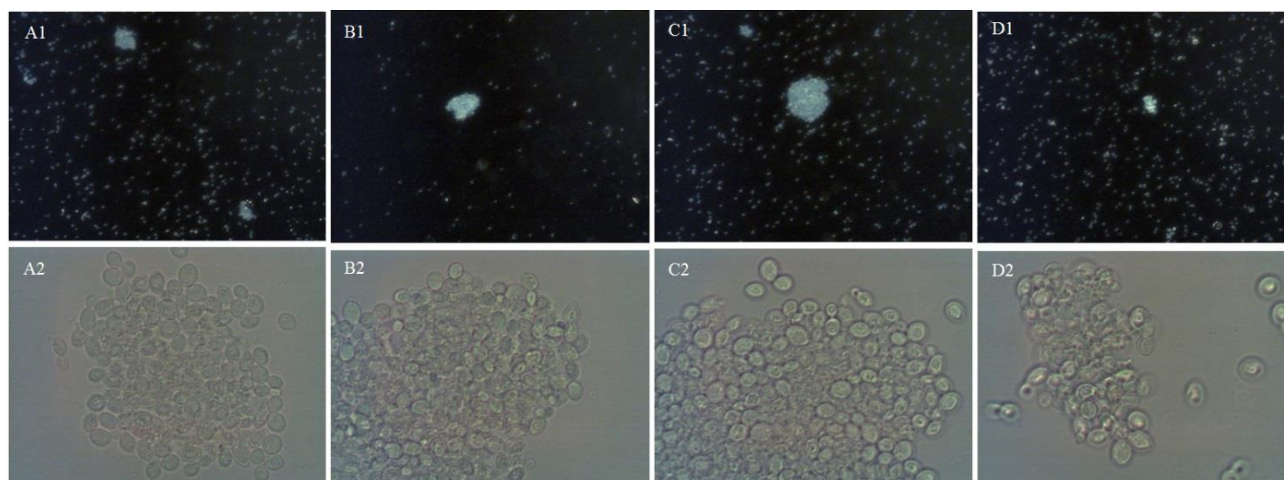

Fig. 27 Yeast agglutination generated by *E. faecalis*. A, *E. faecalis* OG1RF; B, *pts*1 mutant; C, *pts*2 mutant; D, *pts*1/2 mutant; 1, 100 fold; 2, 1000 fold

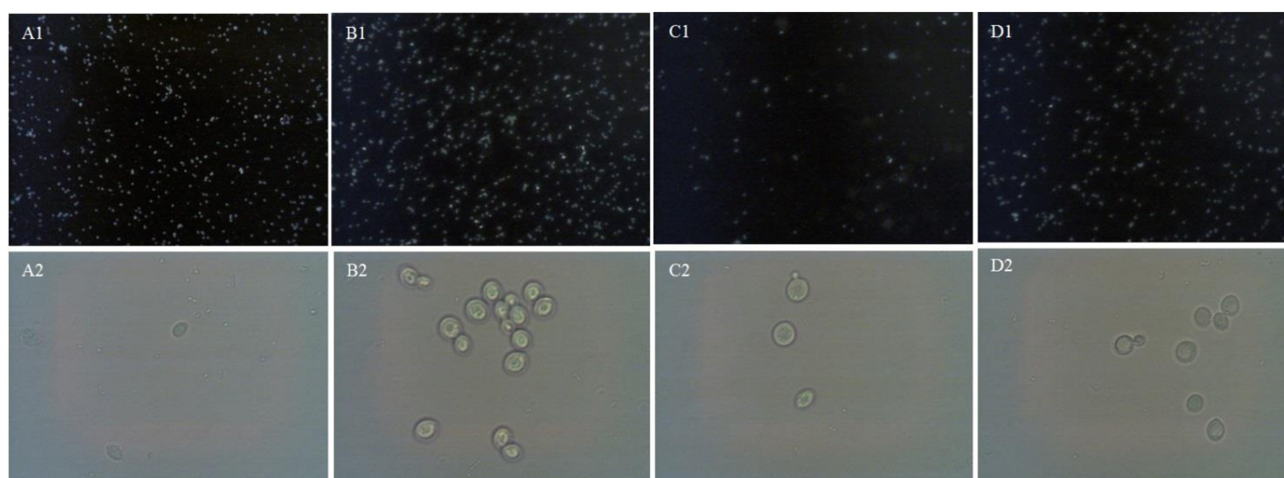

Fig. 28 Yeast agglutination generated by *E. faecalis* and inhibited by inhibitor. A, *E. faecalis* OG1RF; B, *pts*1 mutant; C, *pts*2 mutant; D, *pts*1/2 mutant; 1, 100 fold; 2, 1000 fold

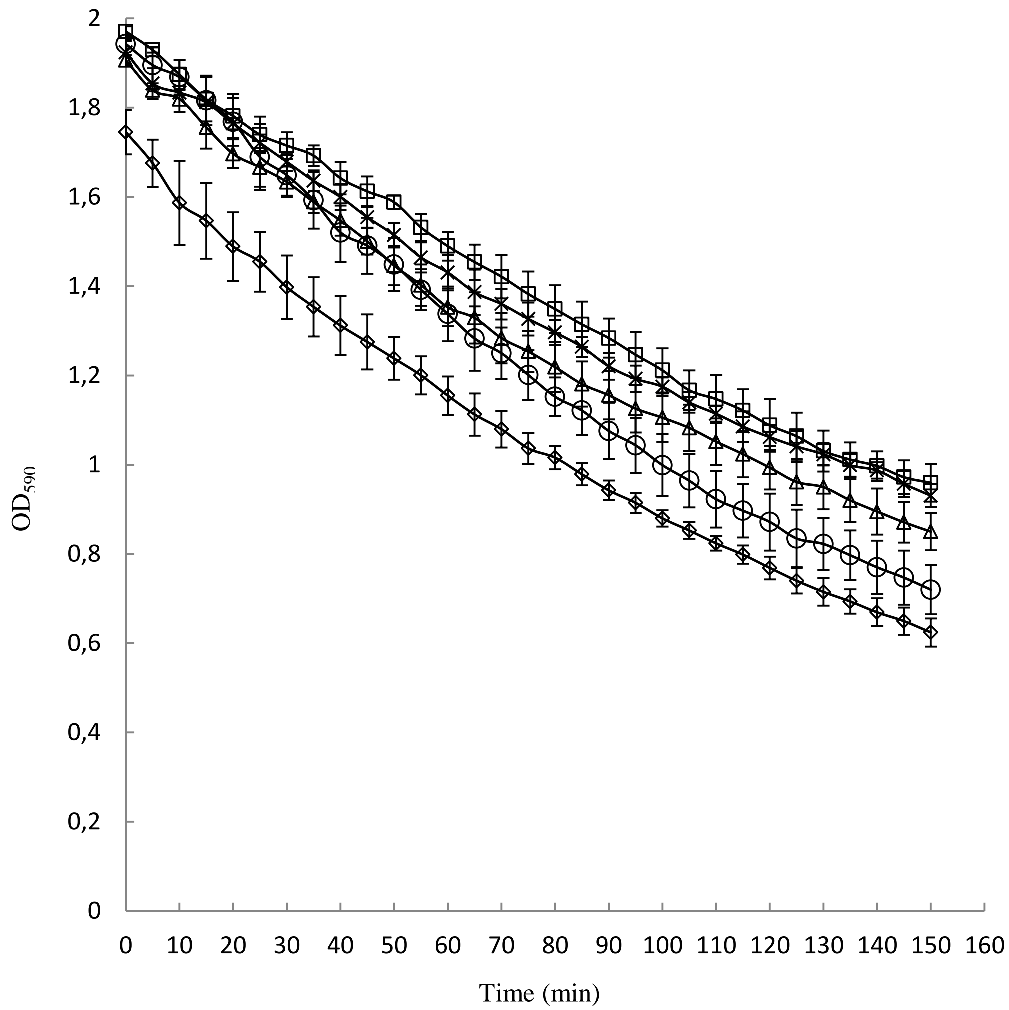

Fig. 29 Agglutination of *S. cerevisiae* by enterococci
—◇— *S. cerevisiae*; —□— *S. cerevisiae*+OG1RF; —△— *S. cerevisiae*+*pts*1 mutant;
—×— *S. cerevisiae*+*pts*2 mutant; —○— *S. cerevisiae*+*pts*1/2 mutant

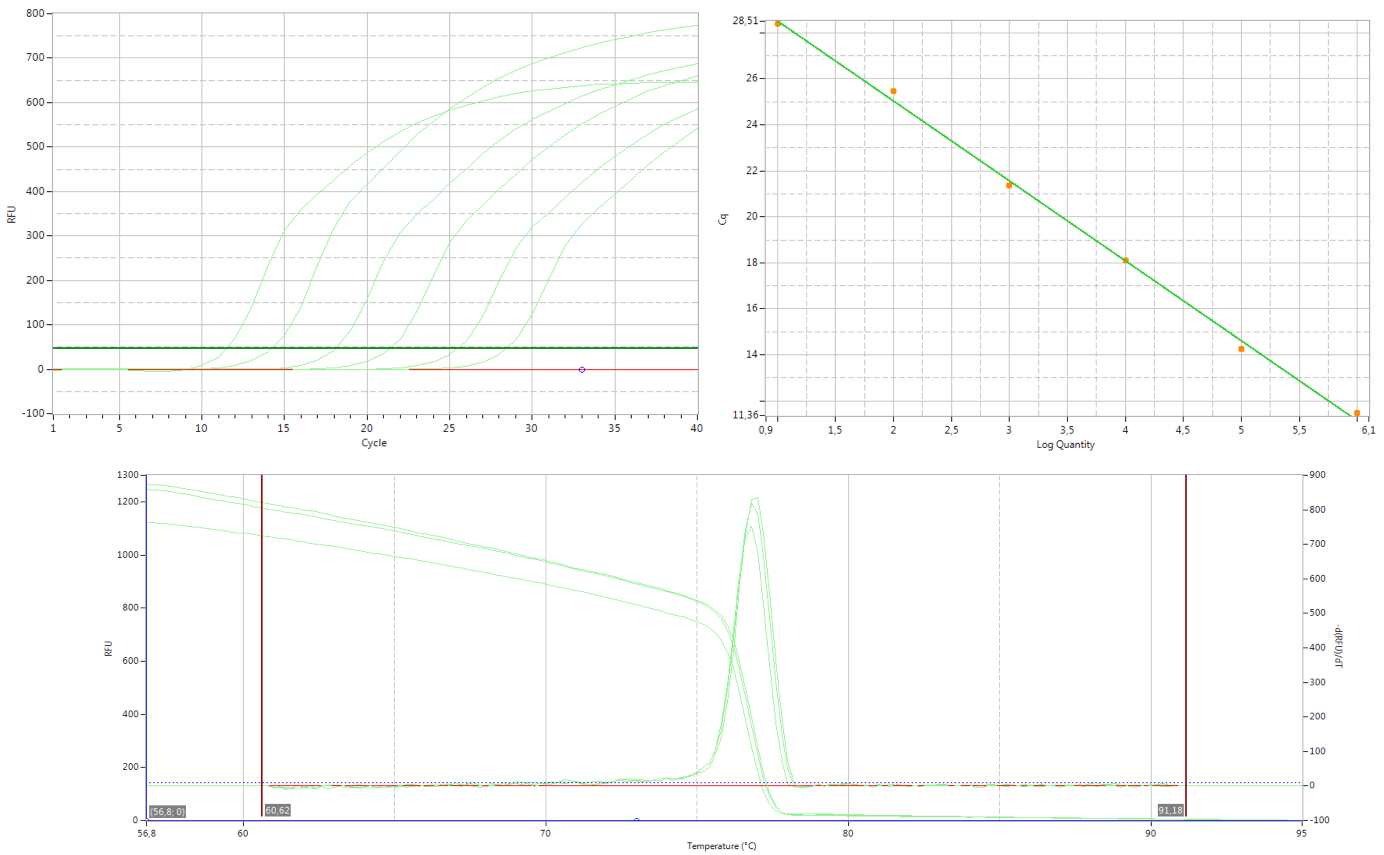

Fig. 30 Amplification curve, standard curve and melting curve of 23s rna (internal control)
Efficiency = 95%; y = -3,44x + 29,629; R2 = 0,9994

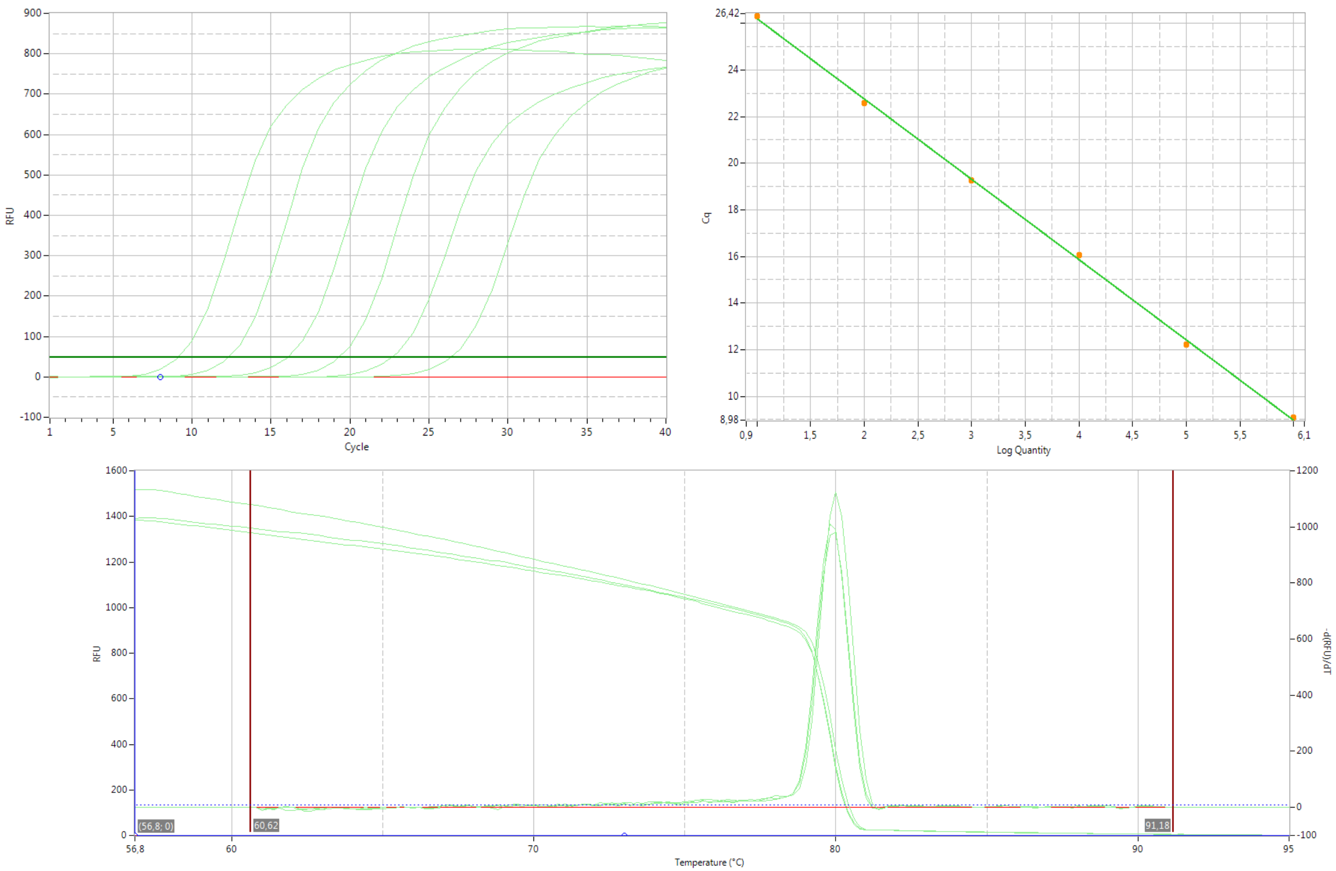

Fig. 31 Amplification curve, standard curve and melting curve of *gyrA* (internal control)
Efficiency = 94%; y = -3,477x + 32,005; R2 = 0,9978

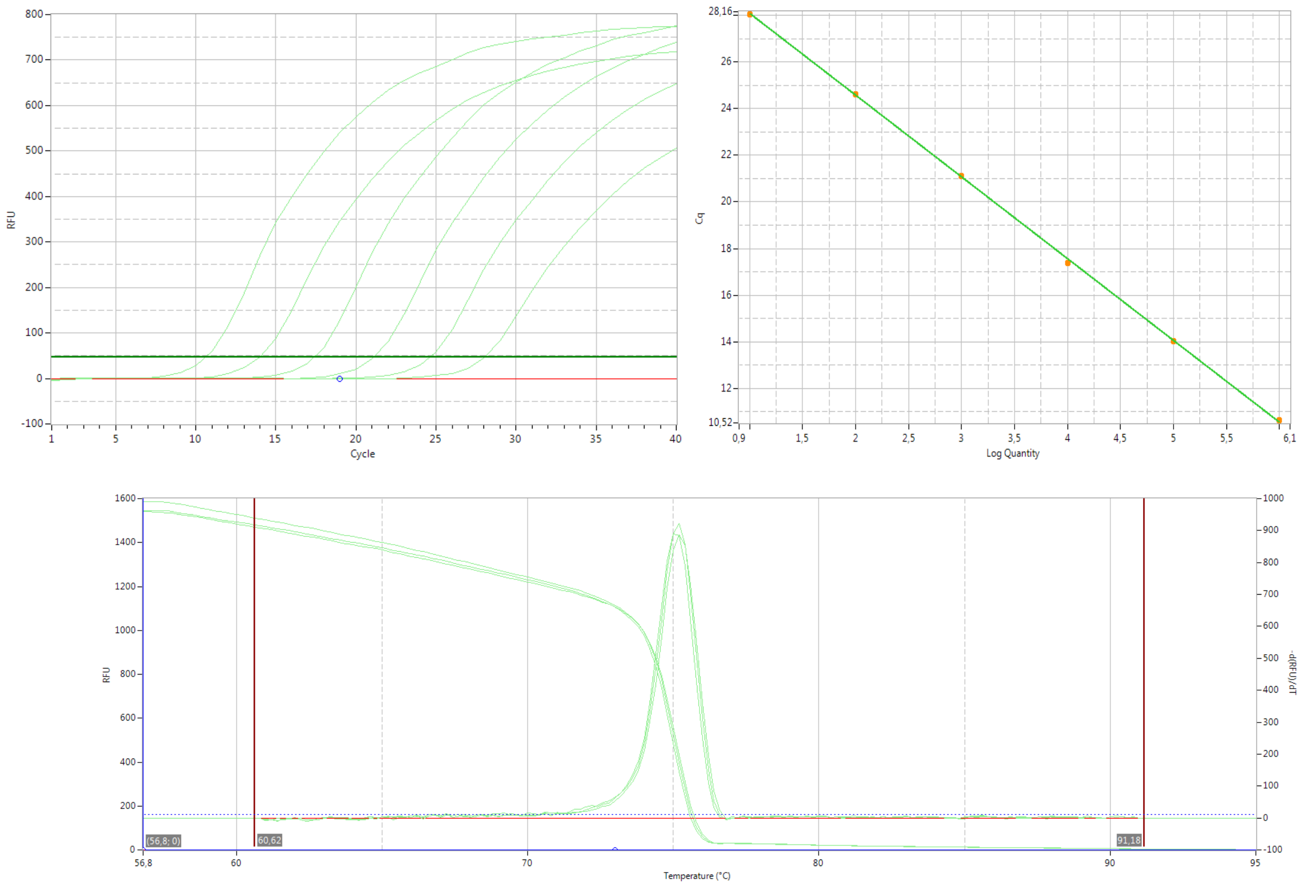

Fig. 32 Amplification curve, standard curve and melting curve of *ahpC*
Efficiency = 93%; y = -3,506x + 31,576; R2 = 0,9998

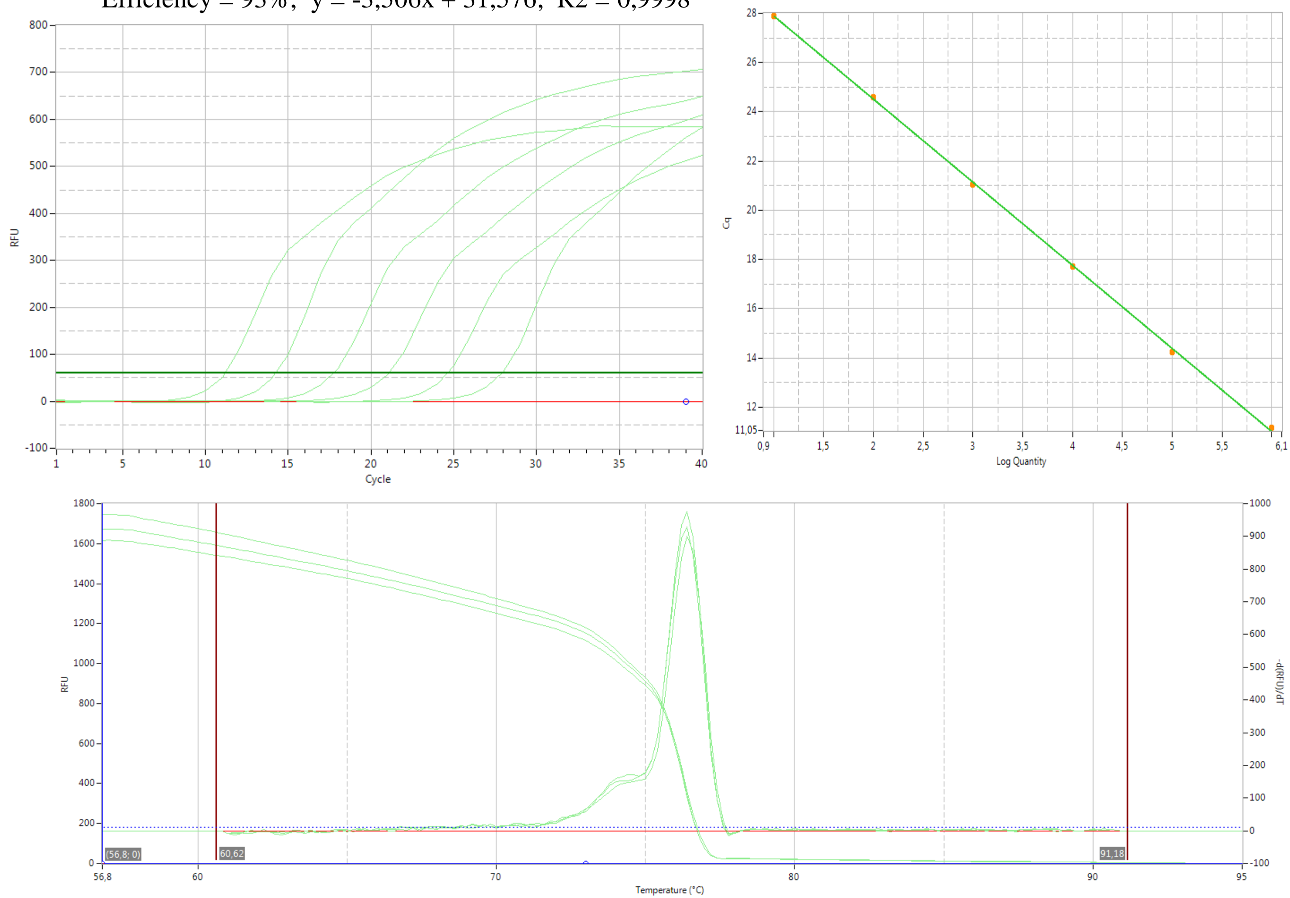

Fig. 33 Amplification curve, standard curve and melting curve of *gor*
Efficiency = 98%; y = -3,376x + 31,253; R2 = 0,9997

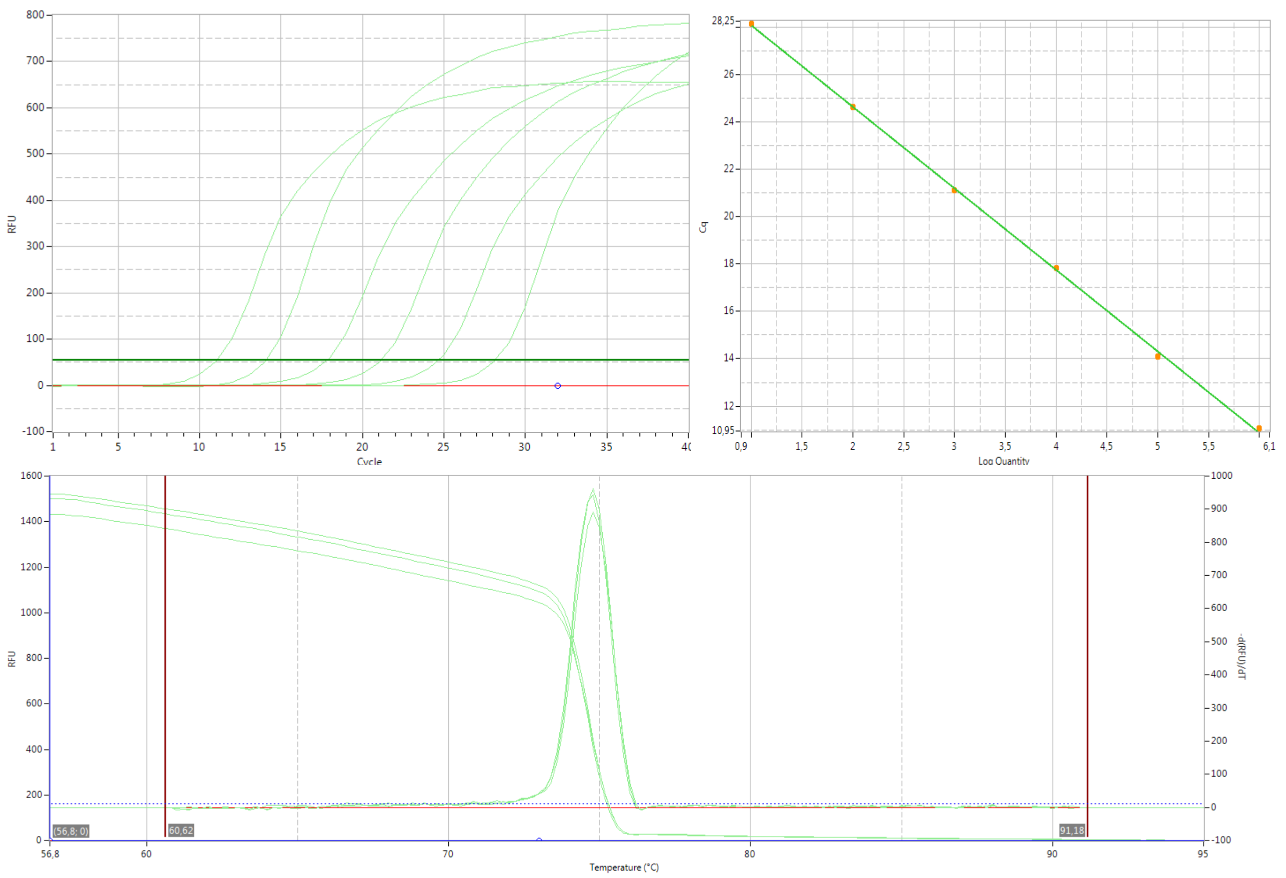

Fig. 34 Amplification curve, standard curve and melting curve of *hypR*
Efficiency = 95%; y = -3,441x + 31,519; R2 = 0,9995

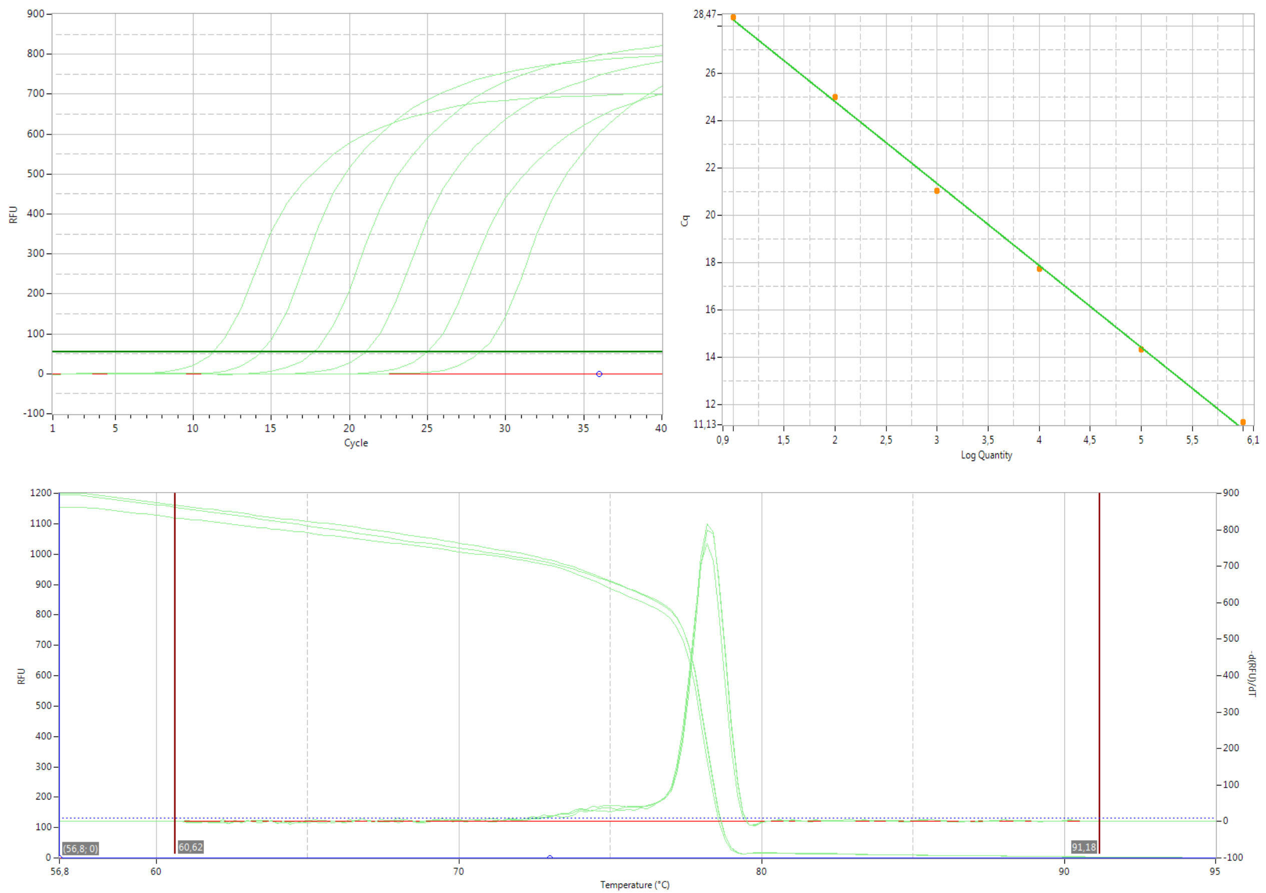

Fig. 35 Amplification curve, standard curve and melting curve of *katA*
Efficiency = 95%; y = -3,459x + 31,713; R2 = 0,9988

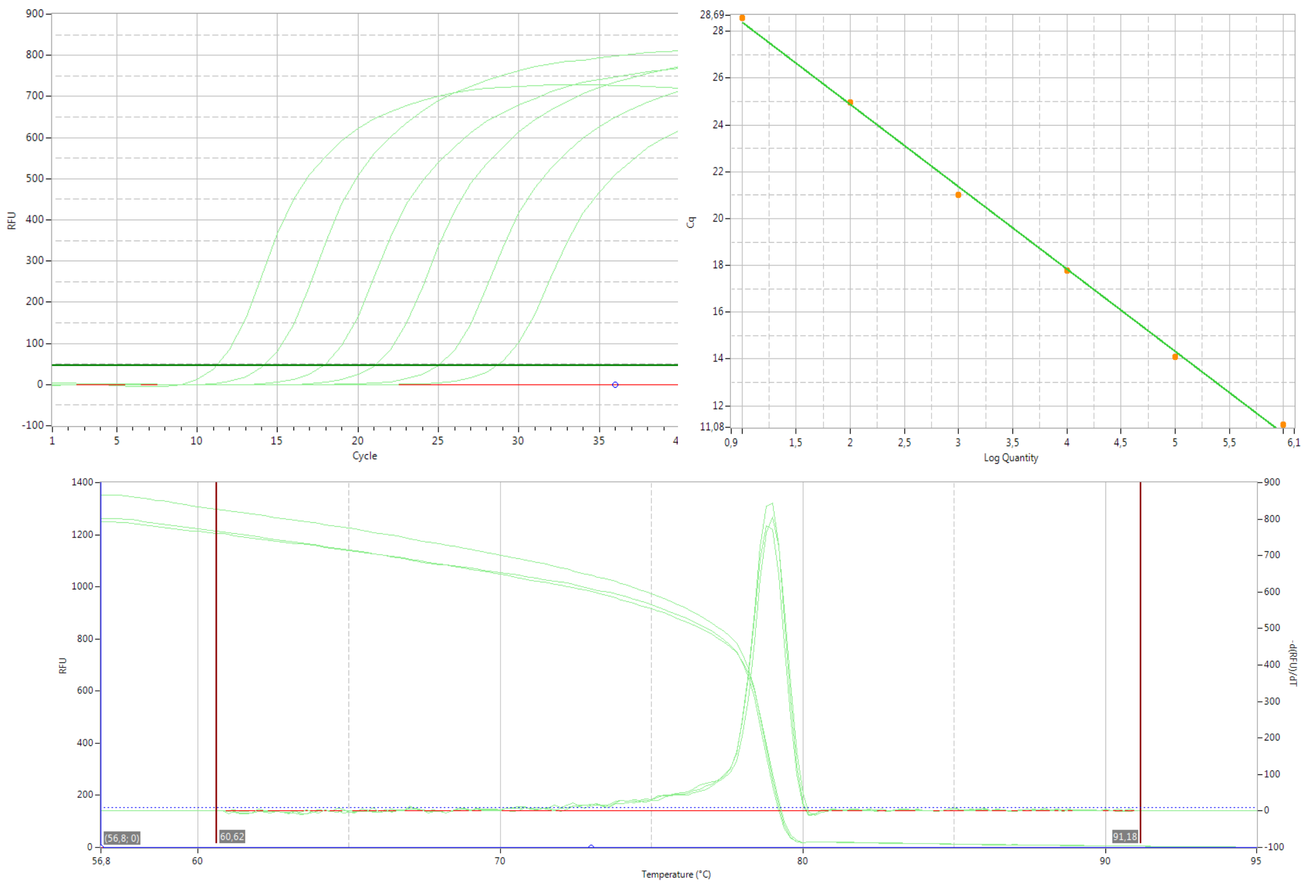

Fig. 36 Amplification curve, standard curve and melting curve of *npr*
Efficiency = 93%; y = -3,509x + 31,885; R2 = 0,9983

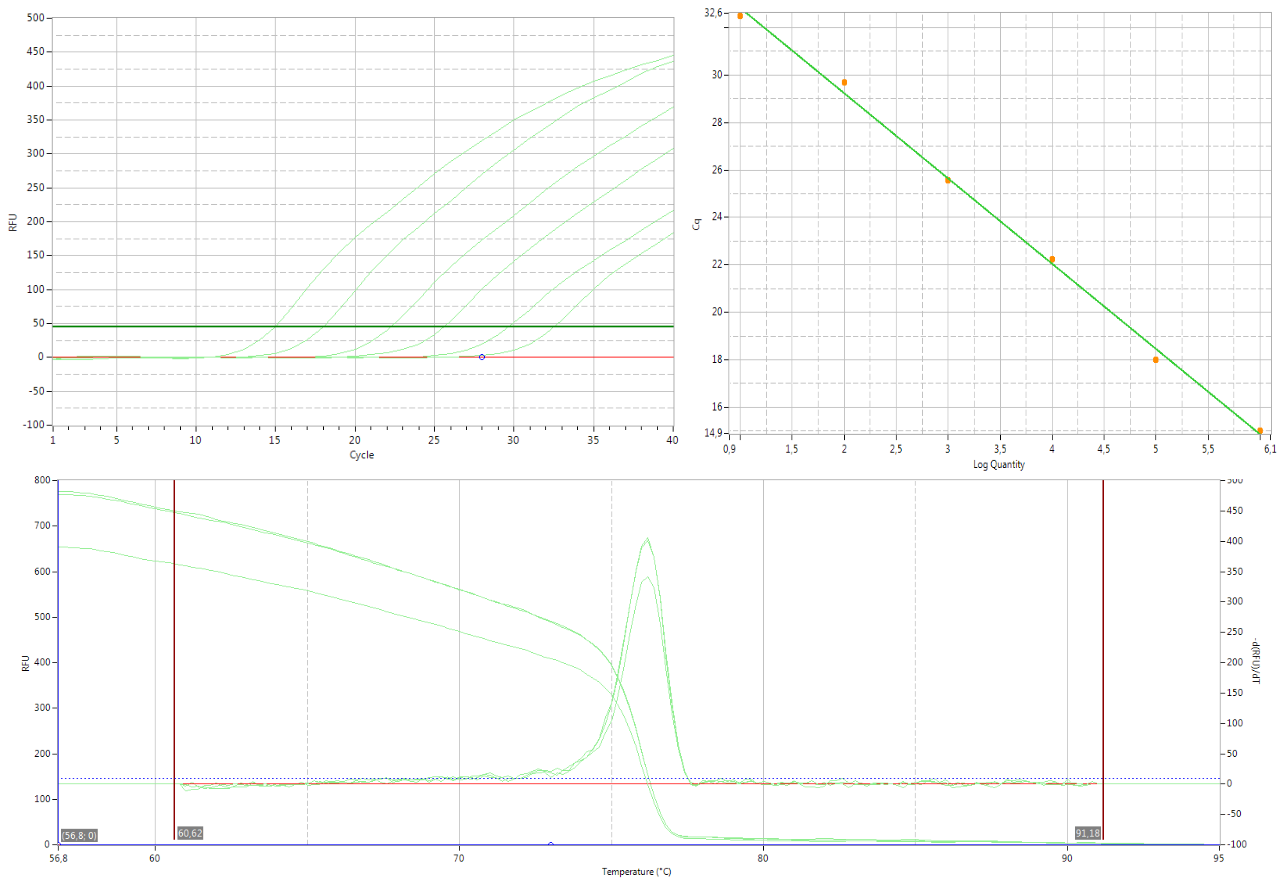

Fig. 37 Amplification curve, standard curve and melting curve of *tpx*
Efficiency = 90%; y = -3,595x + 36,419; R2 = 0,9975

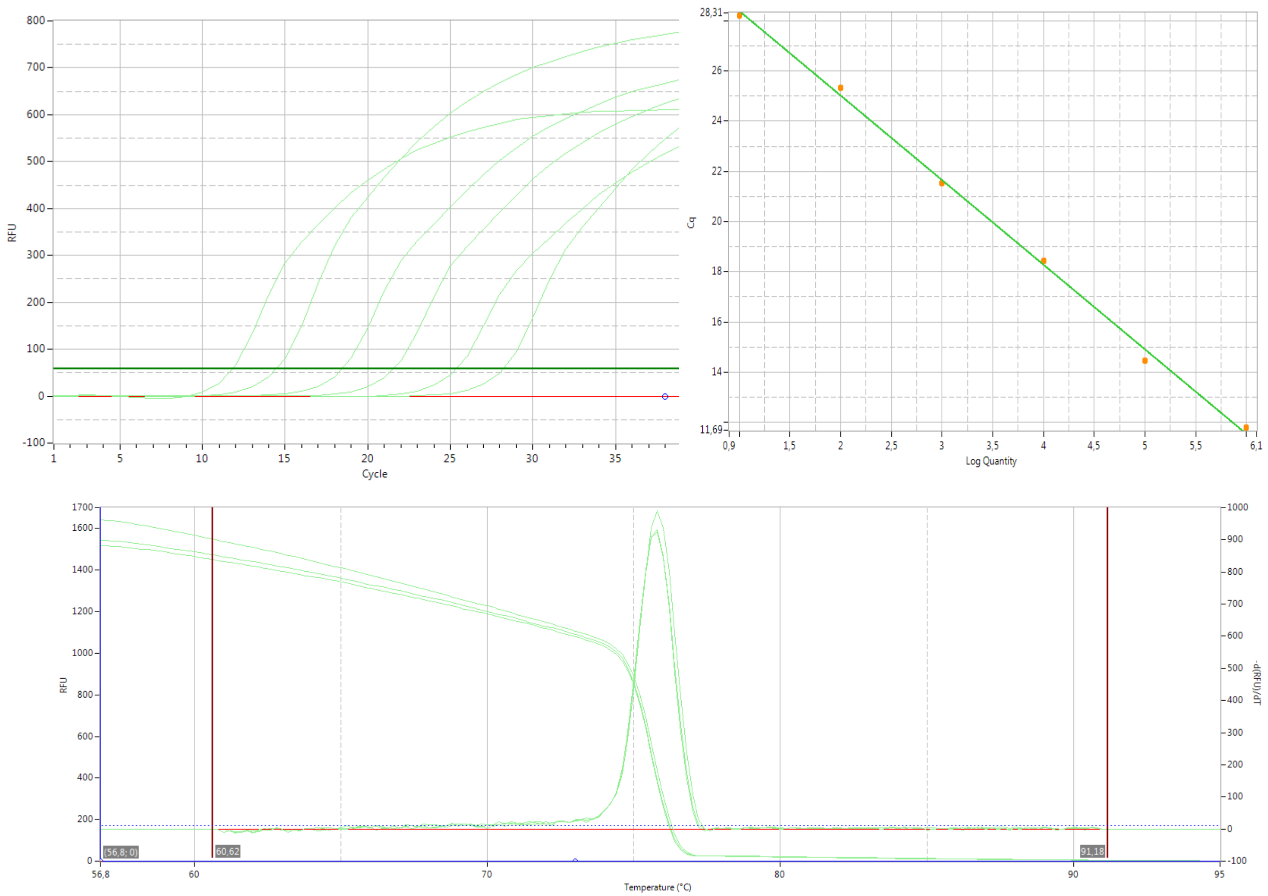

Fig. 38 Amplification curve, standard curve and melting curve of *rex*
Efficiency = 98%; y = -3,366x + 31,736; R2 = 0,9978

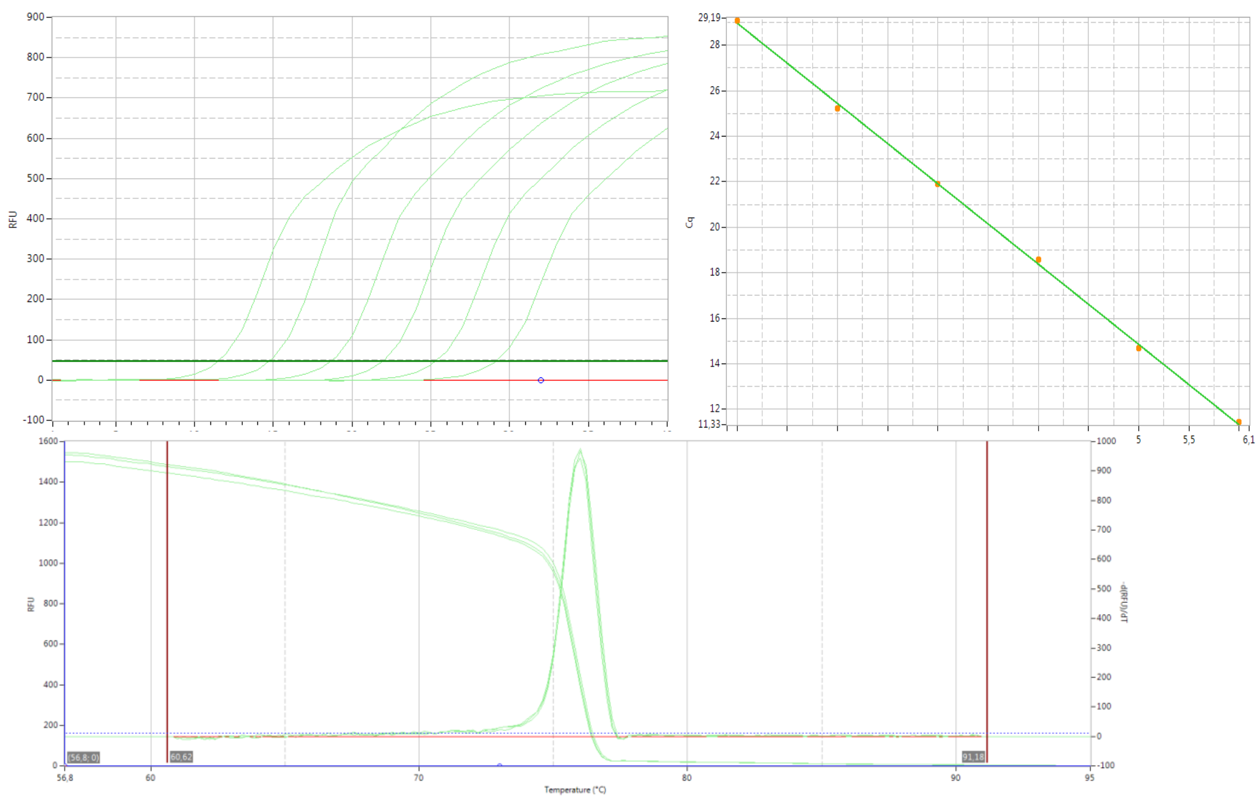

Fig. 39 Amplification curve, standard curve and melting curve of *lysR*
Efficiency = 92%; y = -3,523x + 32,484; R2 = 0,9994

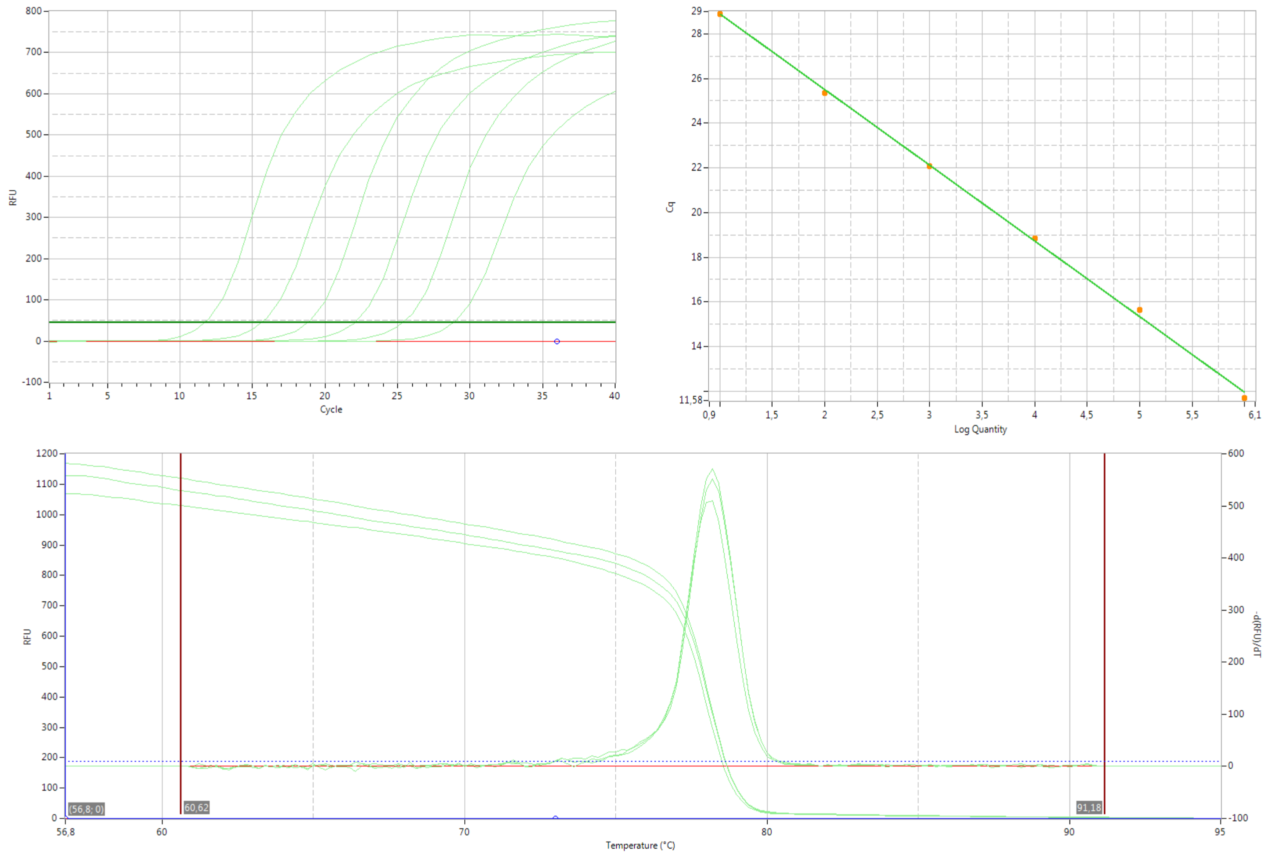

Fig. 40 Amplification curve, standard curve and melting curve of *ccpA*
Efficiency = 97%; y = -3,386x + 32,263; R2 = 0,999

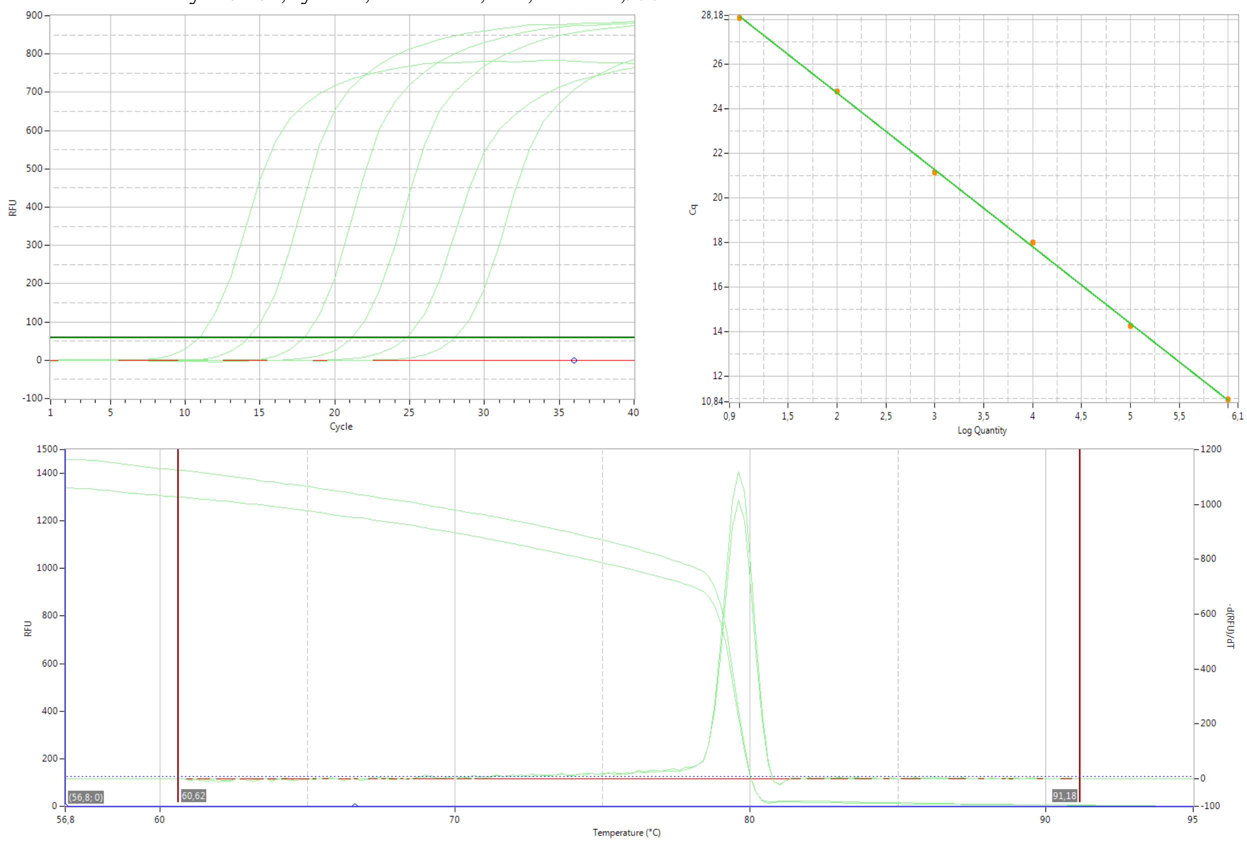

Fig. 41 Amplification curve, standard curve and melting curve of *hprK*
Efficiency = 95%; y = -3,44x + 31,573; R2 = 0,9997